CORE
Metadata, citation and similar papers at core.ac.uk
Provided by Universidade do Minho: RepositoriUM

Universidade do Minho
Escola de Ciências

Ana Patrícia Serra Peyroteo Guedes

Essential oils from plants and *in vitro* shoot cultures of *Hypericum androsaemum* L., *H. perforatum* L. and *H. undulatum* Schousboe ex. Wild

Fevereiro de 2009

Universidade do Minho
Escola de Ciências

Ana Patrícia Serra Peyroteo Guedes

Essential oils from plants and *in vitro* shoot cultures of *Hypericum androsaemum* L., *H. perforatum* L. and *H. undulatum* Schousboe ex. Wild

Tese de Doutoramento em Ciências
Ramo do Conhecimento Biologia

Trabalho efectuado sob a orientação de
Professor Doutor Manuel Fernandes Ferreira

Fevereiro de 2009

Acknowledgements

Ao Professor Doutor Manuel Fernandes Ferreira, orientador científico deste trabalho, agradeço o apoio, incentivo e disponibilidade, bem como o espírito científico que sempre me transmitiu ao longo destes anos.

Agradeço às Professoras Maria Helena Cardoso e Margarida Casal, Directoras do Departamento de Biologia, e à Professora Manuela Côrte-Real, Directora do Centro de Biologia, durante a execução deste trabalho, por promoverem todas as condições necessárias para o desenvolvimento do mesmo.

À Eng. Ana Vicente, da Direcção Regional de Agricultura e Pescas da Região Norte, agradeço a simpatia com que sempre me atendeu, mas sobretudo a disponibilidade para ceder e colher todo o material biológico que lhe solicitei.

À Associação Pedras Brancas, Covide - Terras de Bouro, agradeço o material biológico que me disponibilizaram.

Agradeço também a todos os colegas de trabalho e funcionários do Departamento de Biologia da Universidade do Minho, o apoio prestado no decorrer deste trabalho.

A todos os colegas que, ao longo destes anos, comigo trabalharam no Laboratório de Biotecnologia Vegetal de Plantas, obrigada pelo companheirismo sempre dispensado.

À Doutora Paula Gomes, amiga e companheira de laboratório, obrigada pelo apoio, disponibilidade e incentivo, ainda que nos últimos anos fisicamente ausente. Não imagina a falta que me fez…

Ao Paulo e ao Luís agradeço o apoio, o companheirismo, a amizade e os constantes desabafos nas horas mais difíceis.

À Alice, Marta e Mafalda agradeço o apoio e amizade na última fase da realização deste trabalho.

Ao Franklin agradeço a sua contribuição directa na concepção de um dos capítulos desta tese.
Aos meus amigos de sempre, Sandra, João, Herlânder, Zé, Ricardo, Tó e Sofia, um agradecimento muito especial por estarem sempre ao meu lado. A Vida não teria graça sem vocês…

À empresa Águas do Fastio, agradeço o apoio e as facilidades concedidas na fase final deste trabalho.

Às pessoas mais importantes da minha vida e que sempre acreditaram em mim, mesmo quando duvidei de que seria capaz, poucas são as palavras para exprimir o quanto lhes sou grata….

Aos meus sogros e cunhados, agradeço o apoio e incentivo ao longo destes anos.

À minha irmã, Paula, agradeço todo o apoio, amizade, alegria e partilha de experiências. És a melhor "chefinha"…

Aos meus sobrinhos, a luz da minha vida, obrigada por colorirem os meus dias, mesmo os mais cinzentos…

Aos meus Pais, agradeço pela paciência, carinho, incentivo e apoio incondicional sem os quais as dificuldades na realização deste trabalho teriam sido ainda maiores…

Ao Nuno agradeço-te por seres como és…

Summary

Apical buds and nodal segments, from plants grown in Nature were used as primary explants to establish *in vitro* shoot cultures of *Hypericum androsaemum* and *Hypericum undulatum*, respectively. *Hypericum perforatum* shoot cultures were established from nodal segments of axenic seedlings grown from seeds germinated aseptically on MS medium devoid of growth regulators. Shoot multiplication was performed by subculturing nodal segments on MS medium devoid of growth regulators, in the cases of *H. perforatum* and *H. undulatum*, and on MS medium supplemented with IAA and KIN, in the case of *H. androsaemum*. A modified Mg medium was used in parallel with the MS medium both devoid of growth regulators, in the case of *H. undulatum*. After 60 days on MS basal medium, *H. undulatum* cultures were characterized by a higher number of shoots and roots, comparing with the cultures grown on Mg basal medium.

Essential oils (EO) from *in vivo* plants and *in vitro* cultures of *H. androsaemum*, *H. perforatum* and *H. undulatum* were isolated by hydrodistillation in a Clevenger type apparatus and analyzed by Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS).

More than 70 compounds were identified in EO from plants of *H. androsaemum* L. cultivated in two places (Arouca and Arcos de Valdevez) and harvested with intervals of 2 months, over one year. Seasonal variations on the content of sesquiterpene hydrocarbons, the most represented group of compounds, were registered (42.8-72.7% in plants grown in Arouca and 43.4-78.5% in plants grown in Arcos de Valdevez). A high number of intermediate to long chain *n*-alkanes and 1-alkenes was recorded in EO of *H. androsaemum* plants grown in Arouca, in the month of February, as well as plants grown in Arcos de Valdevez, during the spring and in the end of winter. In most of the EO of this species, (*E*)-caryophyllene, β-gurjunene and γ-elemene were the major compounds independently of the experimental field. Ripened seed capsules and stems were the *H. androsaemum* organs with the highest and the lowest EO contents, respectively. Sesquiterpene hydrocarbons dominated the EO of leaves and stems of *H. androsaemum*, while monoterpene hydrocarbons dominated the EO of the ripened seed capsules. From the five most represented compounds in the EO of *H. androsaemum* no one was common to all the three organs (leaves, stems and ripened seed capsules). Almost 80% of the total EO of *in vitro* shoots of *H. androsaemum* was represented by sesquiterpene hydrocarbons, with γ-elemene as the only major constituent common to EO from *in vitro* shoots and from in Nature cultivated plants.

Essential oils from aerial parts of *H. perforatum* plants of two cultivars (common cultivar and cv. 'Topaz'), grown in two experimental fields and sampled over one year, revealed high levels of sesquiterpene hydrocarbons, and low levels of oxygenated compounds. Germacrene D, *(E)*-caryophyllene and *β*-selinene were the major compounds. The highest EO content was found in flowers (~12-17 mg/g of dry biomass), in which sesquiterpene hydrocarbons was the major compound group and 2-methyloctane the most represented compound (22-29%). Alkanes which represented no more than 9% of the total EO from in Nature cultivated plants, was the second major group in the EO of *in vitro* shoots, in which *n*-nonane accounted for more than 24% of the total EO.

Essential oils of plants and *in vitro* shoots of *H. undulatum* Schousboe ex Willd had *n*-nonane as the major constituent, accounting for more than 40% in most of them. This compound was that most contributed for the high level of *n*-alkanes group in the EO of the different plant organs, notwithstanding sesquiterpene hydrocarbons constituted the dominant group. The highest yield of *H. undulatum* EO was obtained from leaves, followed by ripened seed capsules, flowers and stems. The EO contents observed in *in vitro H. undulatum* shoots (4.9-10.5 mg/g of dry weight, for MS basal medium and 4.1-9.5 mg/g of dry weight, for Mg basal medium) were higher than those observed in aerial parts of in Nature growing plants. Although variations in the composition of the EO from shoots grown on two different basal media had been registered, over the 60 days of culture, the group of alkanes was the major one independently of the culture conditions. The highest contents of *n*-nonane were recorded in the EO from shoots grown on Mg basal medium.

In order to get hairy root cultures of *H. androsaemum*, *H. perforatum* and *H. undulatum*, the influence of several factors (effect of explant pre-culture, bacterial density, explant wounding, addition of acetosyringone to the bacterial suspension and co-culture medium, as well as co-culture period) were evaluated, using the *A. rhizogenes*-mediated transformation as the main approach. Notwithstanding the several assays performed, hairy roots production was not achieved in any of the tested explants (leaves, internodal segments and roots).

Resumo

No âmbito do presente trabalho, foram estabelecidas culturas *in vitro* de *Hypericum androsaemum*, *Hypericum perforatum* e *Hypericum undulatum*. As culturas *in vitro* de *H. androsaemum* e *H. undulatum* foram obtidas, respectivamente, a partir de gemas apicais e segmentos nodais de plantas desenvolvidas na Natureza. Os explantes primários de *H. perforatum* foram obtidos de plântulas desenvolvidas a partir de sementes germinadas em condições de assépsia em meio de cultura MS sem fitorreguladores. Segmentos nodais foram utilizados na multiplicação de rebentos caulinares em meio MS suplementado com IAA e KIN, no caso de *H. androsaemum*, meio base MS, no caso de *H. perforatum* e meios base MS e Mg, no caso de *H. undulatum*. Após 60 dias de cultura, as plântulas de *H. undulatum* desenvolvidas em meio MS apresentavam maior número de rebentos e raízes, do que as plântulas obtidas em meio Mg.

Os óleos essenciais (OE) de plantas *in vivo* e culturas *in vitro* de *H. androsaemum*, *H. perforatum* e *H. undulatum* foram isolados por hidrodestilação em aparelho tipo Clevenger e analisados por Cromatografia Gasosa (CG) e Cromatografia Gasosa acoplada a Espectrometria de Massa (CG-EM).

Mais de 70 compostos foram identificados nos OE de plantas de *H. androsaemum* L., cultivadas em dois locais distintos (Arouca e Arcos de Valdevez), e colhidas com a periodicidade de 2 meses. Ao longo do ano, foram registadas variações de composição traduzidas em variações dos teores percentuais dos grupos de compostos, designadamente dos hidrocarbonetos sesquiterpénicos, grupo maioritário cuja expressão variou entre 42.8% e 72.7% nos OE de plantas desenvolvidas em Arouca e entre 43.4% e 78.5% nos OE de plantas desenvolvidas em Arcos de Valdevez. Nos OE de plantas desenvolvidas em Arcos de Valdevez, durante a Primavera e no final do Inverno foi registada a acumulação de um número superior de *n*-alcanos, de cadeia intermédia a longa e, de 1-alcenos, ao passo que nos OE de plantas desenvolvidas em Arouca a maior diversidade de *n*-alcanos de cadeia intermédia a longa e 1-alcenos foi registada em Fevereiro. (*E*)-Cariofileno, *β*-gurjuneno e *γ*-elemeno foram os compostos maioritários na maioria das amostras de OE desta espécie, em ambos os campos experimentais. Os teores de OE mais elevados foram registados nos frutos e os mais baixos nos caules desta espécie. Nos OE das folhas e dos caules predominaram os hidrocarbonetos sesquiterpénicos, ao passo que nos OE dos frutos predominaram os hidrocarbonetos monoterpénicos. Dos cinco compostos maioritários dos OE de cada um dos órgãos em estudo,

nenhum era comum aos três, reflectindo a dissemelhança entre eles. Cerca de 80% do total dos OE das culturas *in vitro* de *H. androsaemum* era constituído por hidrocarbonetos sesquiterpénicos, sendo o *γ*-elemeno o único composto deste grupo maioritário nos OE dos rebentos caulinares e das plantas cultivadas.

Os OE da parte aérea de plantas das cultivares comum e 'Topaz' de *H. perforatum*, cultivadas em dois campos experimentais distintos revelaram teores elevados de hidrocarbonetos sesquiterpénicos e teores baixos de compostos oxigenados. Os compostos maioritários dos OE de ambas as cultivares foram o germacreno D, o *(E)*-cariofileno e o *β*-elemeno. Os OE de flores proporcionaram rendimentos superiores (~12-17 mg/g de biomassa seca) aos das partes aéreas das plantas. A sua composição era maioritariamente constituída por hidrocarbonetos sesquiterpénicos embora o composto maioritário fosse um alcano, o 2-metiloctano cujo teor variou de 22% a 29%. O constituinte maioritário dos rebentos caulinares foi o *n*-nonano (24% do OE), sendo os alcanos o segundo grupo de compostos maioritário neste tipo de culturas *in vitro* de *H. perforatum*.

Nos OE de plantas e rebentos caulinares de *H. undulatum* Schousboe ex Willd., o *n*-nonano foi o composto maioritário, constituindo mais de 40% do seu total na maioria das amostras analisadas. De facto, este composto foi o responsável pela elevada expressão percentual do grupo dos *n*-alcanos nos diferentes órgãos de *H. undulatum*. Nas folhas, porém, os hidrocarbonetos sesquiterpénicos foram o grupo predominante. Os teores mais elevados de OE foram registados nas folhas, seguindo-se os frutos, flores e caules. Os rendimentos em OE dos rebentos caulinares variaram de 4,9 a 10,5 mg/g de biomassa seca nas culturas mantidas em meio base MS e de 4,1 a 9,5 mg/g de biomassa seca nos rebentos caulinares desenvolvidos em meio base Mg, sendo superiores aos registados para os OE das partes aéreas das plantas. Embora se tenham verificado variações na composição dos OE de rebentos caulinares mantidos nos dois meios de cultura, o grupo dos *n*-alcanos foi o maioritário em ambos os casos. Os teores mais elevados de *n*-nonano, composto maioritário, foram registados nos rebentos caulinares desenvolvidos em meio base Mg.

Na tentativa de se induzir a formação de hairy roots de *H. androsaemum*, *H. perforatum* e *H. undulatum* foi testada a influência de diversos factores (pré-cultura dos explantes, densidade bacteriana, ferimento dos explantes, adição de acetoseringona à suspensão bacteriana e ao meio de co-cultura, e período de co-cultura) na transformação das referidas espécies mediada por *A. rhizogenes* A4. Apesar dos diversos ensaios realizados, não se verificou a produção de hairy roots em nenhum dos explantes utilizados (folhas, segmentos internodais e raízes).

Index

Abbreviations

AMS: amorpha-4,11-diene synthase

AS: Acetosyringone

BAP: 6-Benzylaminopurine

BC: Before Christ

ºC: degrees Celsius

carb: Carbenicillin

cef: Cefotaxime

DMAPP: Dimethylallyl diphosphate

DNA: Deoxyribonucleic acid

DRAEDM: Direcção Regional de Agricultura de Entre Douro e Minho

DRAPN: Direcção Regional de Agricultura e Pescas do Norte

dsT-DNA: double stranded transfer DNA

DXP: 1-deoxy-D-xylulose-5-phosphate

dw: dry weight

e.g.: *exempli gratia*

EO: Essential Oils

FID: Flame ionisation detector

Fl: Flowers

FPP: Farnesyl diphosphate

FPS: Farnesyl diphosphate synthase

g: gram

GA_3: Gibberellin

GC: Gas Chromatography

GC-MS: Gas Chromatography-Mass Spectrometry

GGPP: Geranyl geranyl diphosphate

GPP: Geranyl diphosphate

h: hour

He: Helium

HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA

HpA: *Hypericum perforatum* plants cultivated in Arcos de Valdevez

HpM: *Hypericum perforatum* plants cultivated in Merelim

IAA: Indol-3-acetic acid

i.e.: in example

IPP: Isopentenyl diphosphate

ITD: Ion Trap Detector

kb: kilo base pairs

KI: Kovats retention time

KIN: kinetin

L: litre

m: metre

MEP: 2-C-methyl-D-erythritol-4-phosphate

Mg: Basal medium modified from that of $N_{30}K$

mg: milligram

µg: microgram

MH: Monoterpene Hydrocarbons

MIC: Minimal Inhibitory Concentration

min: minute

mL: millilitre

µl: microlitre

mm: millimetre

µm: micrometre

MO: Oxygenated Monoterpenes

MS: Murashige & Skoog medium

MVA: Acetate Mevalonate

NAA: α-Naphtaleneacetic acid

NAPRALERT: Natural Products Alert Database

NLS: Nuclear Location Signals

OD: Optical density

OE: Óleos essenciais

OPS: Organizaciõn Panamericana de La Salud

RI: Retention Index

Ri: Root-inducing

Rip seed capsules: Ripened seed capsules

s: second

SH: Sesquiterpene Hydrocarbons

SO: Oxygenated Sesquiterpenes

ssDNA: single strand DNA

ssT-DNA: single stranded transfer DNA

T-DNA: transfer DNA

Ti: Tumour-inducing

tr: trace amount

UV: Ultra Violet radiation

VAp: Vegetative aerial parts

Vir: Virulence proteins

vir: virulence genes

WHO: World Health Organisation

Chapter 1

GENERAL INTRODUCTION

1.1- Medicinal plants and their uses in a Historical perspective

From the plant species living on Earth (250.000 to 500.000) only 1 to 10% are used as sources of nutrients in animal and human diets (Cowan, 1999; Rao *et al.*, 2002). However, it is possible that more are used for medicinal purposes (Cowan, 1999). The use of medicinal and aromatic plants is reported since ancient times, as evidenced by archaeological records from Chinese, Egyptian, Mesopotamian, Greek and Roman origins. First civilizations had already cured people with plants about 5000 years ago (i.e. 3000 years BC). Thus, either for alimentation, therapeutic or welfare uses, plants exploitation is as old as mankind (Margaris *et al.*, 1982). However, with the development of science and technology, the use of plants as medicines declined, becoming mostly restricted to the culinary and cosmetics industry (Margaris *et al.*, 1982). Nowadays however, the interest regarding plants' curative properties is re-emerging, motivated by (i) the cost of complex pharmaceuticals synthesis and (ii) the consumers´ concern regarding the impact of synthetic chemicals on health and environment (Marasco *et al.*, 2007). As a consequence, about 60% of anti-tumour and anti-infectious drugs, currently on the market or yet under clinical trials, are from natural origin. Furthermore, 11% of the 252 drugs considered as basic and essential by the World Health Organisation (WHO) are exclusively of plant origin. On the other hand a significant number of synthetic drugs are obtained from natural precursors, through semi-synthesis (Rates, 2001). Several important healing drugs, currently used, are directly extracted from plants, such as digoxin from *Digitalis* spp., quinine and quinidine from *Cinchona* spp., vincristine and viblastine from *Catharanthus roseus*, atropine from *Atropa belladonna*, morphine and codeine from *Papaver somniferum* (Rates, 2001). In the past decade the market for herbal remedies rose at a rate of about 4-10% a year in North America and Europe (Saxena *et al.*, 2007). According to some authors, however, the use of natural products might be unauthorised by legal authorities dealing with efficacy and safety procedures, due to a lack of quality in production, trade and prescription of phytomedicinal products (Rates, 2001). To overcome this issue, many countries such as Canada and USA are introducing legislation to improve safety and efficacy of these products (Saxena *et al.*, 2007). Therefore, in the last years there has been an increase in the studies concerning this subject, involving a multi-disciplinary research. Different sciences, such as Botany, Agronomy, Phytochemistry, Pharmacognosy, and Pharmaceutical Technology play an important role in the medicinal plant research and allow the development of new drugs (Briskin, 2000; Rates, 2001). Most of the medicinal plant species used is still harvested in the wild, which can be problematic due to biodiversity loss, variation in plant quality, and misleading uses as a consequence of

improper plant identification. Thus, contribution of Plant Physiology and Biotechnology is essential for providing new approaches for the safe production of plant secondary metabolites. In the last years there has been an increasing interest in nutraceuticals or functional foods, in which phytochemical constituents can have long-term health promoting or medicinal qualities (Briskin, 2000). According to the OPS (Organizaciõn Panamericana de La Salud), medicinal plant is any plant used in order to relieve, prevent or cure a disease or to alter physiological and pathological process. Medicinal plant can, also, be any plant employed as a source of drugs or their precursors (Arias, 1999). Medicinal effects might be exerted without the plant having any nutritional role in the human diet. In contrast, nutraceuticals have an important nutritional role in the diet and the benefits to health only arise after a long-term use as food (Briskin, 2000). In addition, a phytopharmaceutical preparation or herbal medicine is defined as any manufactured medicine obtained exclusively from plants, such as aerial and non-aerial parts, juices, resins and oils, either in the crude state or as a pharmaceutical formulation (Rates, 2001). Plants are not only exclusively sources of potential therapeutic and/or pharmacological metabolites, as they are also a valuable source of a wide range of compounds used as agrochemicals, flavours, fragrances, colours, biopesticides and food additives (Rao *et al.*, 2002).

1.2- Secondary Plant Metabolism

Plants produce a broad range of bioactive chemical compounds that can be classified as primary metabolites and secondary metabolites, depending on its biosynthetic origin, biochemical role and general occurrence. Primary metabolic routes produce primary metabolites, which are essential for all life forms. These compounds include carbohydrates, lipids, proteins, chlorophyll and nucleic acids, being involved in plant cells structure and building processes as well as maintenance and survival (Briskin, 2000).

Secondary metabolites were firstly mentioned by Kossel in 1891, who defined that type of compounds as opposed to primary metabolites (Bougard *et al.*, 2001). Thirty years later, Czapek (1921) reported that secondary metabolites could be produced by "secondary modifications" such as deamination, from the nitrogen metabolism (Bougard *et al.*, 2001). In the middle of the 20th century, with the advances of analytical techniques, such as chromatography, more compounds were identified, giving origin to Phytochemistry as a new discipline. Although some of the compounds were demonstrated to be pigments, others display important roles in plant life, still unknown however at the begin of the 3rd millenium (Bougard

et al., 2001). Two schools of thought regarding their function emerged in the 1970s: for the first one, secondary metabolites, such as cardiac glycosides, cannabinoids, anthocyanins and pyrrolizidine alkaloids, displayed important ecological roles, whereas for the second one, "no special physiological meaning" should be attributed to metabolites such as alkaloids (Kutchan, 2001). In fact, secondary metabolites were first considered as "waste products" apparently useless to the plant. According to some authors secondary metabolites are "intermediates or products found in restricted taxonomic groups that are synthesized from general metabolites by a wider variety of pathways, but with no essential role for growth and life of the producing organism" (Bennett *et al.*, 1989). Advances of biochemical techniques and the rise of molecular biology showed, however, that those metabolites play a major role in the adaptation of plants to their environment. Indeed, secondary metabolites largely contribute to plant fitness by interacting with the ecosystems. For instance, they play an important role in the plants defence against pathogens, insects and even other plants. This protecting effect is mainly related to their antibiotic, antifungal and antiviral properties, as well as their antigerminative or toxic activity against other plants (Bougard *et al.*, 2001). Additionally, they also participate in the responses to abiotic stresses such as changes in temperature, water status, light levels and UV radiation exposure. Some of them also allow the attraction of beneficial organisms such as pollinators or symbionts (Briskin, 2000).

Maybe the following definition of secondary metabolites, is more appropriated, taking into consideration the state of the art of the matter in the beginning of the 3rd millenium (Verpoorte, 2000):

"Secondary metabolites are compounds with a restricted occurrence in taxonomic groups, that are not necessary for a cell (organism) to live, but play a role in the interaction of the cell (organism) with its environment, ensuring the survival of the organism in its ecosystem"

Besides the secondary metabolites have various functions in plants, it is likely that some of them may be pharmacologically active on humans and useful as medicines (Briskin, 2000). Recently, some of those molecules were shown to act by resembling endogenous metabolites, ligands, hormones, signal transduction molecules, or neurotransmitters. In fact, plant secondary metabolites have beneficial medicinal effects on humans because of their similarities in their target sites (Briskin, 2000). Therefore, these metabolites are valued therapeutics, such as (i) salicin, an analgesic and antipyretic compound isolated from *Salix* species and used as a

template for the synthesis of acetylsalicylic acid (aspirin); (ii) the anticancer drug taxol (paclitaxel) isolated from *Taxus brevifolia*; and (iii) the strongly analgesic, narcotic, and addictive compound morphine, isolated from opium. Nevertheless, these beneficial effects are not usually attributed to one single metabolite but are the result from combinations of secondary molecules produced by the plant. Moreover, some secondary metabolites extracted and purified from plants have other commercial applications, such as drugs, dyes, flavours, fragrances and insecticides among others (Verpoorte *et al.*, 2002).

Plant secondary metabolites are usually grouped in three large molecule classes based on their biosynthetic pathways: (i) terpenoids, (ii) phenolics and (iii) alkaloids. Some of the compounds of the first two groups are present in all plants while others, like some specific alkaloids are only found in certain species of plant families. This narrow distribution of secondary plant metabolites makes them important taxonomical markers, being the basis for Chemotaxonomy and Chemical Ecology (Briskin, 2000; Bougard *et al.*, 2001). Terpenoids and alkaloids are the largest groups of secondary compounds. In 1998, more than 88.000 compounds were registered in the NAPRALERT database (Natural Products Alert Database), from which 33.000 were terpenoids and 16.000 were alkaloids (Verpoorte, 2000). Surprisingly, only 3 biosynthetic pathways generate most of such abundant and diverse compounds: (i) isoprenoid pathway, (ii) shikimate pathway and (iii) polyketide pathway. In fact, plants produce similar basic structures that undergo modifications, resulting in specific molecules. Such modifications involve introduction of substituents, like hydroxyl, methoxy, aldehyde and carboxyl groups; oxidative reactions resulting in loss of certain fragments of the molecule, or even, rearrangements leading to new molecules (Verpoorte, 2000). This chemical diversity found within plants is consistent with the idea that such molecules represent adaptive characters that have been under natural selection during evolution (Theis *et al.*, 2003; Wink, 2003).

Because of their myriad of functions, secondary metabolites have been used for centuries in traditional medicine. However, if most of these molecules are already identified, many of their relevant biosynthetic steps are still to elucidate. Indeed, the enzymes involved in the most of the several pathways are not fully characterised, and/or the corresponding genes are not identified. Furthermore, many of the regulatory processes are still unknown, even for the well-characterised pathways. The total length and corresponding number of genes of these pathways is an additional constraint in this field of investigation (Peters *et al.*, 2004). In the past 15 years, enormous efforts have been made to better understand the molecular mechanisms which regulate secondary metabolites production and accumulation leading to a considerable improvement of our knowledge. These advances, together with the concomitant development of

Metabolic Engineering (improvement of cellular activities by manipulation of enzymatic, transport and regulatory functions of the cell with the use of recombinant DNA technology), have allowed to prevail over the limiting steps (*e.g.* limiting enzyme activities) (Bougard *et al.*, 2001; DellaPenna, 2001; Dixon, 2005). Another promising approach is the exploitation of transcription factors that turn on or off the whole secondary pathways (DellaPenna, 2001; Canter *et al.*, 2005). Plant metabolic engineering is nowadays being successfully applied to improve the production of constitutively produced secondary metabolites, which are of interest for human health. It is also seen as a way to considerably modify secondary metabolites patterns, by modulation of enzymes located downstream to the synthesis (Bougard *et al.*, 2001).

In the next years, new genomic approaches and efficient gene isolation methods will undoubtedly expand the range and precision of manipulations via transgenesis, providing potentially superior material for the breeder. Advances in genetic engineering will also make possible the use of crops such as corn and tobacco as drug factories. Plants used as bioreactors (biopharming) may soon represent one of the most important developments in the US agriculture, as pharmaceutical and chemical industries use field crops to produce therapeutic proteins, drugs, and vaccines (*e.g.* Cholera vaccine in tobacco plants by Chlorogen, Inc.) (Elbehri, 2005). According to Anon, 2002 *"plant-based biopharming is a uniquely powerful tool for the mass production of biotechnology-derived pharmaceuticals and biologics and is expected to become prevalent and established in years to come"* (Joshi et al., 2005).

1.3- Essential oils

Since ancient times medicinal and aromatic plants have been highly valued for both their fragrances and/or their medicinal and culinary uses. Essential oils (EO) are aromatic oily extracts obtained from different parts of the plant, such as flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots. The extraction methods used can be expression, fermentation, enfleurage or extraction. Steam distillation is the most commonly method of extraction for commercial production of EO (Dimandja *et al.*, 2000; Chan, 2001; Burt, 2004). It is believed that the concept of essential oil derive from the name coined in the 16th century by Paracelsus von Hohenheim, the Swiss reformer of medicine (Burt, 2004). As they are volatile, once extracted, EO need to be stored in airtight containers, in the dark, to prevent compositional changes. EO are miscible in organic solvents but not in water. Generally, its composition is complex. The overall olfactive character of the oil is often determined by a subtle interplay

between numerous components. In nature, EO play an important role in the protection of the producing plants as antibacterials, antivirals, antifungals, insecticides and also against herbivores by reducing their appetite for such plants. They also may attract some insects to favour the dispersion of pollens and seeds, or repel undesirable others (Cowan, 1999; Burt, 2004; Bakkali *et al.*, 2008). Because of its specific characteristics, aromatic plants and its EO have been used since early times in a variety of activities, from religious ceremonies and adornments, to remedies and personal use (Sangwan *et al.*, 2001). Nowadays, about 300 out of 3000 EO commercially known prevail, mainly on the flavours and fragrances markets (Burt, 2004). The public's desire for the natural flavourings and fragrances favour their use instead of synthetic chemicals. Natural aromas are also economically preferred since naturally occurring aroma chemicals often exist in an enantiomerically pure form that is more expensive to obtain synthetically (McCaskill *et al.*, 1997). Additionally, specific components of EO can be used as chiral auxiliaries in synthetic organic chemistry and microbial transformations of common structures, to give compounds of enhanced economical value (Sangwan *et al.*, 2001). This preference of consumers for natural products as well as the wider spectrum of applications of these extracts in different industries will be responsible for the expected continuous expansion of world trade in EO.

1.3.1- Local of Production

Plant volatiles are mostly synthesized, accumulated and released to the environment by a variety of epidermal or mesophyll structures, whose morphology tends to be characteristic of the taxonomic group. Such highly-specialized anatomical structures are usually present in leaves, roots, stem, floral part and fruits (Sangwan *et al.*, 2001). The uniqueness of essential oil bearing plants relies in the differentiation of specialised structures (*e.g.* epidermal hairs or trichomes) of synthesis and secretion of these metabolites. Among them, glandular trichomes, the best studied, morphologically vary between species but several types can occur on a single leaf (Sangwan *et al.*, 2001). Glandular tissues are capable of devoting great amounts of carbon and energy to the production of secondary metabolites. Considering the source of carbon and energy in the secretory tissues, a distinction should be made between nonphotosynthetic and photosynthetic glands. Many essential oil producing glandular tissues are nonphotosynthetic, being dependent on the carbon imported from the underlying leaf cells to support metabolism (Haudenschild *et al.*, 1998; McCaskill *et al.*, 1999). For example, nonphotosynthetically

secretory cells isolated from glandular trichomes of peppermint showed to be capable of converting sucrose into mono- and sesquiterpenes, characteristic compounds of its EO (McCaskill *et al.*, 1992). Such cells seemed to express the genes encoding several enzymes needed to the monoterpene production. On the other hand, there are other species in which the trichomes are photosynthetic and then able to fix carbon dioxide for the production of secondary metabolites (McCaskill *et al.*, 1999).

EO are not however, exclusively produced and stored in glandular trichomes. Other secretory structures such as microhairs, glandular hairs, secretory cavities and specialised internal cells have also been described in several species (Haudenschild *et al.*, 1998; Sangwan *et al.*, 2001). Apparently, these specialised structures evolved in order to allow the production and storage of high amounts of toxic compounds that should be kept apart from the mainstream of plant metabolism due to their properties. This type of anatomical organization also places EO production at the most appropriate location for emission or for "first contact" by herbivores and pathogens, since they have an important role in attracting pollinators and in plant defence mechanisms (Haudenschild *et al.*, 1998; Theis *et al.*, 2003).

1.3.2- Chemical Composition

The production of EO widely occurs across the Plant Kingdom, without being restricted to any specific taxonomic group (Sangwan *et al.*, 2001). Thus, apart from the great diversity of EO produced, they are mostly composed by (i) terpenoids of low molecular weight, such as monoterpenoids and sesquiterpenoids, (ii) phenylpropanoids, (iii) benzoids, (iv) fatty-acid derivatives and (v) non-terpene aliphatics (including nitrogen- and sulphur-containing compounds) (Dudareva *et al.*, 2005; Tholl *et al.*, 2006).

1.3.2.1- Terpenes

Terpenes are lipophilic compounds whose carbon skeleton derives from a fundamental five-carbon building block, isoprene. They are highly variable in structure, ranging from simple linear hydrocarbon chains to highly complex derived polycyclic molecules (Hallahan, 2000; Lucker *et al.*, 2007). Classification of this family of compounds is based on the number of isoprene units in the structure. The smallest terpenes are called hemiterpenes and contain a

single isoprene unit. Isoprene, an alkene released from actively photosynthesising tissues of C_3 plants, is the best known hemiterpene. Terpenes with two isoprene units (ten carbon atoms) are called monoterpenes, which often provide major sensory notes in flavours and fragrances derived from plant essential oil (Peters *et al.*, 2004). A huge variety of plant monoterpenoids are elaborated from basic skeletons (acyclic monoterpenoids, cyclohexanoid and cyclopentanoid monoterpenoids) through oxidation, reduction, isomerisation and ring-cleavage reactions. Some 38 distinct skeletons can be identified within these monoterpenoids (Hallahan, 2000). Sesquiterpenes, diterpenes and triterpenes are molecules with three, four and six isoprene units, respectively. All of these compounds often play an important role in plant defence as phytoalexins, a group of *de novo* synthesized molecules as a response to pathogenic attack (Peters *et al.*, 2004; Lucker *et al.*, 2007). Phytoalexins are not a constitutive part of the plant's chemical composition, but are induced upon tissue damage with an extremely localized response (*e.g.* oryzalexins in rice; capsidiol from tobacco and green pepper) (Peters, 2006; Araceli *et al.*, 2007; Macías *et al.*, 2007). Terpenes containing eight isoprene units are known as tetraterpenes, while terpenes with more than eight isoprene units are called polyterpenes. In this later class are included the very long polymers of natural rubber (Peters *et al.*, 2004). In addition to the role in plant defence mechanism, terpenoids are also involved in central processes of the plant metabolism, such as photosynthesis (the phytol side chain of chlorophyll and carotenoid pigments), electron transport (ubiquinone and plastoquinone), cell membrane architecture (phytosterols) and regulation of cellular development (gibberellins, abcisic acid, brassinosteroids) (McCaskill *et al.*, 1998; Trapp *et al.*, 2001). By acting as attractants of pollinators and seed-dispersing animals, terpenoids are important in plant reproduction (Pichersky *et al.*, 2002). Apart from their ecological functions, mono-, sesqui- and more rarely diterpenoids or their derivatives are frequently used as flavour and fragrance compounds, nutraceuticals, pharmaceuticals and as industrial raw materials.

1.3.2.2- Terpenes Biosynthesis

In the early nineties a major advance in the terpenoids chemistry was achieved when Wallach (1914) formulated the "isoprene rule". According to this principle, terpenoids could be assembled by a repetitive joining of isoprene units. It provided the first unified concept for a common structural relationship among terpenoid natural products (McGarvey *et al.*, 1995). Later on, this concept was refined by Ruzicka (1953), who hypothesised the "biogenetic

isoprene rule", which ignores the precise character of the biological precursors, assuming that they are "isoprenoid" in structure (Barkovich *et al.*, 2001). This rule states that terpenes are formed by the repetitive joining of isoprene units linked "head to tail". However, some groups of compounds, such as steroids and related compounds, violated this rule showing that the "head to tail" principle could be modified and the original skeleton cleaved or rearranged (Barkovich *et al.*, 2001). These additional modifications to the isoprenoid backbones are responsible for the structural diversity in the terpenoid class. Notwithstanding this great diversity of structures, terpenoid compounds seem to have a common biosynthetic origin. All terpenoids are synthesized through the condensation of isopentenyl diphosphate -IPP and its allylic isomer dimethylallyl diphosphate (DMAPP). Nowadays, two independent pathways are responsible for the biosynthesis of IPP and DMAPP, the classical acetate mevalonate (MVA) pathway and the non-mevalonate pathway, also called the 1-deoxy-D-xylulose-5-phosphate (DXP) or the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway as can be seen in Figure 1 (McCaskill *et al.*, 1997; Eisenreich *et al.*, 1998; Tholl *et al.*, 2004).

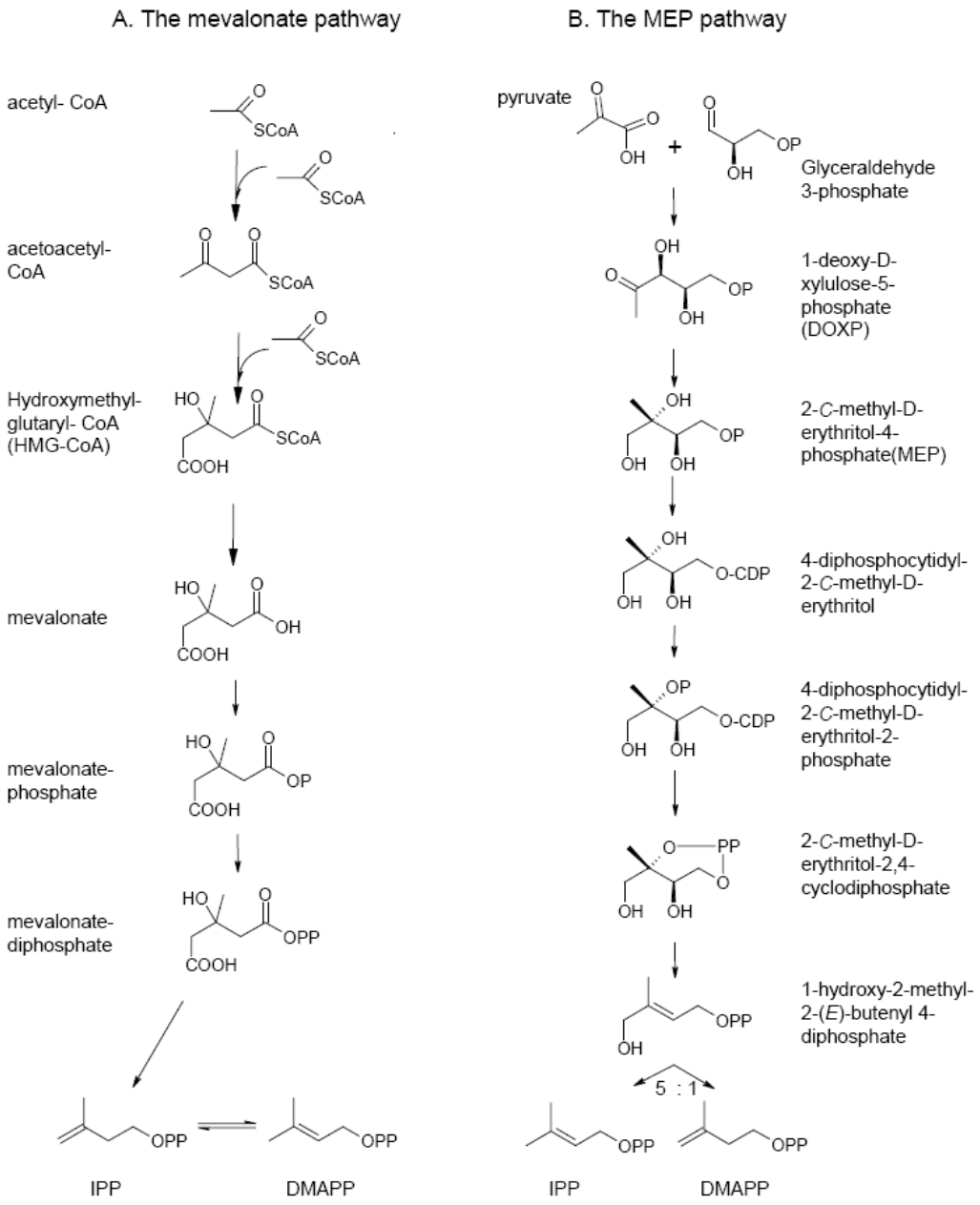


Figure 1– Two metabolic pathways (MVA and MEP pathways) leading to the general terpenoid precursors IPP and DMAPP (Lucker, 2002).

The initial steps of mevalonate pathway involve the condensation of three molecules of acetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is subsequently reduced to mevalonic acid by HMG-CoA reductase. Two sequential kinases then, form the mevalonate pyrophosphate, and IPP is finally produced by decarboxylative elimination. HMG-CoA reductase is highly regulated and is an important regulatory step in the IPP synthesis (McGarvey *et al.*, 1995; McCaskill *et al.*, 1997). For many years, it was generally assumed that

all terpenoids were synthesized *via* the MVA pathway because radioactive ^{14}C-mevalonate was shown to be incorporated in all kinds of terpenoids (McGarvey *et al.*, 1995; Verpoorte, 2000). However, in many cases the incorporation of mevalonate was low or preferentially incorporated in one group of terpenoids and not in others. Additionally, the biosynthesis of certain terpenoids was shown to be insensitive to inhibitors of the mevalonate pathway enzymes (Hallahan, 2000). Such evidences were clearly inconsistent with the MVA pathway suggesting the possible presence of an alternative route for the formation of some terpenoids. Indeed, in 1999, Rohmer reported a MVA-independent pathway operating in bacteria, algae and higher plants. After being elucidated in prokaryotes, the presence of this alternative pathway was demonstrated in phototrophic eukaryotes by performing incorporation experiments with ^{13}C labeled glucose isotopomers in *Ginkgo biloba* embryos in order to understand the biosynthesis of diterpenoids from the ginkgolide and bilobalide series (Rohmer, 1999). This experimental protocol opened the way to a whole array of new investigations. In the MEP pathway a transketolase-like decarboxylation from pyruvate and glyceraldehyde-3-phosphate, catalyzed by the 1-deoxy-D-xylulose-5-phosphate synthase, produces the five carbon intermediate, 1-deoxy-D-xylulose-5-phosphate. Subsequent rearrangement and reduction of the intermediate DXP, catalyzed by the 1-deoxy-D-xylulose-5-phosphate reductoisomerase, yields 2-C-methyl-D-erythritol-4-phosphate. Further transformations, probably involving redox and dehydration reactions, as well as an additional phosphorylation, result in the formation of IPP and DMAPP (Eisenreich *et al.*, 1998; Lange *et al.*, 1998; McCaskill *et al.*, 1998). DXP synthase and DXP reductoisomerase seem to be important because overexpression of their corresponding genes altered the levels of several terpenoids on different plant species (Mahmoud *et al.*, 2001; Rodriguez-Concepcion *et al.*, 2002). These two biosynthetic pathways (MVA and MEP) both operate in plant cells for the formation of terpenoids, although they are located in distinct sub-cellular compartments (Rodriguez-Concepcion *et al.*, 2002). MVA pathway operates in the cytosol producing sesquiterpenes, triterpenes and polyterpenes. On the other hand all the enzymes cloned from plants that are involved in MEP pathway have plastid targeting signals, demonstrating that this pathway occurs in the plastids. In these organelles, the MEP pathway provides precursors for the production of isoprene, monoterpenes, diterpenes and tetraterpenes (Eisenreich *et al.*, 2001; Bick *et al.*, 2003; Lucker *et al.*, 2007). Many experimental data based on labelled precursor flux studies and terpenoids engineered plants, indicate an exchange of precursors and intermediates between the cytosol and the choloroplast, corroborating the idea of cooperation of both pathways in terpenoids biosynthesis (McCaskill *et al.*, 1998; Eisenreich *et al.*, 2001; Rodriguez-Concepcion *et al.*, 2002; Bick *et al.*, 2003; Dudareva *et al.*, 2004). This crosstalk explains in

part why the alternative pathway went undetected for several decades despite numerous studies using mevalonate as a precursor (Eisenreich *et al.*, 2001).

After the production of IPP, regardless the original synthetic pathway, chain elongation is needed in order that terpenoids can be produced. IPP is isomerized to DMAPP by the enzyme isopentenyl pyrophosphate isomerase (Barkovich *et al.*, 2001). The head to tail condensation of one molecule of IPP with one molecule of DMAPP yields the C10 compound geranyl diphosphate (GPP), which is the immediate precursor of the monoterpenes (Figure 2). The addition of another IPP unit to GPP generates farnesyl diphosphate (FPP), the precursor of sesquiterpenes, triterpenes and sterols (Figure 2). The latter two groups both require the initial condensation of two units of FPP to form the squalene molecule. Addition of IPP to FPP provides geranyl geranyl diphosphate (GGPP), the precursor of diterpenes and tetraterpenes, consisting of two units of GGPP formed *via* phytoene (McCaskill *et al.*, 1997; Verpoorte, 2000; Tholl *et al.*, 2004).

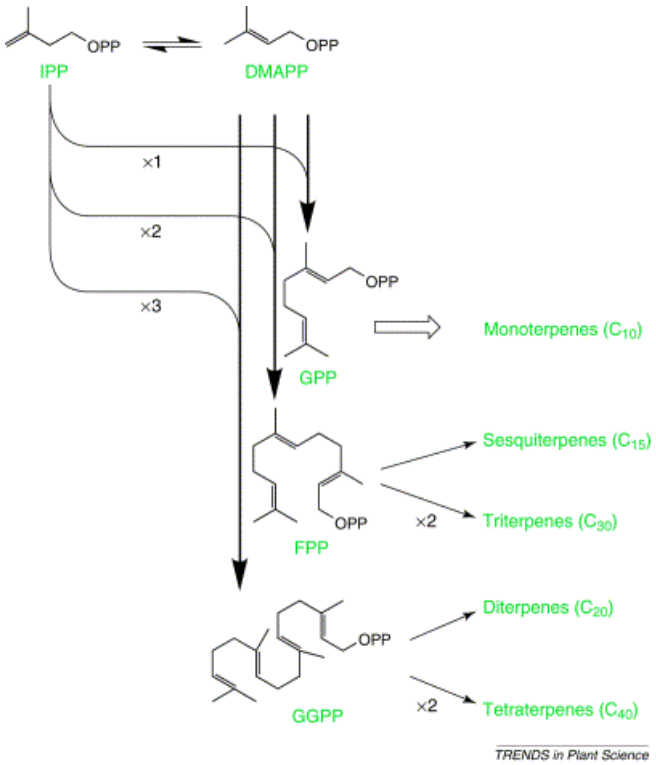


Figure 2– Overview of isoprenoid biosynthesis. IPP- isopentenyl diphosphate; DMAPP- dimethylallyl diphosphate; GPP- geranyl diphosphate; FPP- farnesyl diphosphate ; GGPP- geranyl geranyl diphosphate (Mahmoud *et al.*, 2002).

The electrophilic condensation reactions are catalysed by prenyltransferases, being GPP-synthase, FPP-synthase and GGPP-synthase, according to the corresponding end products, responsible for the major terpene classes (Gershenzon *et al.*, 1993; McCaskill *et al.*, 1997). The

reactions catalysed by these prenyltransferases are believed to be multi-step and sequential in which intermediate elongation products are not released from the enzyme surface in appreciable amounts. Plant prenyltransferases appear to be similar in size, requiring only a divalent metal ion for catalysis and sharing common primary structural elements including an aspartate-rich motif involved in substrate binding (Chen *et al.*, 1994). Terpenoid synthases or cyclases use the prenyl diphosphates precursors to generate the enormous diversity of carbon skeletons characteristic for terpenoids. These hydrocarbon structures can be either acyclic, cyclic or consist of multiple ring systems. After terpene synthases originate the primary terpene skeletons from the prenyl diphosphate substrates, a variety of other enzymes can modify these molecules (*e.g.* hydroxylases, dehydrogenases, reductases and glycosyl, methyl and acyl transferases). Terpene synthases may be involved in the regulation of the pathway flux since they function at the metabolic branch points and catalyse the first step leading to the various terpene classes (Gershenzon *et al.*, 1993). The terpene synthases possess quite similar properties, and operate with electrophilic reaction mechanisms just like the prenyltransferases (Trapp *et al.*, 2001).

1.3.3- Biological effects

EO or their isolated constituents have proven antimicrobial, antiviral, antiparasitic and insecticidal properties (Burt, 2004; Bakkali *et al.*, 2008). Antimicrobial activities of EO have been demonstrated against a wide variety of microorganisms. Several studies attribute this action to a number of specific terpenoids, although, additive, antagonistic, and synergistic effects have been observed between components of EO (Panizi *et al.*, 1993; Helander *et al.*, 1998; Chao *et al.*, 2000; Burt, 2004). Given that EO comprise a large number of components, it is most likely that their antibacterial activity is not due to one specific mode of action but involves several targets in the bacterial cell (Chao *et al.*, 2000; Skandamis *et al.*, 2001; Burt, 2004). It is believed that most EO exert their antimicrobial activities by interacting with processes associated with the bacterial cell membrane, including electron transport, ion gradients, protein translocation, phosphorylation, and other enzyme-dependent reactions (Cowan, 1999; Ultee *et al.*, 1999; Dorman *et al.*, 2000). EO have a high affinity for lipids of bacterial cell membranes due to their hydrophobic nature, and their antibacterial properties are evidently associated with their lipophilic character. Burt (2004) suggested that Gram-positive bacteria appear to be more susceptible to the antibacterial properties of plant EO compounds than Gram-negative bacteria. This may be expected as Gram-negative bacteria have an outer layer surrounding their cell wall that acts as a permeability barrier, limiting the access of

hydrophobic compounds. However, some reports also showed that the small molecular weight of the EO components can allow them to penetrate the inner membrane of some Gram-negative bacteria (Nikaido, 1994; Dorman *et al.*, 2000). Other biological effects are attributed to EO, such as phototoxicity, since some of them contain photoactive molecules (like furocoumarins) and carcinogenicity, because some EO or rather some of their constituents may be considered as secondary carcinogens after metabolic activation (*e.g.* estrogen secretions induced by *Salvia sclarea* EO can lead to estrogen-dependent cancers). Anti-mutagenic and antioxidant properties have also been attributed to EO (Bakkali *et al.*, 2008). The above mentioned EO cytotoxic capacity, based on a pro-oxidant activity, can make them excellent antiseptic and antimicrobial agents for personal use (internal use *via* oral consumption), and insecticidal products useful for the preservation of crops and food stocks. Thus, EO present a great potential to make their way from the traditional into the modern medical domain (Bakkali *et al.*, 2008).

1.3.4- Factors affecting Essential Oils production

Several factors affect EO production and accumulation. Some of them are inherent to the plant development and physiology, while others are extrinsic to the plant. One of the most important characteristics of oil accumulation is its dependence on the developmental stage of the plant as well as the organ in which they are produced. Indeed, it was shown a close co-ordination between leaf, flower and fruit ontogeny and EO accumulation for many aromatic plants. Organ maturation is often associated to an increase in the yield of the volatiles, as well as in differences in their EO composition. EO composition and yield is also organ dependent (Sangwan *et al.*, 2001; Figueiredo *et al.*, 2008). The type of secretory structure might also influence the EO yield and composition. For instance, plants with external secretory structures can release secretions while the organ is developing because of trichome cuticle disruption. On the other hand, plants with internal secretory structures more often maintain a more stable yield and composition (Figueiredo *et al.*, 2008). Other factors such as pollinator activity cycle, mechanical and chemical injuries, genetic factors and evolution are known to influence EO production (Figueiredo *et al.*, 2008). Geographical and seasonal variations also affect the EO production. The knowledge of how these two factors influence yields and composition is very important since the right time of harvest may be of major importance from an agronomic and economic point of view. EO are also very dependent on environmental conditions like climate, pollution, edaphic factors, pests and diseases (Figueiredo *et al.*, 2008). The method used to extract EO may also affect its chemical profile (Bakkali *et al.*, 2008). Nowadays, EO

production has also been negatively affected by political instability and insufficient capacity for investment in production and quality, leading to a big difference in prices of raw materials of different origins (Figueiredo *et al.*, 2008).

1.4- *In vitro* cultures

Despite the remarkable progress in synthetic organic chemistry of the twentieth century, over 25% of prescribed medicines in the industrialised countries derive direct or indirectly from plants. This is mainly because of their complex structural features which make them difficult to synthesize (Hostettmann *et al.*, 2002). Nowadays, the rapid disappearance of tropical forests and other important areas of vegetation as well as the increasing consumption of herbal medicines is causing habitat destruction and a rapid loss of medicinal plant wealth (Hostettmann *et al.*, 2002; Canter *et al.*, 2005). On the other hand, the increase of world population is pressuring on the available cultivable land to produce food and fulfil the needs. Therefore, for production of pharmaceuticals and chemicals from plants, the available land should be used efficiently (Rao *et al.*, 2002). In fact, some valuable medicinal plants are already under the threat of extinction (Rout *et al.*, 2000). Misidentification, genetic and phenotypic variability as well as contaminants are also problems inherent to harvesting from the wild (Canter *et al.*, 2005). Thus, modern technologies must be developed to avoid a possible loss of genetic diversity. Advances in Plant Biotechnology showed that cultivation of plant tissue in synthetic media offers an alternative way to produce metabolites of interest. Actually, plant tissue culture is being widely used for the commercial propagation of a large number of plant species, including many medicinal plants (Rout *et al.*, 2000). This technology is based in the potential capacity of cells to regenerate the entire organism, expressing its totipotency. The main advantages of secondary metabolites production through *in vitro* plant culture over conventional agricultural practices include:

- reliability of the production, since plant growth is simpler, more predictable as it is independent from environmental factors like climate, pests, geographical and seasonal constraints;
- potential for fast growth rates with the possibility for large-scale cultivation of cells, organs and even entire plants in bioreactors for easier and higher product recoveries;
- efficiency of the phytochemical extraction and isolation, as compared to extraction from complex whole plants;

- possibility to produce novel compounds *in vitro*, which are absent in the *in vivo* parent plant material;
- *in vitro* cultures can be used as a source of useful precursors from which novel compounds can be produced;
- it offers a defined production system, which ensures the continuous and homogenous supply of products, uniform quality and yield, being possible to avoid interfering compounds that occur in the field-grown plant;
- use of improvement strategies for better yields and cost-benefit ratios by growth medium, microenvironment manipulations and by using metabolic engineering at the cellular level (Zafar *et al.*, 1992; Rao *et al.*, 2002; Lila, 2005).

The production of secondary metabolites from undifferentiated plant cells and *callus* cultures has been studied intensively since the 1960's. Indeed, successful protocols of cell suspension cultures can offer a repeatable method to produce secondary metabolites from elite mother plants with easily controlled conditions and with a continuous supply of material (Tisserat *et al.*, 2005). In a few cases, cell cultures showed to produce higher levels of secondary metabolites than the differentiated mother plant itself (Bougard *et al.*, 2001). However, cell lines instability, low yields, slow growth, cell clumps formation and scale-up problems may occur, affecting the correct production (Zafar *et al.*, 1992; Bougard *et al.*, 2001; Verpoorte *et al.*, 2002). Additionally, in some cases secondary metabolites production is controlled in a tissue-specific manner, and the undifferentiated cell culture is not a good alternative for the high capacity production of the compound (Bougard *et al.*, 2001; Verpoorte *et al.*, 2002). This situation often occurs when secondary metabolites are produced in specialized plant tissues or glands (Lila, 2005). Therefore other approaches such as the induction of root, shoot and embryo cultures have been developed. Rout and co-workers (2000), referred three broad categories in which experimental approaches for medicinal plant propagation can be divided. The most common one, known as micropropagation, consist in the isolation of organised meristems like shoot tips or axillary buds and their culture to regenerate complete plants. Another approach consists in differentiation of adventitious shoots on leaf, root and stem segments or on *callus* derived from those organs. According to some authors, theoretically, the most efficient approach consists in inducing somatic embryogenesis from cells and *callus* cultures (Rout *et al.*, 2000). However, by this way high ratios of somaclonal

variation are expected. *In vitro* plant technology was also thought to be applied in the industrial field between 1976 and 1986. However, since that time, this technology has led to only a few applications for the production of commercial compounds, mainly attributed to several bottlenecks such as the economic feasibility of plant cell and organ cultures (Bougard *et al.*, 2001). Indeed, this technology involves high-cost bioreactors associated with aseptic conditions that are expensive to maintain. Therefore, in the last decades several attempts have been made to address the problem. Automation of micropropagation *via* organogenesis or somatic embryogenesis in modified bioreactors has been advanced as a possible way of reducing costs and it is a promising technology for industrial plant propagation (Paek *et al.*, 2005). Another constraint of the application of this technology in secondary metabolites production is related with the insufficient knowledge of biosynthetic routes and enzymology, since secondary metabolites are produced following long biosynthetic pathways that can involve dozens of enzymes (Bougard *et al.*, 2001). Despite a number of advantages listed above, plant cell and tissue culture technologies suffer from some drawbacks such as the possibility of culture conditions may trigger new pathways producing novel but useless products and the inability to predict yields of secondary metabolites *in vitro* beforehand. In fact, often the compounds of interest are not produced *in vitro* or if so they may be present in extremely low quantities. On the other hand, some methods developed for cell cultures of a particular plant species cannot be extrapolated to a wide range of plants and culture systems (Zafar *et al.*, 1992). To overcome such problems associated with *in vitro* plant cultures, different strategies have been adopted for enhancing secondary metabolites production in these systems. Some of them are described in Table 1 (Rao *et al.*, 2002).

Table 1- Strategies to enhance production of secondary metabolites in plant cell cultures (Rao *et al.*, 2002).

1. Obtaining efficient cell lines for growth
2. Screening of high-growth cell line to produce metabolites of interest
a. Mutation of cells
b. Amenability to media alterations for higher yields
3. Immobilization of cells to enhance yields of extracellular metabolites and to facilitate biotransformations
4. Use of elicitors to enhance productivity in a short period of time
5. Permeation of metabolites to facilitate downstream processing
6. Adsorption of the metabolites to partition the products from the medium and to overcome feedback inhibition
7. Scale-up of cell cultures in suitable bioreactors

1.5- Hairy root cultures

1.5.1- Agrobacterium *sp. in Plant Genetic Engineering*

Thirty-two years ago the landmark paper written by Mary-Chilton and co-workers reported the presence of a small piece of bacterial plasmid DNA in the DNA isolated from crown gall tumors (Chilton *et al.*, 1977). This finding caused an important impact on plant biology, agriculture and biotechnology. Indeed, this discovery brought a major revolution in agricultural practice and crop production as it motivated the study and manipulation of the mechanisms of the genetic transfer mediated by *Agrobacterium* sp., what became the best tool for genetic plant transformation. Genetically engineered crops with improved agronomic traits have made the transition from laboratory benches and greenhouses to fields all over the world. Genetic engineering technologies have evolved as a science and continue to provide the tools for making the crops of tomorrow (Moeller *et al.*, 2008). Nowadays, genetic techniques are also applied to medicinal and aromatic plants in order to improve the efficiency of secondary metabolites biosynthesis. Advances in the cloning of genes involved in relevant pathways, genomic tools and resources, and the recognition of a higher order of regulation of secondary plant metabolism operating at the whole plant level are important in definition of strategies for the effective manipulation of secondary products in plants (Gómez-Galera *et al.*, 2007). Early genetic transformation experiments applied to medicinal plants were carried out using *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* (Gómez-Galera *et al.*, 2007). *Agrobacterium*-mediated transformation is an indirect method to transfer gene. However DNA can be directly introduced in plant cells by microinjection into the nucleus, or fusion of protoplasts with lipossomes carrying DNA, or particle bombardment (Lindsey *et al.*, 1995; Vasil, 2008). Nevertheless, plant transformation mediated by *Agrobacterium*, a soil phytopathogenic bacterium, has become the main technology used to introduce a foreign gene into a plant host, due to the simplicity of the process and the correct targeting of the transgene (Veluthambi *et al.*, 2003). The authors also enumerate another set of advantages that *Agrobacterium*-based DNA transfer system offers comparing to other methods, such as a linked transfer of genes of interest along with the transformation marker; a higher frequency of stable transformation with many single copy insertions; reasonably low incidence of transgene silencing; and the ability to transfer long stretches of T-DNA (>150kb). The inability of *Agrobacterium* to transfer DNA to monocotyledonous plants was considered, for a long time, the major limitation of the technique. However, with effective modifications in Ti plasmid

vectors and finer modifications of transformation conditions, a number of monocots like rice (Hiei *et al.*, 1997; Tyagi *et al.*, 2000), wheat (Supartana *et al.*, 2006) and barley (Tingay *et al.*, 1997; Shrawat *et al.*, 2007) have been successfully transformed. Nowadays, *Agrobacterium* T-DNA transfer is viewed as "universal" since it has been successfully used in the transformation of non-plant eukaryotic organisms, such as yeast (Piers *et al.*, 1996), filamentous fungi (Zhang *et al.*, 2008) and human cells (Lacroix *et al.*, 2006).

1.5.2- Mechanism of genetic transfer mediated by Agrobacterium *sp.*

Transfer of DNA mediated by *Agrobacterium* sp. is usually done using two species of the genus, *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens*. Both species infect wounded plant tissues; *A. rhizogenes* induces the production of roots with a characteristic phenotype, "hairy roots", while *A. tumefaciens* induces the formation of crown gall tumours (Lindsey *et al.*, 1995; Caboni *et al.*, 1996; Zupan *et al.*, 1996; Spencer *et al.*, 1997). Evidences suggest that the mechanism of action of both bacteria in plant cell infection is similar (Tepfer *et al.*, 1987). The interaction between *Agrobacterium* species and plants involves a complex series of chemical signals communicated between the pathogen and the host. These signals include neutral and acidic sugars, phenolic compounds, opines, virulence proteins (Vir) and the T-DNA that is transferred from the bacterium to the plant cell (Gelvin, 2000). The process of gene transfer from *Agrobacterium* to plant cells implies several essential steps: bacterial colonization; induction of bacterial virulence system by specific host signals; generation of T-DNA transfer complex; T-DNA transfer and integration into plant genome (Riva *et al.*, 1998; Tzfira *et al.*, 2006). The infection mechanism is initiated with the attraction of the bacteria to the wounded tissues of the host plant followed by its attachment to the plant cell surface. This is an essential step in the process since a loss of tumour-inducing capacity was observed in non-attaching *Agrobacterium* mutants (Riva *et al.*, 1998). The attraction phenomenon is mediated by chemoattractive metabolites, mainly small phenolic compounds involved in phytoalexin and lignin biosynthesis, that are released from wounded plants (*e.g.* acetosyringone). Thus, *Agrobacterium* subverts part of the plant's defence mechanism and uses these compounds to signal the presence of a potentially susceptible plant. Signal metabolites are recognized by a sensor protein, VirA protein, triggering the expression of a subset of *Agrobacterium* virulence genes (*vir*) located on the bacterial Ti (tumour inducing) plasmid and leading to the excision of a single-stranded copy of T-DNA. T-DNA contains two types of genes: the oncogenic genes, encoding for the enzymes involved in the synthesis of auxins and cytokinins and responsible for tumour formation; and the genes encoding for the synthesis of opines. Opines, produced by

condensation between amino acids and sugars, are synthesised and excreted by the gall cells and consumed by *Agrobacterium* as carbon and nitrogen sources. Outside the T-DNA are located the genes for opine catabolism, the genes involved in the process of T-DNA transfer from the bacterium to the plant cell and the genes involved in bacterium-bacterium plasmid conjugative transfer (Riva *et al.*, 1998). Figure 3 shows the major molecular events of the transformation process mediated by *Agrobacterium* from the recognition and attachment of the bacterium to the host cell until T-DNA integration in the plant genome.

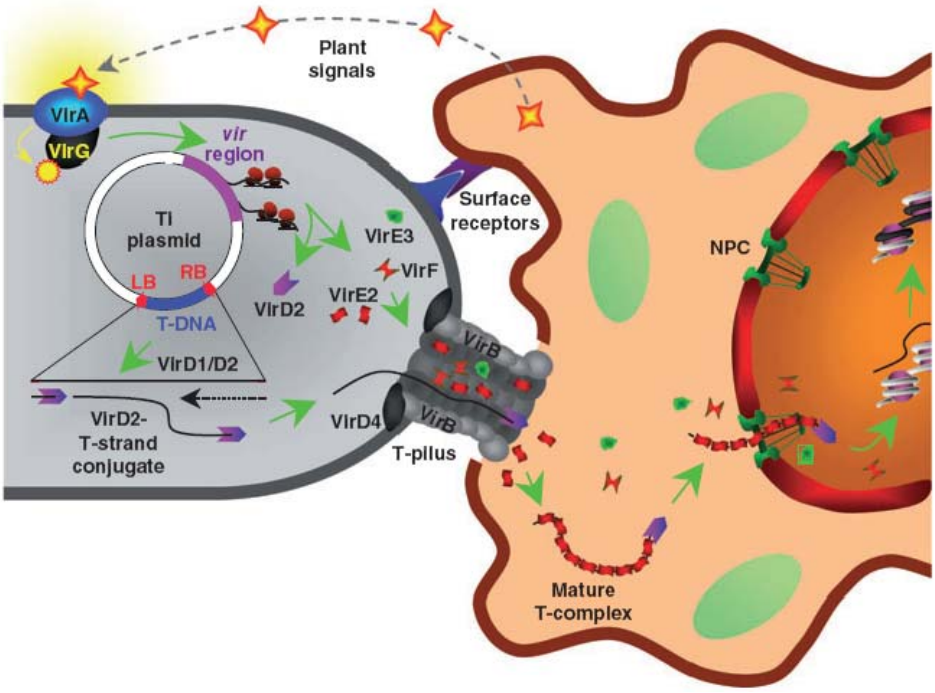


Figure 3 - Schematic diagram of the major molecular events and structures for the *Agrobacterium*-mediated genetic transformation (extracted from Citovsky *et al.*, 2007).

Activated VirA protein has the ability to transfer its phosphate to the cytoplasmic DNA binding protein VirG, resulting in the activation of *vir* gene transcription. Most of the induced Vir proteins are directly involved in T-DNA processing from the Ti-plasmid and the subsequent transfer of T-DNA from the bacterium to the plant (Lindsey *et al.*, 1995; Rossi *et al.*, 1996; Nester *et al.*, 1997; Riva *et al.*, 1998; Gelvin, 2000; Gelvin, 2003). Any DNA placed between the T-DNA borders will be transferred to the plant cell as single strand DNA (ssDNA). T-DNA border sequences are 25 bp in length and highly homologous in sequence, that delimits T-region in a directly repeated orientation. Border sequences are recognized by VirD1 and VirD2 proteins, which are able of nicking the Ti-plasmid by endonucleotidic cleavage. The nick sites are assumed as the initiation and termination sites for T-strand recovery. Following nicking,

VirD2 remains covalently attached to the 5'-end of the resulting DNA molecule, serving as a pilot protein to guide the T-strand from the bacterium into the plant cell. So, T-DNA enters the plant cell as a protein/nucleic acid complex composed of a single VirD2 molecule attached to the ssT-DNA. This complex has to pass through three membranes, the plant cell wall and cellular spaces. The protein/nucleic acid transfer seems to take place through a pilus, composed of numerous proteins encoded by the *virB* operon, and VirD4. T-pilus may not only serve as a hook to bring the bacterium and plant cell into close proximity, but it is likely that it interacts directly with the plant cell (Riva *et al.*, 1998; Gelvin, 2000; Zupan *et al.*, 2000; Gelvin, 2003). According to the most accepted model, the ssT-DNA/virD2 complex is coated by VirE2 protein, a DNA binding protein that cooperatively associates with any ssDNA sequence. VirE2 is believed to prevent the attack of nucleases and, additionally, extends the ssT-DNA strand reducing the complex diameter to approximately 2nm, making the translocation through membrane channels easier. Although, this is the model of T-DNA transfer most accepted, recent researches suggest that the ssT-strand/VirD2 complex may be transferred from the bacterium to the plant cell separate from VirE2 protein. An alternative model, in which a naked ssT-DNA/VirD2 complex is transferred from the bacterium and once inside the plant cell, is coated by VirE2 has been proposed (Riva *et al.*, 1998). However, it has already been demonstrated that in the export of VirE2 to the plant cell is essential another protein, VirE1. VirF also seems to be exported to the plant cell. Nevertheless, experimental data suggest that this protein functions in the plant rather than in the bacterium, by stimulating the plant cell division, and becoming more susceptible to transformation (Gelvin, 2000). Once inside the plant cell, the ssT-DNA/VirD2 complex is targeted to the nucleus, being VirD2 and VirE2 responsible for mediating the complex uptake to this organelle. This is so because VirE2 contains two plant nuclear location signals (NLS) and VirD2 one. The nuclear import is probably mediated also by specific NLS-binding proteins, which are present in the cytoplasm (Riva *et al.*, 1998; Zupan *et al.*, 2000; Tzfira *et al.*, 2006). Indeed, the first physical barrier on the nuclear import of the T-DNA complex is the nuclear pore, which the bacterium overcomes by using a host protein, which interacts directly with the bacterial VirD2 protein, that leads to directed translocation of the T-DNA complex through this pore (Ballas *et al.*, 1997). The final step of T-DNA transfer is its integration into the plant genome although the molecular mechanism involved is still under debate. Generally, however, it is accepted that T-DNA integration relies on the ability of the host DNA repair machinery to convert the T-strand molecule into double-stranded T-DNA (dsT-DNA) integration intermediates, to recognize these molecules as broken DNA, and to incorporate them into the host genome (Tzfira *et al.*, 2006).

Once, inside the nucleus, the T-DNA complex needs to travel to its point of integration and be stripped of its escorting proteins before integration into the host genome. This seems to happen due to the interaction of T-DNA complex with the host transcription machinery that guides the T-DNA complex to the site of integration in the host chromatin. Furthermore, biological evidences indicate that *Agrobacterium* harnesses the plant-targeted proteolysis machinery to uncoat the T-strand of its cognate proteins (Citovsky *et al.*, 2007; Dafny-Yelin *et al.*, 2008). VirE2, which has also been reported as essential for nuclear import of the T-complex, does not interact directly with the host nuclear-import machinery. It does, however, interact with VirE2-interacting protein 1 (VIP1), a putative transcription factor whose cellular function remains largely unknown, but that functions as an adaptor between the host nuclear-import machinery and the host-cell chromatin and VirE2. Recent data suggest a possible mechanism by which *Agrobacterium* induces and uses the defence signalling of the plant cell to deliver its T-DNA complex into the host-cell nucleus. *Agrobacterium* also uses the host transcription and translation machineries that are responsible for expression of opines (Citovsky *et al.*, 2007; Dafny-Yelin *et al.*, 2008).

1.5.3- Hairy roots applications in production of secondary metabolites

Transformation is currently used for genetic manipulation of more than 120 species of at least 35 families, including the major economic crops, vegetables, ornamental, medicinal, fruit, tree and pasture plants, using *Agrobacterium*-mediated or direct transformation methods (Riva *et al.*, 1998). However, efficient methodologies of *Agrobacterium*-mediated gene transfer are sometimes difficult to achieve. The optimization of *Agrobacterium*-plant interaction is probably the most important aspect to be considered. For instance, the synthesis of phenolic *vir* gene inducers by the plant, bacterial attachment, T-DNA transfer into the plant cytoplasm, T-DNA nuclear translocation, and T-DNA integration can limit the transformation efficiency of a particular plant. The type of explant must also suits for regeneration allowing the recovery of whole transgenic plants (Riva *et al.*, 1998; Gelvin, 2000).

The production of secondary metabolites can be enhanced, thanks to genetic transformation methods, namely those mediated by *Agrobacterium* sp.. Indeed, the genetic manipulation of terpenoids has already been reported. In *Mentha piperita*, the over-expression of genes involved in the increase of (-)-menthol pathway flux, a major monoterpene of this plant species, resulted into the increase in monoterpene yield and profile. Other strategy used to

enhance the quality of the peppermint EO was the reduction of undesirable monoterpenes production (*e.g.* molecules which are intermediates in the (-)-menthol biosynthesis pathway) (Lange *et al.*, 1999; Mahmoud *et al.*, 2002). In the same way, the production of a sesquiterpene, artemisinin, was improved by over-expression of its biosynthesis key enzymes-related genes (e.g. farnesyl diphosphate synthase (FPS), amorpha-4,11-diene synthase (AMS)) in *Artemisia annua* (Matsushita *et al.*, 1996; Mercke *et al.*, 2000; Han *et al.*, 2005). Transformation of tissues or plants with *A. rhizogenes*, as well as transformation with *rol* genes, may produce, in addition to hairy roots, alterations in the plants' secondary metabolism. Hairy roots have been found to be suitable for the production of secondary metabolites because of their stable and high productivity in hormone-free culture conditions (Tripathi *et al.*, 2003). Nowadays, many medicinal plants have been successfully transformed with *A. rhizogenes* with higher yields of secondary metabolites. Transformed hairy roots have been shown interesting in *in vitro* systems in production of some type of secondary metabolites, including some of terpenic origin. Indeed, transformed roots of *Artemisia annua* produced higher yields of the sesquiterpene, artemisinin, than the whole plants (Souret *et al.*, 2002) while in the hairy roots of *Tripterygium wilfordii* var. *regelii*, two new compounds were isolated (an abietane diterpenoid and a sesquiterpenoid) (Nakano *et al.*, 1998). On the other hand, in some cases the profile of EO produced by the hairy roots may be different from the EO profile found in roots of non-transformed in Nature growing plants, with the potential for production of new compounds. Such differences were observed in the EO isolated from hairy roots and non-transformed roots of cultivated plants from *Achillea millefolium* (Lourenço *et al.*, 1999). The variation in the density of secretory structures is also an alternative or additional approach to modulate the EO yields, as suggested by the identification of genes that regulate the differentiation of non-glandular trichomes in *Arabidopsis thaliana* (Veronese *et al.*, 2001). However, it is of upmost importance to better understand the impact of the introduced genes to the whole production pathways in order to evaluate the potential risks of side-effects of these methods. Investigation must continue to improve the production of valuable molecules in a sustainable and cost effective way (Gómez-Galera *et al.*, 2007).

1.6- Aims of this Work

In our country, *H. androsaemum* (Hipericão do Gerês), *H. perforatum* and *H. undulatum* are used in folk medicine for the therapeutic properties of their extracts. *H. perforatum* is the most characterized species of this genus in phytochemical terms. Most of the phytochemical studies on this genus are related to phenolic compounds, lacking data in the literature on its EO. However, these volatile mixtures constitute a pool of potential pharmaceutical substances with medical and commercial value, requiring a detailed identification of its constituents as well as the understanding of the factors (physiological, environmental) that might influence their relative concentration. On the other hand, excepting for *H. perforatum*, *in vitro* cultures of the other two species have not been explored, yet.

In this context, the objectives of this work for each *Hypericum* species were:

- characterize the essential oils profiles isolated from *in vivo* plants;
- establish and maintain shoot *in vitro* cultures;
- characterize the essential oils profiles produced by *in vitro* shoot cultures;
- "optimize" an *Agrobacterium rhizogenes*-mediated transformation protocol.

1.7- References

Anon (2002). cited by Joshi & Lopez 2005.

Araceli, A.-C., C.-M. Elda, L.-G. Edmundo & G.-P. Ernesto (2007). Capsidiol production in pepper fruits (*Capsicum annuum* L.) induced by arachidonic acid is dependent of an oxidative burst. *Physiological and Molecular Plant Pathology 70*(1-3), 69-76.

Arias (1999). cited by Rates (2001).

Bakkali, F., S. Averbeck, D. Averbeck & M. Idaomar (2008). Biological effects of essential oils - A review. *Food and Chemical Toxicology 46*(2), 446-475.

Ballas, N. & V. Citovsky (1997). Nuclear localization signal binding protein from *Arabidopsis* mediates nuclear import of *Agrobacterium* VirD2 protein. *Proceedings of the National Academy of Sciences of the United States of America 94*(20), 10723-10728.

Barkovich, R. & J. Liao (2001). Metabolic Engineering of Isoprenoids *Metabolic Engineering 3*, 27-39.

Bennett, J. & R. Bentley (1989). What's in a name?—Microbial secondary metabolism. *Advances in Applied Microbiology 34*, 1-28.

Bick, J. & M. Lange (2003). Metabolic cross talk between cytosolic and plastidial pathways of isoprenoid biosynthesis: unidirectional transport of intermediates across the chloroplast envelope membrane. *Archives of Biochemistry and Biophysics 415*, 146-154.

Bougard, F., A. Gravot, S. Milesi & E. Grontier (2001). Production of Plant Secondary Metabolites: a Historical Perspective. *Plant Science 161*, 839-851.

Briskin, D. (2000). Medicinal Plants and Phytomedicines. Linking Plant Biochemistry and Physiology to Human Health. *Plant Physiology 124*, 507-514.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology 94*, 223– 253.

Caboni, E., P. Lauri, M. Tonelli, G. Falasca & C. Damiano (1996). Root induction by *Agrobacterium rhizogenes* in walnut. *Plant Science 118*, 203-208.

Canter, P., H. Thomas & E. Ernst (2005). Bringing medicinal plants into cultivation: opportunities and challenges for biotechnology. *Trends in Biotechnology 23*(4), 180-185.

Chan, K. (2001). Quantitation of monoterpenoid compounds with potential medicinal use in biological fluids. *Journal of Chromatography A 936*, 47-57.

Chao, S. C., D. G. Young & C. J. Oberg (2000). Screening for inhibitory activity of essential oils on

selected bacteria, fungi and viruses. *Journal of Essential Oil Research 12*, 639–649.

Chen, A., P. Kroon & C. Poulter (1994). lsoprenyl diphosphate synthases: Protein sequence comparisons, a phylogenetic tree, and predictions of secondary structure. *Protein Sci. 3*, 600-607.

Chilton, M., M. Drummond, D. Merlo, S. D, A. Montoya, M. Gordon & E. Nester (1977). Stable incorporation of plasmid DNA into higher plant cells: molecular basis of crown gall tumorigenesis. *The Cell 11*(2), 263-271.

Citovsky, V., S. V. Kozlovsky, B. Lacroix, A. Zaltsman, M. Dafny-Yelin, S. Vyas, A. Tovkach & T. Tzfira (2007). Biological systems of the host cell involved in *Agrobacterium* infection. *Cellular Microbiology 9*(1), 9-20.

Cowan, M. (1999). Plant Products as Antimicrobial Agents. *Clinical Microbiology Reviews 12*(4), 564-582.

Dafny-Yelin, M., A. Levy & T. Tzfira (2008). The ongoing saga of *Agrobacterium*-host interactions. *Trends in Plant Science 13*(3), 102-105.

DellaPenna, D. (2001). Plant Metabolic Engineering. *Plant Physiology 125*, 160-163.

Dimandja, J.-M., S. Stanfill, J. Grainger & D. P. Jr (2000). Application of comprehensive two-dimensional gas chromatography (GC x GC) to the qualitative analysis of essential oils. *Journal High Resol. Chromatography 23*, 208-214.

Dixon, R. (2005). Engineering of plant natural product pathways. *Current Opinion in Plant Biology 8*, 329–336.

Dorman, H. J. D. & S. G. Deans (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J. Appl. Microbiol. 88*, 308–316.

Dorman, H. J. D. & S. G. Deans (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology 88*, 308–316.

Dudareva, N. & F. Negre (2005). Practical applications of research into the regulation of plant volatile emission. *Current Opinion in Plant Biology 8*(1), 113-118.

Dudareva, N., E. Pickersky & J. Gershenzon (2004). Biochemistry of Plant Volatiles. *Plant Physiology 135*(4), 1893-1902.

Eisenreich, W., F. Rohdich & A. Bacher (2001). Deoxyxylulose phosphate pathway to terpenoids. *Trends Plant Science 6*, 78-84.

Eisenreich, W., M. Schwarz, A. Cartayrade, D. Arigoni, M. Zenk & A. Bacher (1998). The deoxyxylulose phosphate pathway of terpenoid biosynthesis in plants and microorganisms. *Chemistry & Biology 5*, R221-R233.

Elbehri, A. (2005). Biopharming and the Food System: Examining the Potential Benefits and Risks. *AgBioForum 8*(1), 18-25.

Figueiredo, A., J. Barroso, L. Pedro & J. Scheffer (2008). Factors affecting secondary metabolite production in plants: volatile components and essential oils. *Flavour and Fragrance Journal 23*, 213–226.

Gelvin, S. (2000). *Agrobacterium* and plant genes involved in T-DNA transfer and integration. *Annu. Rev. Plant Physiol. Plant Mol. Biol. 51*, 223–256.

Gelvin, S. B. (2003). *Agrobacterium*-Mediated Plant Transformation: the Biology behind the "Gene-Jockeying" Tool. *Microbiol. Mol. Biol. Rev. 67*(1), 16-37.

Gershenzon, J. & R. Croteau (1993). Terpenoid biosynthesis: the basic pathway and formation of monoterpenes, sesquiterpenes and diterpenes. *Lipid Metabolism in Plants*. T. M. Jr. Boca Raton, FL, CRC Press**:** 340–388.

Gómez-Galera, S., A. Pelacho, A. Gené, T. Capell & P. Christou (2007). The genetic manipulation of medicinal and aromatic plants. *Plant Cell Reports 26*(10), 1689-1715.

Hallahan, D. (2000). Monoterpenoid Biosynthesis in Glandular Trichomes of Labiate Plants. *Advances in Botanical Research*, Academic Press Limited. **Vol. 31:** 77-120.

Han, J.-l., H. Wang, H.-c. Ye, Y. Liu, Z.-q. Li, Y. Zhang, Y.-s. Zhang, F. Yan & G.-f. Li (2005). High efficiency of genetic transformation and regeneration of *Artemisia annua* L. via *Agrobacterium tumefaciens*-mediated procedure. *Plant Science 168*(1), 73-80.

Haudenschild, C. & R. Croteau (1998). Molecular Engineering of Monoterpene Production. *Genetic Engineering*. J. Setlow. New York, Plenum Press. **20:** 267-280.

Helander, I. M., H. L. Alakomi, K. Latva-Kala, T. Mattila-Sandholm, I. Pol, E. J. Smid, L. G. M. Gorris & A. von Wright (1998). Characterization of the Action of Selected Essential Oil Components on Gram-Negative Bacteria. *J. Agric. Food Chem. 46*(9), 3590-3595.

Hiei, Y., T. Komari & T. Kubo (1997). Transformation of rice mediated by *Agrobacterium tumefaciens*. *Plant Mol. Biol. 35*, 205–218.

Hostettmann, K. & A. Marston (2002). Twenty years of research into medicinal plants: Results and perspectives. *Phytochemistry Reviews 1*, 275-285.

Joshi, L. & L. Lopez (2005). Bioprospecting in plants for engineering proteins. *Current Opinion in Plant Biology 8*, 223-226.

Kutchan, T. (2001). Ecological Arsenal and Developmental Dispatcher. The Paradigm of Secondary Metabolism. *Plant Physiology 125*, 58-60.

Lacroix, B., T. Tzfira, A. Vainstein & V. Citovsky (2006). A case of promiscuity: Agrobacterium's endless hunt for new partners. *Trends in Genetics 22*(1), 29-37.

Lange, B. & R. Croteau (1999). Genetic engineering of essential oil production in mint. *Current Opinion in Plant Biology 2*, 139–144.

Lange, B., M. Wildung, D. Mccaskill & R. Croteau (1998). A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway. *Proceedings of the National Academy of Sciences of the United States of America 95*, 2100-2104.

Lila, M. (2005). Valuable Secondary Products from *In Vitro* Culture. *Plant develpoment and Biotechnology*. R. Trigiano and D. Gray, CRC Press**:** 285-289.

Lindsey, K. & M. C. K. Jones (1995). *Plant Biotechnology in Agriculture*. Chichester, New York, John Wiley & Sons.

Lourenço, P. M. L., A. C. Figueiredo, J. G. Barroso, L. G. Pedro, M. M. Oliveira, S. G. Deans & J. J. C. Scheffer (1999). Essential oils from hairy root cultures and from plant roots of *Achillea millefolium*. *Phytochemistry 51*(5), 637-642.

Lucker, J. (2002). Metabolic engineering of monoterpene biosynthesis in plants. Wageningen, Wageningen University **PhD Thesis**.

Lucker, J., H. Bouwmeester & A. Aharoni (2007). Metabolic Engineering of Terpenoid Biosynthesis in Plants. *Applications of Plant Metabolic Engineering*. R. Verpoorte, A. W. Alfermann and T. S. Johnson, Springer**:** 219-236.

Macías, F. A., J. L. G. Galindo & J. C. G. Galindo (2007). Evolution and current status of ecological phytochemistry. *Phytochemistry 68*(22-24), 2917-2936.

Mahmoud, S. & R. Croteau (2002). Strategies for transgenic manipulation of monoterpene biosynthesis in plant. *Trends Plant Science 7*, 366–373.

Mahmoud, S. S. & R. B. Croteau (2001). Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase. *Proceedings of the National Academy of Sciences of the United States of America 98*(15), 8915-8920.

Marasco, E. K. & C. Schmidt-Dannert (2007). *Biosynthesis of Plant Natural Products and Characterization of Plant Biosynthetic Pathways in Recombinant Microrganisms*, Springer.

Margaris, N., A. Koedam & D. Vokou (1982). *Aromatic Plants: basic and applied aspects*. Hague, London, Boston, Martinus Nijhoff Publishers.

Matsushita, Y., W. Kang & B. V. Charlwood (1996). Cloning and analysis of a cDNA encoding farnesyl diphosphate synthase from *Artemisia annua*. *Gene 172*(2), 207-209.

McCaskill, D. & R. Croteau (1997). Prospects for the Bioengineering of Isoprenoid Biosynthesis. *Advances in Biochemical Engineering/ Biotechnology*. T. Scheper. Berlin Heidelberg, Springer-Verlag. **55:** 108-146.

McCaskill, D. & R. Croteau (1998). Some caveats for bioengineering terpenoid metabolism in plants. *Trends in Biotechnology 16*(8), 349-355.

McCaskill, D. & R. Croteau (1999). Strategies for bioengineering the development and metabolism of glandular tissues in plants. *Nature Biotechnology 17*, 31-36.

McCaskill, D., J. Gerhenzon & R. Croteau (1992). Morphology and monoterpene biosynthetic capabilities of secretory cell clusters isolated from glandular trichomes of peppermint (*Mentha piperita* L.). *Planta 187*, 445-454.

McGarvey, D. J. & R. Croteau (1995). Terpenoid Metabolism. *Plant Cell 7*(7), 1015-1026.

Mercke, P., M. Bengtsson, H. J. Bouwmeester, M. A. Posthumus & P. E. Brodelius (2000). Molecular Cloning, Expression, and Characterization of Amorpha-4,11-diene Synthase, a Key Enzyme of Artemisinin Biosynthesis in *Artemisia annua* L. *Archives of Biochemistry and Biophysics 381*(2), 173-180.

Moeller, L. & K. Wang (2008). Engineering with Precision: Tools for the New Generation of Transgenic Crops. *BioScience 58*(5), 391–401.

Nakano, K., C. Yoshida, W. Furukawa, Y. Takaishi & K. Shishido (1998). Terpenoids in transformed root culture of *Tripterygium wilfordii*. *Phytochemistry 49*(6), 1821-1824.

Nester, E. W., J. Kemmer, W. Deng, Y.-W. Lee, K. Fullner, X. Liang, S. Pan & J. D. Heath (1997). *Agrobacterium*: A natural genetic engineer exploited for plant biotechnology. 8th International Symposium on Molecular Plant-Microbe Interactions, Tennessee.

Nikaido, H. (1994). Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science 264*, 382–388.

Paek, K. Y., D. Chakrabarty & E. J. Hahn (2005). Application of bioreactor systems for large scale production of horticultural and medicinal plants. *Plant Cell, Tissue and Organ Culture 81*, 287–300.

Panizi, L., G. Flamini, P. L. Cioni & I. Morelli (1993). Composition and antimicrobial properties of essential oils of four Mediterranean Lamiaceae. *Journal of Ethnopharmacology 39*, 167–170.

Peters, R. J. (2006). Uncovering the complex metabolic network underlying diterpenoid phytoalexin biosynthesis in rice and other cereal crop plants. *Phytochemistry 67*(21), 2307-2317.

Peters, R. J. & R. B. Croteau (2004). Metabolic Engineering of Plant Secondary Metabolism. *Handbook of Plant Biotechnology*. P. Christou and H. Klee. Chichester, Jonh Wiley & Sons, Ltd.

Pichersky, E. & J. Gershenzon (2002). The formation and function of plant volatiles: perfumes for pollinator attraction and defense. *Current Opinion in Plant Biology* 5(3), 237-243.

Piers, K., J. Heath, X. Liang, K. Stephens & E. Nester (1996). *Agrobacterium tumefaciens*-mediated transformation of yeast. *Proceedings of the National Academy of Sciences of the United States of America 93*, 1613–1618.

Rao, S. R. & G. A. Ravishankar (2002). Plant cell cultures: Chemical factories of secondary metabolites. *Biotechnology Advances 20*, 101–153.

Rates, S. (2001). Plant as source of drugs. *Toxicon 39*, 603-613.

Riva, G. d. l., J. González-Cabrera, R. Vázquez-Padrón & C. Ayra-Pardo (1998). *Agrobacterium tumefaciens*: a natural tool for plant transformation. *EJB Electronic Journal of Biotechnology 1*(3), 1-16.

Rodriguez-Concepcion, M. & A. Boronat (2002). Elucidation of the Methylerythritol Phosphate Pathway for Isoprenoid Biosynthesis in Bacteria and Plastids. A Metabolic Milestone Achieved through Genomics. *Plant Physiol. 130*(3), 1079-1089.

Rohmer, M. (1999). The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. *Natural Products Reports 16*, 565-574.

Rossi, L., A. M. Bravo Angel, B. Tinland & B. Hohn (1996). The role of virulence proteins in nuclear

targeting and integration of T-DNA. *Crown Gall: Advances in Understanding Interkingdom Gene Transfer*. W. Ream and S. B. Gelvin. Minnesota, APS Press**:** 30-43.

Rout, G. R., S. Samantaray & P. Das (2000). In vitro manipulation and propagation of medicinal plants. *Biotechnology Advances 18*(2), 91-120.

Sangwan, N., A. Farooqi, F. Shabih & R. Sangwan (2001). Regulation of essential oil production in plants. *Plant Growth Regulation 34*, 3-21.

Saxena, P., I. Cole & S. Murch (2007). Approaches to Quality Plant Based Medicine: Significance of Chemical Profiling. *Applications of Plant Metabolic Engineering*. R. Verpoorte, A. W. Alfermann and T. S. Johnson**:** 311-330.

Shrawat, A. K., D. Becker & H. Lörz (2007). *Agrobacterium tumefaciens*-mediated genetic transformation of barley (*Hordeum vulgare* L.). *Plant Science 172*(2), 281-290.

Skandamis, P., K. Koutsoumanis, K. Fasseas & G.-J. E. Nychas (2001). Inhibition of oregano essential oil and EDTA on *Escherichia coli* O157:H7. . *Italian Journal Food Science 13*(1), 65–75.

Souret, F., P. Weathers & K. Wobbe (2002). The mevalonate-independent pathway is expressed in transformed roots of *Artemisia annua* and regulated by light and culture age. *In-vitro Cell Dev Biol-Plant 38*, 581-588.

Spencer, J. S., E. Dowd & W. Faas (1997). The genuiness of two mint essential oils. *Perfurmer & Flavorist 22*, 37-45.

Supartana, P., T. Shimizu, M. Nogawa, H. Shioiri, T. Nakajima, N. Haramoto, M. Nozue & M. Kojima (2006). Development of simple and efficient in Planta transformation method for wheat (*Triticum aestivum* L.) using *Agrobacterium tumefaciens*. *Journal of Bioscience and Bioengineering 102*(3), 162-170.

Tepfer, M. & F. Casse-Delbart (1987). *Agrobacterium rhizogenes* as a vector for transforming higher plants. *Microbiological Sciences 4*(1), 24-28.

Theis, N. & M. Lerdau (2003). The Evolution of Function in Plant Secondary Metabolites. *International Journal of Plant Sciences 164*(3 Suppl.), S93–S102.

Tholl, D., W. Boland, A. Hansel, F. Loreto, U. Röse & J.-P. Schnitzler (2006). Practical approaches to plant volatile analysis. *The Plant Journal 45*, 540-560.

Tholl, D., F. Chen, J. Gershenzon & E. Pickersky (2004). *Arabidopsis thaliana*, a model system for investigating volatile terpene biosynthesis, regulation, and function. *Secondary Metabolism in Model Systems*. J. Romeo, Elsevier. **38**.

Tingay, S., D. McElroy, R. Kalla, S. Fieg, M. Wang, S. Thornton & R. Brettell (1997). *Agrobacterium tumefaciens*-mediated barley transformation. *Plant Journal 11*, 1369–1376.

Tisserat, B., S. Vaughn & R. Silman (2005). Influence of Various Tissue Culture Technologies on Essential Oil Metabolism. *Plant Bioactives in Traditional Medicine*. D. Majumdar, J. Govil, V. Singh and R. Sharma. Houston, Studium Press, LLC. **9:** 321-334.

Trapp, S. & R. Croteau (2001). Genomic Organization of Plant Terpene Synthases and Molecular Evolutionary Implications. *Genetics 158*, 811-832.

Tripathi, L. & J. Tripathi (2003). Role of biotechnology in medicinal plants. *Tropical Journal of Pharmaceutical Research 2*(2), 243-253.

Tyagi, A. K. & A. Mohanty (2000). Rice transformation for crop improvement and functional genomics. *Plant Science 158*(1-2), 1-18.

Tzfira, T. & V. Citovsky (2006). *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. *Current Opinion in Biotechnology 17*, 1-8.

Ultee, A., E. P. W. Kets & E. J. Smid (1999). Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. *Appl. Environ. Microbiol. 65*, 4606–4610.

Vasil, I. (2008). A history of plant biotechnology: from the Cell Theory of Schleiden and Schwann to biotech crops. *Plant Cell Reports 27*, 1423–1440.

Veluthambi, K., A. Gupta & A. Sharma (2003). The current status of plant transformation technologies. *Current Science 84*(3), 368-380.

Veronese, P., X. Li, X. Niu, S. C. Weller, R. A. Bressan & P. M. Hasegawa (2001). Bioengineering mint crop improvement. *Plant Cell, Tissue and Organ Culture 64*, 133-144.

Verpoorte, R. (2000). Secondary Metabolism. *Metabolic Engineering of Plant Secondary Metabolism*. R. Verpoorte and A. Alfermann. Dordrecht, The Netherlands, Kluwer Academic Publishers**:** 1-30.

Verpoorte, R., A. Contin & J. Memelink (2002). Biotechnology for the production of plant secondary metabolites. *Phytochemistry Reviews 1*, 13–25.

Wink, M. (2003). Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry 64*, 3–19.

Zafar, R., V. Aeri & A. Datta (1992). Application of plant tissue and cell culture for production of secondary metabolites. *Fitoterapia LXIII*, 33-43.

Zhang, P., B. Xu, Y. Wang, Y. Li, Z. Qian, S. Tang, S. Huan & S. Ren (2008). *Agrobacterium tumefaciens*-mediated transformation as a tool for insertional mutagenesis in the fungus *Penicillium marneffei*. *Mycological Research 112*(8), 943-949.

Zupan, J., T. Muth, O. Draper & P. Zambryski (2000). The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. *The Plant Journal 23*(1), 11-28.

Zupan, J. R., V. Citovsky & P. Zambryski (1996). *Agrobacterium* VirE2 protein mediates nuclear uptake of single-stranded DNA in plant cells. *Proc. Natl. Acad. Sci. USA 93*, 2392-2397.

Chapter 2

HYPERICUM SP.: ESSENTIAL OIL COMPOSITION AND BIOLOGICAL ACTIVITIES

2.1- Chapter overview

Phytochemical characterization of several species of *Hypericum* genus has been studied for a long time. Several reviews, most of them on *H. perforatum*, have already been published concerning the characterization of alcoholic and water extracts as well as their biological activities (Bombardelli *et al.*, 1995; Nahrstedt *et al.*, 1997; Hölzl *et al.*, 2003). Studies on the essential oils of *H. perforatum* and other species of this genus have already been published, some of them reporting the positive biological activities of these essential oils. Additionally, variations on the essential oils of *Hypericum* species induced by seasonal variation, geographic distribution, phenological cycle and type of the organ in which essential oils are produced and/or accumulated have also been reported. However, so far, no review paper has been published gathering all the reported data on essential oils of plants of this genus. Thus in this chapter we intended to collect and summarize as many information as possible concerning composition and biological activities of essential oils of *Hypericum* species. This chapter was written by invitation of a book series editor to be a chapter of the book entitled Advances in Medicinal and Aromatic Plant Research.

Bombardelli, E. & P. Morazzoni (1995). *Hypericum perforatum*. *Fitoterapia 66*, 43-68.

Hölzl, J. & M. Petersen (2003). Culture and biotechnology of *Hypericum*. *Chemical constituents of* Hypericum *ssp.* E. Ernst. London and New York, Taylor & Francis**:** 77-93.

Nahrstedt, A. & V. Butterweck (1997). Biologically active and other chemical constituents of the herb of *Hypericum perforatum* L. *Pharmacopsychiatry 30*, 129-134.

2.2- Manuscript

This chapter comprises the following manuscript:

Ana P. Guedes, G. Franklin and Manuel Fernandes-Ferreira. *Hypericum* sp.: Essential oil composition and biological activities. **In: Advances in Medicinal and Aromatic Plant Research** (em edição), Devarajan Thangadurai D., Fernandes-Ferreira M. (Eds.). Regency Publications, New Delhi; Bioscience Publications, India; and ABD Publishers, Jaipur

Hypericum sp.: Essential oil composition and biological activities

A. P. Guedes, G. Franklin and M. Fernandes-Ferreira

CITAB- Centro de Investigação e de Tecnologias Agro-Alimentares e Biológicas, Department of Biology, University of Minho, Campus de Gualtar, 4720-057 Braga, Portugal

1- Introduction

Hypericum is an important genus of the family Clusiaceae (Guttiferae), which includes about 450 species of trees, shrubs and herbs. Some of the general morphological characteristics of this genus are: (i) opposite simple entire exstipulated leaves; (ii) (4-)5-merous flowers consisting of green sepals and free yellow petals (occasionally, the sepals as well as the petals can be red-tinged); (iii) free of more or less united ovary and; (iv) stamens organized in 5 fascicles. The main feature of some of the important species is the presence of translucent and often black or red schizogenous hypericin glands. Although, these glands are present in the plant parts like stem, flowers and leaves, their distribution differ between species. Hypericin was first identified in *Hypericum* species and serves as a chemotaxonomic marker of the genera. For more details about the taxonomy and distribution of this genus, refer Robson (2006) (Robson, 2006).

Many species of the *Hypericum* genus have a long traditional value as medicinal plants. Ancient Greeks knew about the medicinal properties of this genus. Greek herbalist Dioscorides recommends four species of *Hypericum* (Upericon, Askuron, Androsaimon, and Koris) for sciatica and burns. He referred that *H. crispum* and *H. barbatum* have antidiuretic and antimalarial properties. Theophrastus recommends *H. lanuginosum* for external application, while Pliny says it should be taken in wine against poisonous reptiles. *H. coris*, another Greek species, was mentioned by Hippocrates and Pliny. Yet another species, *H. perforatum* (St. John's wort) has been appreciated for its medicinal value since at least as early as the 1st century A.D. and was known by Hippocrates, Pliny, and Dioscorides who included it in the *De materia medica* (Bombardelli *et al.*, 1995; Upton, 1997)

Currently, several species of this genus has been used in ailments as knowledge-based medicine in many countries. In Portuguese folk medicine, some *Hypericum* species are used the major of them being *H. androsaemum*, *H. perforatum*, and *H. undulatum*. *H. androsaemum* (Hipericão do Gerês) is known for its diuretic effect and infusions are used for kidney and

bladder ailments, whereas *H. undulatum* extract is used for liver problems. Flowers decoction of *H. undulatum* is also traditionally used for migraine, bladder ailments; and as renal antispasmodic and hepatic protector (Ferreira *et al.*, 2006). Moreover, decoctions of the flowering aerial parts of *H. perforatum* have been used to treat liver troubles (Camejo-Rodrigues *et al.*, 2003), depression, liver disorders and rheumatism (Nogueira *et al.*, 1998). In the Canary Islands (Spain), infusions prepared from the flowers, leaves and fruits of various species of the genus *Hypericum* have been used as a vermifuge, diuretic, as well as a wound healing, sedative, antihysterical and antidepressive agent (Prado *et al.*, 2002; Rabanal *et al.*, 2002). In Italy, leaves and stems of *H. hircinum* have been widely used against cough (Pieroni *et al.*, 2004). *H. perforatum*, a well-known species of this genus, has been included in the traditional pharmacopeia of many countries. For instance, in the 1997 German List of Prescription Drugs described more than 50 preparations from *H. perforatum*. In the German Drug Prescription Report 1996, about 131 million of daily doses of products prepared from this species were prescribed. In Serbia, aerial parts of *H. perforatum* were cited to be used internally and externally for all ailments (Jaric *et al.*, 2007). Different parts of the plant and different preparations are externally applied or orally administrated for several ailments, including digestive, urinary, respiratory and cardiac diseases in Turkey (Kültür, 2007). In Bulgarian phytotherapy, aerial parts of *H. perforatum* have been recommended for treatment of gastric and duodenal ulcer; as regenerative and anti-inflammatory agent in digestive tract diseases and as epithelotonic agent (Ivanova *et al.*, 2005). In Italy, leaves and stems of *H. hircinum* have been widely used against cough (Pieroni *et al.*, 2004).

In the last decade, several pharmacological studies have been performed using crude extracts to evaluate the traditional knowledge. Results of those studies have revealed that extract of *Hypericum* exert several pharmacological properties including antidepressant, antimicrobial and wound healing effects (Ishiguro *et al.*, 1990; Decosterd *et al.*, 1991; Jayasuriyab *et al.*, 1991; Rocha *et al.*, 1995; Öztürk *et al.*, 1996; Sokmen *et al.*, 1999; Daudt *et al.*, 2000; Mukherjee *et al.*, 2000; Pistelli *et al.*, 2000; Avato *et al.*, 2004).

Plants produce and accumulate different types of compounds. Molecules with an important role in basic life functions such as cell division, growth, respiration, storage, and reproduction were described as primary metabolites (Bougard *et al.*, 2001). Several other compounds which are not essential for the above functions are known as secondary metabolites. For a long time, secondary metabolites have been considered as waste by-products of plant metabolism. Recent improvement of biochemical techniques and the rise of molecular biology have shown that these molecules play a major role in the adaptation of plants to their

environment including biotic and abiotic challenges. They also act on animals (anti-feeding properties), pathogens (phytoalexins) and other plants (allelopathy) (Bougard *et al.*, 2001). Plant secondary metabolites include a vast array of compounds that to date sum up to more than 200,000 defined structures.

Studies on medicinal plants are rapidly increasing because of the search for new active molecules, and for the improvement in the production of plants or molecules for the herbal pharmaceutical industries (Poutaraud *et al.*, 2005). As a genus with several medicinal plant species, *Hypericum* has recently drawn the attention of phytochemists and pharmacologists. However, it is clear from the literature that *H. perforatum* is the only most characterized species of this genus in terms of phytochemistry and pharmacology. Under the stimulus of great scientific interest and economic value acquired by *H. perforatum*, studies with other plants of *Hypericum* genus have also been carried out revealing their antidepressant, analgesic, anti-inflammatory, antioxidant, antimicrobial and wound healing properties (Öztürk *et al.*, 1996; Apaydin *et al.*, 1999; Daudt *et al.*, 2000; Mukherjee *et al.*, 2001; Trovato *et al.*, 2001; Öztürk *et al.*, 2002; Prado *et al.*, 2002; Rabanal *et al.*, 2002; Sánchez-Mateo *et al.*, 2002; Cakir *et al.*, 2003; Couladis *et al.*, 2003; Heilmann *et al.*, 2003; Rabanal *et al.*, 2005).

Phytochemical characterisation of several species of this genus have revealed the presence of a variety of compounds, but not limited to phenylpropanes, flavonol derivatives, biflavones, proanthocyanidines, xanthones, phloroglucinols and naphtodianthrones [for review see (Bombardelli *et al.*, 1995; Nahrstedt *et al.*, 1997; Hölzl *et al.*, 2003)] and essential oils. In spite of the large size of *Hypericum* genus, the composition of volatile compounds is known in only a small number of species, the most studied of which is *H. perforatum* (Saroglou *et al.*, 2007). The present review summarizes information available on the chemical composition of essential oils isolated from *Hypericum* genus. Additionally, the biological activities of this genus with special emphasis on essential oil composition are also discussed.

2- Chemical Composition of *Hypericum* Essential Oils

Interest in essential oils has revived in recent decades, with the popularity of aromatherapy, a branch of alternative medicine which claims that the specific aromas carried by essential oils have curative effects. Oils are volatilized or diluted in a carrier oil and used in massage or burned as incense. About 300 essential oils out of 3000 known are commercially important mainly for their flavours and fragrances (Burt, 2004).

Essential oils are aromatic oily extracts obtained from the plants. Composition of essential oils can differ between different parts of the plants. Steam distillation is the most commonly used method of extraction for commercial production of essential oils (Burt, 2004), although other extraction methods such as expression, fermentation, enfleurage or extraction are also in practice. In the steam distillation, the water is heated and the steam passes through the plant material, thus vaporizing the volatile compounds. The vapors flow through a coil where they condense back to liquid, which is then collected in the receiving vessel. Because of the mode of extraction, mostly by distillation from aromatic plants, the extract contains a variety of volatile molecules such as terpenes and terpenoids, phenol-derived aromatic components and aliphatic components (Bakkali *et al.*, 2008).

Essential oils are very complex natural mixtures which can contain about 20–60 components at quite different concentrations. Hence, they are generally characterized by two or three major components at fairly high concentrations compared to other components that are present in trace amount (Bakkali *et al.*, 2008).

Characterization of essential oils from species of *Hypericum* genus revealed the presence of monoterpenoid and sesquiterpenoid compounds, as well as alkanes and aldehydes as the main compounds in the most of them (Mathis *et al.*, 1964a; Mathis *et al.*, 1964b; Mathis *et al.*, 1964c).

(E)-Caryophyllene was one of the most represented compounds in most of the studied *Hypericum* essential oils. Germacrene D was another major sesquiterpene hydrocarbon common to several species. Caryophyllene oxide, spathulenol and globulol, are the oxygenated-sesquiterpenes present in the top three most represented compounds of some *Hypericum* species. Likewise, α-pinene and β-pinene were the two mostly represented monoterpene hydrocarbons in essential oils from *Hypericum*. Interestingly, in *H. barbatum* the three most represented compounds are monoterpene hydrocarbons (α-pinene, β-pinene and limonene) (Saroglou *et al.*, 2007). In the essential oils of *H. ericoides*, α-curcumene, α-pinene, γ-muurolene and δ-cadinene were the most represented compounds (Cardona *et al.*, 1983). *n*-Alkanes were found as the major compounds in few species of the genus. In *H. foliosium*, *H. hirsutum*, *H. myrianthum*, *H. richeri* and *H. triquetrifolium*, *n*-nonane is among the three most represented compounds. Some of these species also had high proportions of *n*-undecane (Santos *et al.*, 1999; Bertoli *et al.*, 2003; Ferraz *et al.*, 2005; Ferretti *et al.*, 2005; Saroglou *et al.*, 2007). The main essential oil constituents identified in *Hypericum* species are summarised in Table 1.

Table 1 - Main essential oil constituents of *Hypericum* species.

Species	Origin	Main constituents	Reference
H. alpinum	Serbia	*β*-Pinene (13.3%) *γ*-Terpinene (7.7%) (-)-(*E*)-Caryophyllene (6.5%)	(Saroglou *et al.*, 2007)
H. acmosepalum	China	*β*-Selinene (16.3%) *ar*-Curcumene (12.6%)	(Demirci *et al.*, 2005)
H. aegypticum* ssp. *aegypticum	Libya	Ishwarane (14.4%) Eudesm-11-en-4-ol stereoisomer (9.6%) Eudesm-11-en-4-ol stereoisomer (10.7%)	(Crockett *et al.*, 2007)
H. aegypticum* ssp. *marrocanum	Northwestern Africa	Caryophyllene oxide (29.2%) *β*-Caryophyllene (15.1%) Caryophylladienol-II (9.7%)	(Crockett *et al.*, 2007)
H. androsaemum	Portugal	$C_{15}H_{24}$ (27.6%) Germacrene D (12.3%) *β*-Caryophyllene (14.0%)	(Nogueira *et al.*, 1998)
	Portugal	(*E*)-Caryophyllene (9.4-15.1%) *γ*-Elemene (8.0-17.9%) *β*-Gurjunene (6.1-15.5%)	(Guedes *et al.*, 2003)
	Portugal	(*E*)-Caryophyllene (9.0-17.0%) *γ*-Elemene (9.3-17.3%) *β*-Gurjunene (7.9-14.8%)	(Guedes *et al.*, 2004)
H. balearicum	Balearic Islands	*α*-Pinene (28.5%) *β*-Pinene (20.4%) *β*-Eudesmol (11.2%)	(Crockett *et al.*, 2007)
H. barbatum	Serbia	(-)-*α*-Pinene (17.1%) (-)-*β*-Pinene (17.0%) (-)-Limonene (6.0%)	(Saroglou *et al.*, 2007)
H. beanii	China	Caryophyllene oxide (18.7%) *β*-Selinene (16.3%) *γ*-Muurolene (11.3%)	(Demirci *et al.*, 2005)

Species	Origin	Main constituents	Reference
H. brasiliense	Brazil	β-Caryophyllene (29.5%) α-Humulene (12.7%) Caryophyllene oxide (9.9%)	(Abreu *et al.*, 2004)
H. bupleuroides	Turkey	β-Sesquiphellandrene (33.2%) β-Caryophyllene (20.2%) Selina-3,7(11)-diene (7.0%)	(Demirci *et al.*, 2006)
H. calcynum	China	β-Pinene (29.2%) α-Terpineol (11.5%)	(Demirci *et al.*, 2005)
H. carinatum	Brazil	β-Caryophyllene (21.0%) α-*trans*-Bergamotene (10.0%) Caryophyllene oxide (9.5%)	(Ferraz *et al.*, 2005)
H. choisyanum	China	*cis*-Eudesma-6,11-diene (11.4%)	(Demirci *et al.*, 2005)
H. connatum	Brazil	Caryophyllene oxide (40.1%) β-Caryophyllene (13.1%) Humulene oxide II (10.5%)	(Ferraz *et al.*, 2005)
H. coris	France	α-Curcumene (40.1%) γ-Cadinene (14.7%) δ-Cadinene (6.6%)	(Schwob *et al.*, 2002)
H. delphicum	Arabian penninsula	Caryophyllene oxide (31.5%) β-Caryophyllene (18.2%) *n*-Undecane (17.5%)	(Crockett *et al.*, 2007)
H. foliosum	Portugal	*n*-Nonane (28.7-72.6%) Limonene (6.9-45.8%) Terpinolene (0.5-18.8%) β-Caryophyllene (1.1-6.9%)	(Santos *et al.*, 1999)
H. forrestii	China	Caryophyllene oxide (12.7%) α-Pinene (10.4%)	(Demirci *et al.*, 2005)
H. heterophyllum	Turkey	Isocaryophyllene (17.1%) α-Pinene (11.6%) δ-Cadinene (9.5%)	(Cakir *et al.*, 2004)

Species	Origin	Main constituents	Reference
H. hirsutum	Serbia	*n*-Nonane (24.8%) Undecane (13.3%) (-)-(*E*)-Caryophyllene (5.4%)	(Saroglou *et al.*, 2007)
	Serbia	*n*-Undecane (32.2%) Patchoulene (11.8%) Caryophyllene oxide (9.3%)	(Gudžic *et al.*, 2007)
H. humifusum	Portugal	α-Pinene (80.6%) β-Pinene (4.7%) Germacrene D (2.1%)	(Nogueira *et al.*, 1998)
	Portugal	α-Pinene (44.7-77.2%) β-Pinene (4.7-7.7%) β-Caryophyllene (1.2-9.3%) Germacrene D (1.9-6.1%)	(Nogueira *et al.*, 2008)
H. kouytchense	China	*cis*-β-Guaiene (10.7%) γ-Muurolene (12.4%)	(Demirci *et al.*, 2005)
H. lancasteri	China	β-Selinene (11.4%) Eudesmadienone (10.8%)	(Demirci *et al.*, 2005)
H. leschenaultii	China	Cuparene (24.8%) γ-Muurolene (16.8%)	(Demirci *et al.*, 2005)
H. linarifolium	Portugal	α-Pinene (31.1%) β-Caryophyllene (11.6%) *n*-Undecane (7.0%)	(Nogueira *et al.*, 1998)
	Portugal	α-Pinene (19.9-31.2%) β-Pinene (5.0-11.0%) β-Caryophyllene (6.6-11.6%)	(Nogueira *et al.*, 2008)
H. linarioides	Turkey	δ-Cadinene (6.9%) γ-Muurolene (5.5%) (*Z*)-β-Farnesene (5.2%)	(Cakir *et al.*, 2005)
H. lysimachioides	South eastern Anatolia	Caryophyllene oxide (30.8%) α-Longifolene (6.4%) β-Selinene (6.7%)	(Toker *et al.*, 2006)

Species	Origin	Main constituents	Reference
H. maculatum	Serbia	Spathulenol (6.8%) Globulol (10.2%) *n*-Nonane (5.5%)	(Saroglou *et al.*, 2007)
H. monogynum	China	*n*-Tricosane (13.3%) Myrcene (10.4%)	(Demirci *et al.*, 2005)
H. myrianthum	Brazil	*n*-Undecane (20.7%) *n*-Nonane (17.5%) Dehydro-aromadendrene (8.6%)	(Ferraz *et al.*, 2005)
H. olympicum	Serbia	(*E*)-Anethole (30.7%) *β*- Farnesene (12.4%) *δ*-Cadinene (8.7%)	(Gudžić *et al.*, 2001)
	Greece	Germacrene D (16.0%) (*E*)-Caryophyllene (7.4%) Spathulenol (6.7%)	(Pavlović *et al.*, 2006)
H. patulum	China	*β*-selinene (14.7%)	(Demirci *et al.*, 2005)
H. polyanthemum	Brazil	HP1 Benzopyrans (26.7%) HP2 Benzopyrans (13.2%) *n*-Undecane (7.9%)	(Ferraz *et al.*, 2005)
H. pseudohenryi	China	*β*-Selinene (18.5%)	(Demirci *et al.*, 2005)
H. pulchrum	Portugal	*α*-Pinene (35.7-49.8%) *β*-Pinene (9.0-12.5%) Germacrene D (2.4-5.4%)	(Nogueira *et al.*, 1998)
	Portugal	*α*-Pinene (49.8%) *β*-Pinene (12.5%) Germacrene D (5.4%)	(Nogueira *et al.*, 2008)
H. richeri	Italy	(*Z*)-*β*-Ocimene (19.5%) *n*-Nonane (13.8%) *β*-Bisabolene (8.7%)	(Ferretti *et al.*, 2005)
H. roeperanum	East Africa	*γ*-Curcumene (15.6%)	(Crockett *et al.*, 2007)

Species	Origin	Main constituents	Reference
		(2*E*,6*E*)-Farnesol (7.8%) *ar*-Curcumene (7.7%)	
H. scabrum	Uzbekistan	α-Pinene (11.2%) Spathulenol (7.2%) *p*-Cymene (6.1%)	(Baser *et al.*, 2002)
H. ternum	Brazil	β-Caryophyllene (12.0%) Bicyclogermacrene (10.0%) β-Cadinene (5.0%)	(Ferraz *et al.*, 2005)
H. tetrapterum	Greece	α-Copaene (11.3%) α-Longipinene (9.7%) Caryophyllene oxide (8.9%)	(Pavlović *et al.*, 2006)
H. tomentosum	Portugal	β-Caryophyllene (12.6%) *n*-Undecane (7.5%) α-Humulene (5.2%)	(Nogueira *et al.*, 1998)
H. xmoserianum	China	γ-Muurolene (10.7%) δ-Cadinene (10.2%)	(Demirci *et al.*, 2005)
H. tomentosum	Tunisia	Menthone (17.0%) *n*-Octane (9.9%) β-Caryophyllene (5.3%) α-Pinene (5.2%)	(Hosni *et al.*, 2008)
H. triquetrifolium	Italy	α-Humulene *cis*-Calamene δ-Cadinene α-Pinene (10.33%) Caryophyllene oxide (1.38%)	(Karim *et al.*, 2007)

Essential oils content and composition can be greatly affected by several parameters including seasonal variation (Guedes *et al.*, 2004), phenological cycle (Schwob *et al.*, 2004) and geographic distribution. We have summarised the most represented compounds of the essential oils of *H. perforatum*, *H. perfoliatum* and *H hyssopifolium* based on their geographical distribution in Table 2.

Table 2 - Variation in the essential oil composition of a few *Hypericum* species based on their geographical distribution

Species	Origin	Main constituents of essential oil	Reference
H. perforatum	Serbia	*cis*-Caryophyllene (48.5%) *β*- Farnesene (12.1%) 2-Methyl-dodecane (5.7%)	(Gudžić *et al.*, 1997)
	Serbia	*β*-Caryophyllene (14.2%) 2-Methyl-octane (13.1%) 2-Methyl-decane (7.9%)	(Gudžić *et al.*, 2001)
	Serbia	*α*-Pinene (8.6%) (*Z*)-*β*- Farnesene (6.6%) Germacrene D (6.8%)	(Saroglou *et al.*, 2007)
	Portugal	Germacrene D (20.0%) *β*-Caryophyllene (10.9%) 2-Methyl-octane (9.7%)	(Nogueira *et al.*, 1998)
	Uzbekistan	*β*-Caryophyllene (11.7%) Caryophyllene oxide (6.3%) Spathulenol (6.0%)	(Baser *et al.*, 2002)
	Lithuania	Caryophyllene oxide (6.1-35.8%) Germacrene D (4.5- 31.5%) *β*-Caryophyllene (5.1-19.1%)	(Mockutė *et al.*, 2003)
	Italy	2-Methyl-octane (21.1%) Germacrene D (17.6%) *α*-Pinene (15.8%)	(Pintore *et al.*, 2005)
	Greece	*α*-Pinene (21.0%) 2-Methyl-octane (12.6%) *γ*-Muurolene (6.9%)	(Pavlović *et al.*, 2006)
	India	Ishwarane *α*-cuprenene	(Weyerstahl *et al.*, 1995)
H. perfoliatum	Portugal	*α*-Pinene (50.0%) *n*-Nonane (16.8%) *n*-Undecane (8.8%)	(Nogueira *et al.*, 1998)

Species	Origin	Main constituents of essential oil	Reference
H. perfoliatum (cont.)	Portugal	α-Pinene (39.4-64.3%) *n*-Nonane (11.9-23.8%) β-Pinene(1.9-3.2%)	(Nogueira *et al.*, 2008)
	Algeria	Thymol (22.1%) τ-Cadinol (18.5%) 4,5-Dimethyl-2-ethylphenol (13.0%)	(Touafek *et al.*, 2005)
	Greece	α-Pinene (48.6%) *n*-Nonane (8.5%) δ-Cadinene (4.6%)	(Couladis *et al.*, 2001)
	Greece	α-Pinene (34.2%) β-Pinene (9.2%) δ-Cadinene (8.1%)	(Couladis *et al.*, 2001)
	Greece	α-Pinene (41.3%) β-Pinene (6.5%) δ-Cadinene (6.2%) *n*-Nonane (6.1%)	(Petrakis *et al.*, 2005)
	Tunisia	α-Pinene (13.1%) *allo*-Aromadendrene (11.4%) Germacrene-D (10.6%)	(Hosni *et al.*, 2008)
H. hyssopifolium	South eastern Anatolia	Caryophyllene oxide (20.4%) Spathulenol (13.4%) Caryophyllene alcool (9.0%)	(Toker *et al.*, 2006)
	Turkey	α-Pinene (57.3%) β-Pinene (9.0%) Limonene (6.2%)	(Cakir *et al.*, 2004)
	France	Spathulenol (19.5 %) Tetradecanol (10.2%) Dodecanol (9.3%)	(Schwob *et al.*, 2006)

(E)-Caryophyllene and germacrene D were abundant in essential oils of *H. perforatum* from Portugal and Lithuania, but not in *H. perforatum* growing wild in Greece. Gudžić and co-workers (1997; 2001) reported *(E)*-caryophyllene as the most represented compound in essential oils of *H. perforatum* from Rujan mountain and Vlasina, both in Serbia (Gudžić *et al.*, 1997; Gudžić *et al.*, 2001). However this sesquiterpene did not take part of the three major compounds in the essential oils of this species in Barelic, another region of Serbia (Saroglou *et al.*, 2007). α-Pinene predominance was registered in the essential oils from *H. perforatum* plants cultivated in Italy, Greece and Serbia (Barelic). Essential oils from *H. perforatum* growing wild in Lithuania and Uzbekistan were the only ones in which oxygenated-sesquiterpenes were the major compounds, caryophyllene oxide and spathulenol in those from Uzbekistan and caryophyllene oxide in those from Lithuania. In the essential oils of *H. perforatum* L. from North Indian, ishwarane and α-cuprenene were identified (Weyerstahl *et al.*, 1995). In essential oils of *H. perfoliatum* from Portugal, Greece and Tunisia some homogeneity was found in the major compounds. In all of them α-pinene was one of the major compounds, with *n*-nonane and β-pinene common to some of them. However, data on essential oils from plants cultivated in Algeria show a different composition regarding the three most represented compounds, without any mono- or sesquiterpene hydrocarbon between them. Essential oils of *H. hyssopifolium* from south eastern Anatolia and France shared the predominance of the oxygenated-sesquiterpene spathulenol. However, in Turkey the three most represented compounds in this species were monoterpene hydrocarbons, with α-pinene as the main constituent. Differences in the essential oils profile of plants cultivated in different locations can be attributed to different climatic and pedological conditions.

As shown in Table 3 *Hypericum* essential oil profile could also be dependent on the plant organs from which they are extracted. For example, essential oils extracted from flowers and leaves of *H. triquetrifolium* vary greatly in their composition. Flowers have a high representation of monoterpene hydrocarbons, whereas they are absent in the essential oils of leaves. On the other hand, although the essential oils composition of leaves and flowers were similar, the main constituent, caryophyllene oxide, varied in the concentration in both *H. androsaemum* and *H. perforatum*. Similarly, leaves and flowers of *H. caprifoliatum* varied in the nonane and *(E)*-caryophyllene concentrations.

Table 3 - Organ dependent variation in the essential oil composition in some *Hypericum* species.

Species	Main constituents of the essential oils		Reference
	Leaves/ vegetative part	**Flowers/ flowering top**	
H. androsaemum	Caryophyllene oxide (35.8%) Ishwarane (30.5%) Humulene epoxide II (5.6%)	α-Guaiene (40.2%) Caryophyllene oxide (28.0%) Khusinol (4.2%)	(Morteza-Semnani *et al.*, 2005)
H. caprifoliatum	Nonane (44.6%) β-Caryophyllene (11.2%) Bicyclogermacrene (5.6%)	Nonane (55.8%) β-Caryophyllene (5.9%) Undecane (5.0%)	(Ferraz *et al.*, 2005)
H. perforatum	Caryophyllene oxide (9.3-25.9%) Spathulenol (6.4-15.7%) Tetradecanol (1.1-24.5%)	Caryophyllene oxide (7.7-34%) β-caryophyllene (4.2-14.2%) Viridiflorol (4.5-11%)	(Radusiene *et al.*, 2005)
H. triquetrifolium	Nonane (14.7%) Germacrene D (12.7%) β-caryophyllene (10.9%)	Myrcene (16.4%) α-Pinene (13.3%) Sabinene (13.1%)	(Bertoli *et al.*, 2003)

3- Biological Activities of *Hypericum* Essential Oils

Since the middle ages, essential oils have been widely used for bactericidal, virucidal, fungicidal, antiparasitical, insecticidal, medicinal and cosmetic applications (Bakkali *et al.*, 2008). Nowadays, essential oils are used in pharmaceutical, sanitary, cosmetic, agricultural and food industries (Burt, 2004).

Although *Hypericum* extracts have been studied in terms of their biological activities, very few studies have so far been performed with essential oils. Antimicrobial activities have been the most reported biological activities for the essential oils of *Hypericum* species.

3.1- Antibacterial activity

Essential oils extracted from the flowers of *H. perforatum* grown in the Vlasina region, Serbia had antibacterial activity against *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Sarcina lutea, Bacillus subtilis 841, Salmonella enteritidis* and *Klebsiella pneumoniae* (Gudžić *et al.*, 1997). However, no activity was found against *Pseudomonas aeruginosa*. The volatile fraction of *H. coris* aerial parts, consisting mainly of α-curcumene showed moderate antibacterial activity against *Staphylococcus aureus* whereas, no activity was found against *Escherichia coli* and *Enterococcus hirae* (Schwob *et al.*, 2002). On the other hand, moderate growth inhibitory activity was found in the essential oils of *H. hyssopifolium* against *E. hirae* and *S. aureus*, while no activity was detected against *E. coli* (Schwob *et al.*, 2006). Essential oils of *H. lysimachioides* and *H. hyssopifolium* were highly effective in growth inhibition of nine microorganisms (*Escherichia coli K12, E. coli PBR 322, E. coli PUC 9, Bacillus brevis ATCC, B. cereus DMC65, Streptococcus pyogenes DMC41, Pseudomonas aeruginosa DMC66,* and *Staphylococcus aureus DMC70*) at a concentration of 60 to 80 μg (Toker *et al.*, 2006). Essential oil isolated from the aerial parts of *H. rumeliacum* exhibited moderate activities against all the tested bacteria (*S. aureus*, *S. epidermidis*, *E. coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*), with a minimal inhibitory concentration (MIC) of 3.80–17.20 mg/ml (Couladis *et al.*, 2003). The *H. rumeliacum* essential oil showed its highest activity against a Gram-negative strain of *E. coli,* while *E. cloaceae* appeared to be the most resistant. According to the authors, the antibacterial properties of *H. rumeliacum* oil could be associated with the high percentage of α-pinene and β-pinene.

In a recent study, essential oils of six *Hypericum* species (*H. alpinum*, *H. barbatum*, *H. rumeliacum*, *H. hirsutum*, *H. maculatum* and *H. perforatum*) were tested against several bacteria (*Escherichia coli* strain ATCC 35218, *Proteus mirabilis*, *Agrobacterium tumefaciens*, *Pseudomonas aeruginosa*, *Pseudomonas tolaasii*, *Salmonella enteritidis* strain ATCC 13076, *Staphylococcus aureus* strain ATCC 6538, *Micrococcus luteus*, *Sarcina lutea* strain ATCC 9341 and *Bacillus cereus*) (Saroglou *et al.*, 2007). They found that *H. barbatum* essential oil was proved to be the most active, while the essential oils of *H. alpinum* and *H. hirsutum* were inactive against the clinical species of *P. mirabilis* and *P. aeruginosa*.

3.2- Antifungal activity

Essential oil of *H. perforatum* flowers was found to be effective against the fungus *Aspergillus niger*, but not against *Candida albicans* (Gudžić *et al.*, 1997). Similarly, *H. coris* essential oil consisting mainly of α-curcumene did not show any antimicrobial activity against *Candida albicans*, whereas antimicrobial activity was found against *Saccharomyces cerevisiae* (Schwob et al., 2002). Couladis and co-workers tested the activity of essential oil isolated from the aerial parts of *H. rumeliacum* against three fungi (*Candida albicans*, *C. tropicalis*, and *C. glabrata*) reporting a MIC value of 4.75–6.34 mg/ml (Couladis *et al.*, 2003). Essential oils of six *Hypericum* species were also tested against *Candida albicans* (Saroglou et al., 2007). Among them, *H. barbatum*, *H. rumeliacum*, *H. maculatum* and *H. perforatum* essential oils were effective. The MIC for *H. barbatum* and *H. rumeliacum* was 25 µg/ml whereas, *H. maculatum* and *H. perforatum* showed an MIC of 50 µg/ml, whereas *H. alpinum* and *H. hirsutum* essential oils were inactive (Saroglou *et al.*, 2007).

Cakir and co-workers tested the essential oils of three species of *Hypericum* against several phytopathogenic fungi using agar diffusion method for the possible use in agricultural pest and disease control. A moderate antifungal activity of *H. hyssopifolium* and *H. heterophyllum* was found against *Fusarium acuminatum,* and *Rhizoctonia solani* (strains AG-5 and AG-11) (Cakir *et al.*, 2004). *H. linarioides* essential oils was active against *Rhizoctonia solani* strain AG-9 and *Verticillium albo-atrum* (Cakir et al., 2005).

Although the antimicrobial activity of essential oils from many plant species has been extensively surveyed, their accurate mechanism of action has not been reported in great details. However, it is thought that it might involve membrane disruption by lipophilic compounds, such as terpenoids (Cowan, 1999). It has been demonstrated that α-pinene and β-pinene are able to destroy cellular integrity, and thereby, inhibit respiration and ion transport processes, and they can also increase the membrane permeability in yeast cells and isolated mitochondria (Andrews *et al.*, 1980; Uribe *et al.*, 1985). Some authors postulated a relative inactivity of hydrocarbons, which is correlated to their limited hydrogen capacity and water solubility (Griffin *et al.*, 1999). On the other hand, besides being active, ketones, aldehydes and alcohols have different specificity and activity levels. These facts seem to be associated to the functional group and with hydrogen-bonding parameters. Indeed, it seems that there is a relationship between the compounds' chemical structure and the antimicrobial activities they exert (Griffin *et al.*, 1999). Several data suggest that a great antimicrobial potential could be ascribed to the oxygenated terpenes (Panizi *et al.*, 1993; Adam *et al.*, 1998; Saroglou *et al.*, 2007). A summary of antimicrobial activities of the essential oils of some *Hypericum* species is shown in Table 4.

Table 4a - Antimicrobial activities of *Hypericum* essential oils, determined in different *in vitro* assays as the Minimum Inhibitory Concentration (MIC), performed on standard microorganisms

***Hypericum* sps**	**Test organism**	**Antimicrobial activity (MIC)**	**Reference**
H. alpinum	*Escherichia coli*	50.0 µg/ml	(Saroglou *et al.*, 2007)
	Proteus mirabilis	no activity	
	Agrobacterium tumefaciens	25.0 µg/ml	
	Pseudomonas aeruginosa	no activity	
	Pseudomonas tolaasii	50.0 µg/ml	
	Salmonella enteritidis	50.0 µg/ml	
	Staphylococcus aureus	12.5 µg/ml	
	Micrococcus luteus	12.5 µg/ml	
	Sarcina lutea	12.5 µg/ml	
	Bacillus cereus	12.5 µg/ml	
	Candida albicans	no activity	
H. barbatum	*Escherichia coli*	25.0 µg/ml	(Saroglou *et al.*, 2007)
	Proteus mirabilis	50.0 µg/ml	
	Agrobacterium tumefaciens	25.0 µg/ml	
	Pseudomonas aeruginosa	50.0 µg/ml	
	Pseudomonas tolaasii	25.0 µg/ml	
	Salmonella enteritidis	25.0 µg/ml	
	Staphylococcus aureus	6.25 µg/ml	
	Micrococcus luteus	6.25 µg/ml	
	Sarcina lutea	6.25 µg/ml	
	Bacillus cereus	6.25 µg/ml	
	Candida albicans	25.0 µg/ml	
H. canariense	*Bacillus cereus* var. *mycoides*	0.05 mg/ml	(Rabanal *et al.*, 2005)
	Micrococcus luteus	0.05 mg/ml	

***Hypericum* sps**	**Test organism**	**Antimicrobial activity (MIC)**	**Reference**
H. canariense	*Staphylococcus aureus*	0.05 mg/ml	
(cont)	*Staphylococcus epidermis*	0.11 mg/ml	
	Bordetella bronchiseptica	0.11mg/ml	
H. coris	*Escherichia coli*	no activity	(Schwob *et al.*, 2002)
	Enterococcus hirae	no activity	
	Staphylococcus aureus	CI 100µg/ml	
	Candida albicans	no activity	
	Saccharomyces cerevisiae	CI 100µg/ml	
H. glandulosum	*Bacillus cereus* var. *mycoides*	0.22 mg/ml	(Rabanal *et al.*, 2005)
	Micrococcus luteus	0.15 mg/ml	
	Staphylococcus aureus	0.09 mg/ml	
	Staphylococcus epidermis	0.22 mg/ml	
	Bordetella bronchiseptica	0.11mg/ml	
H. grandifolium	*Bacillus cereus* var. *mycoides*	0.05 mg/ml	(Rabanal *et al.*, 2005)
	Micrococcus luteus	0.11 mg/ml	
	Staphylococcus aureus	0.09 mg/ml	
	Staphylococcus epidermis	0.15 mg/ml	
	Bordetella bronchiseptica	0.18 mg/ml	
H. hirsutum	*Escherichia coli*	50.0 µg/ml	(Saroglou *et al.*, 2007)
	Proteus mirabilis	no activity	
	Agrobacterium tumefaciens	50.0 µg/ml	
	Pseudomonas aeruginosa	no activity	
	Pseudomonas tolaasii	50.0 µg/ml	
	Salmonella enteritidis	50.0 µg/ml	
	Staphylococcus aureus	25.0 µg/ml	
	Micrococcus luteus	25.0 µg/ml	

***Hypericum* sps**	**Test organism**	**Antimicrobial activity (MIC)**	**Reference**
H. hirsutum	*Sarcina lutea*	12.5 μg/ml	
(cont.)	*Bacillus cereus*	12.5 μg/ml	
	Candida albicans	no activity	
H. maculatum	*Escherichia coli*	25.0 μg/ml	(Saroglou *et al.*, 2007)
	Proteus mirabilis	50.0 μg/ml	
	Agrobacterium tumefaciens	25.0 μg/ml	
	Pseudomonas aeruginosa	25.0 μg/ml	
	Pseudomonas tolaasii	25.0 μg/ml	
	Salmonella enteritidis	25.0 μg/ml	
	Staphylococcus aureus	12.5 μg/ml	
	Micrococcus luteus	12.5 μg/ml	
	Sarcina lutea	12.5 μg/ml	
	Bacillus cereus	12.5 μg/ml	
	Candida albicans	50.0 μg/ml	
H. perforatum	*Escherichia coli*	25.0 μg/ml	(Saroglou *et al.*, 2007)
	Proteus mirabilis	50.0 μg/ml	
	Agrobacterium tumefaciens	25.0 μg/ml	
	Pseudomonas aeruginosa	50.0 μg/ml	
	Pseudomonas tolaasii	25.0 μg/ml	
	Salmonella enteritidis	25.0 μg/ml	
	Staphylococcus aureus	12.5 μg/ml	
	Micrococcus luteus	12.5 μg/ml	
	Sarcina lutea	12.5 μg/ml	
	Bacillus cereus	12.5 μg/ml	
	Candida albicans	50.0 μg/ml	
	Aspergillus niger	15.0 μg/ml	(Rančcić *et al.*, 2005)

***Hypericum* sps**	**Test organism**	**Antimicrobial activity (MIC)**	**Reference**
H. perforatum (cont.)	*Aspergillus flavus*	15.0 μg/ml	
	Cladosporium cladosporioides	15.0 μg/ml	
	Penicillium funiculosum	15.0 μg/ml	
	Trichoderma viride	15.0 μg/ml	
H. rumeliacum	*Staphylococcus aureus*	7.83 mg/ml	(Couladis *et al.*, 2003)
	Staphylococcus epidermidis	11.2 mg/ml	
	Escherichia coli	3.8 mg/ml	
	Enterobacter cloacae	17.2 mg/ml	
	Klebsiella pneumoniae	9.3 mg/ml	
	Pseudomonas aeruginosa	7.35 mg/ml	
	Candida albicans	6.34 mg/ml	
	C. tropicalis	5.25 mg/ml	
	C. glabrata	4.75 mg/ml	
	Escherichia coli	25.0 μg/ml	(Saroglou *et al.*, 2007)
	Proteus mirabilis	50.0 μg/ml	
	Agrobacterium tumefaciens	25.0 μg/ml	
	Pseudomonas aeruginosa	25.0 μg/ml	
	Pseudomonas tolaasii	25.0 μg/ml	
	Salmonella enteritidis	25.0 μg/ml	
	Staphylococcus aureus	6.25 μg/ml	
	Micrococcus luteus	12.5 μg/ml	
	Sarcina lutea	6.25 μg/ml	
	Bacillus cereus	12.5 μg/ml	
	Candida albicans	25.0 μg/ml	

Table 4b - Antimicrobial activities of *Hypericum* essential oils on standard microorganisms in different *in vitro* assays evaluated by the zone of inhibition (mm).

***Hypericum* sps**	**Test organism**	**Antimicrobial activity (mm)**	**Reference**
H. perforatum	*Candida albicans*	5 mm (2.5µl)	(Rančcić *et al.*, 2005)
	Escherichia coli	7 mm (1µl)	
	Micrococcus luteus	6 mm (1µl)	
	Pseudomonas tolaasii	3 mm (1µl)	
	Salmonella enteritidis	4 mm (1µl)	
	Salmonella typhimurium	4 mm (1µl)	
	Staphylococcus aureus	5 mm (1µl)	
	Staphylococcus epidermidis	4 mm (1µl)	
H. scabrum	*Escherichia coli K12*	18 mm (40µg/disc)	(Kızıl *et al.*, 2004)
	Escherichia coli PBR322	10 mm (40µg/disc)	
	Escherichia coli PUC9	14 mm (60µg/disc)	
	Bacillus brevis ATCC	10 mm (40µg/disc)	
	Bacillus cereus DM65	14 mm (60µg/disc)	
	Streptococcus pyogenes DM41	10 mm (60µg/disc)	
	Pseudomonas aeruginosa DMC66	16 mm (40µg/disc)	
	Staphylococcus aureus DMC70	16 mm (40µg/disc)	
	Candida albicans DM31	18 mm (60µg/disc)	
H. scabroides	*Escherichia coli K12*	16 mm (40µg/disc)	(Kızıl *et al.*, 2004)
	Escherichia coli PBR322	16 mm (40µg/disc)	
	Escherichia coli PUC9	20 mm (40µg/disc)	
	Bacillus brevis ATCC	12 mm (40µg/disc)	
	Bacillus cereus DM65	10 mm (60µg/disc)	
	Streptococcus pyogenes DM41	10 mm (40µg/disc)	
	Pseudomonas aeruginosa DMC66	14 mm (40µg/disc)	

***Hypericum* sps**	**Test organism**	**Antimicrobial activity (mm)**	**Reference**
H. scabroides (cont.)	*Staphylococcus aureus DMC70*	10 mm (40µg/disc)	
	Candida albicans DM31	16 mm (40µg/disc)	
H. triquetrifolium	*Escherichia coli K12*	12 mm (60µg/disc)	(Kızıl *et al.*, 2004)
	Escherichia coli PBR322	10 mm (40µg/disc)	
	Escherichia coli PUC9	10 mm (40µg/disc)	
	Bacillus brevis ATCC	10 mm (40µg/disc)	
	Bacillus cereus DM65	12 mm (40µg/disc)	
	Streptococcus pyogenes DM41	12 mm (60µg/disc)	
	Pseudomonas aeruginosa DMC66	12 mm (40µg/disc)	
	Staphylococcus aureus DMC70	16 mm (40µg/disc)	
	Candida albicans DM31	12 mm (40µg/disc)	
H. hyssopifolium* var. *microcalcynum	*Escherichia coli K12*	14 mm (40µg/disc)	(Toker *et al.*, 2006)
	Escherichia coli PBR322	10 mm (40µg/disc)	
	Escherichia coli PUC9	8 mm (40µg/disc)	
	Bacillus brevis ATCC	16 mm (60µg/disc)	
	Bacillus cereus DM65	10 mm (40µg/disc)	
	Streptococcus pyogenes DM41	14 mm (60µg/disc)	
	Pseudomonas aeruginosa DMC66	12 mm (40µg/disc)	
	Staphylococcus aureus DMC70	12 mm (40µg/disc)	
	Candida albicans DM31	12 mm (40µg/disc)	

Table 4c - Antimicrobial activities of *Hypericum* essential oils on standard microorganisms in different *in vitro* assays evaluated by % of inhibition

***Hypericum* sps**	**Test organism**	**Antimicrobial activity (% Inhibition)**	**Reference**
H. linarioides	*Alternaria solani*	1% (2.5 mg/ml)	(Cakir *et al.*, 2005)
	Fusarium acuminatum	0% (5 mg/ml)	
	Fusarium culmorum	13.6% (5 mg/ml)	
	Fusarium equiseti	0% (5 mg/ml)	
	Fusarium oxysporum	0% (5 mg/ml)	
	Fusarium sambucinum	9.6% (5 mg/ml)	
	Fusarium solani	0% (5 mg/ml)	
	Verticillium albo-atrum	36.0% (5 mg/ml)	
	Rhizoctonia solani AG-5	4.8% (2.5 mg/ml)	
	Rhizoctonia solani AG-9	87.5% (5 mg/ml)	
	Rhizoctonia solani AG-11	11.3% (2.5 mg/ml)	
H. heterophyllum	*Rhizoctonia solani* AG-3	0% (5 mg/ml)	(Cakir *et al.*, 2004)
	Rhizoctonia solani AG-4	29% (1 mg/ml)	
	Rhizoctonia solani AG-5	32% (2.5 mg/ml)	
	Rhizoctonia solani AG-9	12% (5 mg/ml)	
	Rhizoctonia solani AG-11	64% (2.5 mg/ml)	
	Fusarium oxysporum	0% (5 mg/ml)	
	Fusarium culmorum	17% (1 mg/ml)	
	Fusarium sambucinum	4% (1 mg/ml)	
	Fusarium solani	7% (0.5 mg/ml)	
	Fusarium acuminatum	33% (0.5 mg/ml)	
H. hyssopifolium	*Rhizoctonia solani* AG-3	0% (5 mg/ml)	(Cakir *et al.*, 2004)
	Rhizoctonia solani AG-4	11% (0.5 mg/ml)	
	Rhizoctonia solani AG-5	38% (2.5 mg/ml)	

***Hypericum* sps**	**Test organism**	**Antimicrobial activity (% Inhibition)**	**Reference**
H. hyssopifolium	*Rhizoctonia solani* AG-9	6% (5 mg/ml)	
(cont.)	*Rhizoctonia solani* AG-11	46% (5 mg/ml)	
	Fusarium oxysporum	6% (5 mg/ml)	
	Fusarium culmorum	15% (0.5 mg/ml)	
	Fusarium sambucinum	0% (5 mg/ml)	
	Fusarium solani	4% (0.5 mg/ml)	
	Fusarium acuminatum	39% (1 mg/ml)	

It is likely that the antimicrobial properties of the *Hypericum* essential oils are due in a great part to the presence of α-pinene, β-pinene and (*E)*-caryophyllene, as these compounds are known for their antimicrobial effects (Aligiannis *et al.*, 2001; Mourey *et al.*, 2002; Cakir *et al.*, 2004; Costa *et al.*, 2008). As these compounds are widely found in the essential oils of *Hypericum*, it is not surprising to see many reports on antimicrobial activities of species of this genus (Gudžić *et al.*, 1997; Schwob *et al.*, 2002; Couladis *et al.*, 2003; Cakir *et al.*, 2004; Cakir *et al.*, 2005; Schwob *et al.*, 2006; Toker *et al.*, 2006; Saroglou *et al.*, 2007). Anti-inflammatory and anticarcinogenic activities have been attributed to *(E)*-caryophyllene (Zheng *et al.*, 1992; Martin *et al.*, 1993; Kubo *et al.*, 1996; Tambe *et al.*, 1996). The monoterpene hydrocarbon, α-pinene have also shown to exert anti-inflammatory activity in rats (Martin *et al.*, 1993).

3.3- Antiangiogenetic effect

Recently, antiangiogenetic activity of essential oil of *Hypericum perforatum* has been demonstrated using the chicken chorio allantoic membrane (CAM) assay (Demirci *et al.*, 2008). The essential oils at various concentrations (5-50 microgram/pellet) remarkably prevented new blood vessel growth in the *in vivo* chicken embryo compared to standards. Antiangiogenic effect of *H. perforatum* has also been attributed to hyperforin, a phenolic type compound that does not take part of the essential oil (Martínez-Poveda B *et al.*, 2005). This compound has shown to inhibit angiogenesis both *in vitro* and *in vivo*. Thus, hyperforin and essential oil may

have potential use in cancer and metastasis inhibition, as well as in treatment of angiogenesis-related pathologies.

3.4- Antioxidant activity

Antioxidant and inhibitory activity of acetylcholinesterase were shown in assays performed with essential oils of *Hypericum undulatum* (Ferreira *et al.*, 2006). According to the authors, further investigations should be done with this essential oils to evaluate its potential use in preventing or alleviating patients suffering from Alzheimer's Disease (Ferreira *et al.*, 2006).

4- Biological Activities of *Hypericum* Extracts

The most of the pharmacological properties described in the previous section related with *Hypericum* essential oils coincides well with biological activities reported to *Hypericum* crude extracts. Hence, we speculate that at least some of the biological activities exhibited by the crude extract should also be present in the essential oils. Here we summarize some of the important biological activities of *Hypericum* crude extract, which have not so far been demonstrated with essential oils, but it would be worth to be done.

4.1- Antitumor activity

Hartwell (1970) reported 14 entries referring to *Hypericum* as a folk remedy for various neoplastic conditions in a survey on the use of plants for the treatment of cancer (Hartwell, 1970). Thus, in the last years, *Hypericum* extracts have been investigated for their potential use as antitumor agent. Antiproliferative activity of crude methanolic extracts of *H. caprifoliatum* Cham. & Schlecht., *H. carinatum* Griseb., *H. connatum* Lam., *H. myrianthum* Cham. & Schlecht., *H. polyanthemum* Klotzsch ex Reichardt and *H. ternum* A. St. Hil. were tested against two cell lines (HT-29 human colon carcinoma cells and H-460 non-small cell lung carcinoma). The most active crude methanolic extracts were those from *H. caprifoliatum, H. myrianthum* and, to a lesser extent, from *H. connatum* (Ferraz *et al.*, 2005a). *H. perforatum* extracts (commercially available St. John's wort preparations) showed potential anticarcinogenic effect, since they functioned as potent inhibitors of the major human

procarcinogen-activating enzyme, the isoform CYP1A1 (Schwarz *et al.*, 2003). These extracts should be also evaluated for cancer chemopreventive potential. Different antiproliferative effects of *Hypericum* methanolic extracts on leukemia cell lines (K562 and U937), human colon carcinoma cells (HT-29) and non-small cell lung carcinoma (H-460) were also reported (Hostanska *et al.*, 2003a; Ferraz *et al.*, 2005a).

4.2- Antioxidant activity

The actual knowledge that reactive oxygen species are involved in the genesis of several diseases, such as arteriosclerosis, rheumatism, some cancers and ageing has prompted the search for new antioxidants (Heilmann *et al.*, 2003). Thus, there is an increasing demand for phytochemicals with antioxidative activity, not only plant extracts but also isolated compounds. The high amount of phenolic compounds produced by *Hypericum* species makes their extracts very interesting in terms of potential antioxidant activity. *H. barbatum* Jacq., *H. hirsutum* L., *H. linarioides* Bosse, *H. maculatum* Crantz, *H. olympicum* L., *H. perforatum* L., *H. richeri* Vill., *H. rumeliacum* Boiss. and *H. tetrapterum* Fries crude methanolic extracts have been tested for their antioxidant activity (Radulovic *et al.*, 2007). According to the authors, all the extracts of the *Hypericum* species studied possess a significant antioxidant activity; however it was particularly high in the crude methanolic extracts of *H. perforatum* flowers, followed closely by aerial parts of *H. barbatum*. The antioxidant activity of ethanolic extracts of *H. perforatum* has also been demonstrated (Silva *et al.*, 2005). Methanolic extracts of *Hypericum rumeliacum* Boiss. also revealed antioxidant activity (Galati *et al.*, 2008).

H. triquetrifolium, is another species of the genus, whose water and methanol extracts had significant antioxidant activities (Conforti *et al.*, 2002; Tawaha *et al.*, 2007). Ethanol and water extracts of the flowers of *H. venustum* showed a strong reducing power, free radicals and hydrogen peroxide scavenging activity (Spiteller *et al.*, 2008). Amongst 21 species of medicinal plants widely used in Bulgaria, extracts of *H. perforatum* prepared as infusions with water, showed considerable antioxidant activity (Ivanova *et al.*, 2005). Various extracts from *H. undulatum*, such as essential oils, decoctions and ethanolic extracts showed inhibitory activity of acetylcholinesterase and antioxidant activity, demonstrating that it may help to prevent or alleviate patients suffering from Alzheimer's Disease (Ferreira *et al.*, 2006). Water and methanol extracts of *H. triquetrifolium*, from Jordania, revealed high antioxidant activities, concomitant with high amounts of phenolic compounds. Significant antioxidant activity has also been attributed to methanolic extracts of *H. triquetrifolium* (Conforti *et al.*, 2002).

4.3- Antiviral activity

Schmitt and co-workers (2001) tested aqueous and methanolic extracts of *H. connatum*, *H. caprifoliatum* and *H. polyanthemum* for their antiviral activity against feline immunodeficiency virus (FIV). Feline immunodeficiency virus (FIV) is a causative agent of acquired immunodeficiency syndrome (AIDS)-like disease in cats. The fact that FIV has similarity to HIV-1 in many molecular and biochemical properties, and the pathogenesis of FIV infection is thought to be similar to that of HIV infection makes it an attractive model for AIDS research. From the three plants studied only *H. connatum*, used in traditional medicine, showed activity against FIV (Schmitt *et al.*, 2001). Antiviral activity against herpes simplex viruses (HSV) was also shown in crude methanolic extracts of *H. connatum* (Fritz *et al.*, 2007).

4.4- Wound-healing and anti-inflammatory activities

Besides the above mentioned, several other biological activities have been attributed to the *Hypericum* extracts. Wound-healing activity of *H. perforatum* was recently shown, when their alcoholic extracts were incubated with chicken embryonic fibroblasts from fertilized eggs (Öztürk *et al.*, 2007). The authors found that the mechanism of action of these extracts was similar to that of titrated extract of *Centella asiatica*, which is used in Europe in wound healing drugs (Maquart *et al.*, 1999). Indeed, both extracts seemed to stimulate collagen synthesis in fibroblast cultures and increase the tensile strength of tissues.

Analgesic and topical anti-inflammatory activities in mice were also reported for methanol extracts of three other *Hypericum* species, *H. reflexum*, *H. canariense* L, and *H. glandulosum* Ait. (Rabanal *et al.*, 2005; Sánchez-Mateo *et al.*, 2006). Furthermore, the methanol extracts of *H. perforatum*, *H. empetrifolium, H. triquetrifolium* and *H. rumeliacum* have been shown to exhibit anti-inflammatory activity (Apaydin *et al.*, 1999; Öztürk *et al.*, 2002; Galati *et al.*, 2008). Analgesic activity was also attributed to the *H. empetrifolium* extracts (Trovato *et al.*, 2001).

5- Biotechnology in *Hypericum* Essential Oil Improvement

The composition of essential oils depends on climatic and pedological conditions, plant organ and vegetative cycle stage. Thus, it is of uppermost importance to characterize the essential oils composition as well as the influence of the referred parameters on its quality, in order to obtain essential oils of constant composition. According to Bakkali (2008), this could only be possible if essential oils are extracted under the same conditions from the same organ of the plant which has been growing on the same soil, under the same climate and has been picked in the same season.

However, one possible alternative to conventional agriculture practices for the production of essential oils could be the use of *in vitro* cultures (Rao *et al.*, 2002; Lila, 2005). This technology mostly offers the possibility of having a controlled system of production, ensuring a continuous supply of products with uniform quality and yield (Lila, 2005). To the best of our knowledge, essential oils composition upon *in vitro* cultures has been reported only in *H. androsaemum* (Guedes *et al.*, 2003). In the essential oils isolated from shoots of *H. androsaemum,* sesquiterpene hydrocarbons was the major group of compounds, representing more than 80% of the total essential oil. Its major constituent was γ-muurolene (15% of the total essential oil) (Guedes *et al.*, 2003).

Genetic transformation methods can also be used to improve the essential oils content. Transformation is currently used for genetic manipulation of major economic crops, vegetables, ornamental, medicinal, fruit, tree and pasture plants, using *Agrobacterium*-mediated or direct transformation methods (Riva *et al.*, 1998). Genetic transformation on species of *Hypericum* has only been performed in *H. perforatum*. A successful protocol of particle bombardment-mediated transformation for *H. perforatum* L. has been reported (Franklin *et al.*, 2007). According to the authors, this species remains highly recalcitrant towards *Agrobacterium*-mediated transformation. Further data have shown a reduction of *Agrobacterium*-viability when in contact with *H. perforatum* cells. Possibly, this can be due to the activation of defence mechanisms of the plant cells against the bacteria (Franklin *et al.*, 2008). The establishment of successful transformation procedures and the knowledge of biosynthetic pathways of useful secondary metabolites can make possible the modulation of its production. In the last decades, great progress has been made in the elucidation of plant terpenoids biosynthetic pathways at the gene and enzyme levels. Indeed, Metabolic Engineering is seen as a powerful tool to improve secondary metabolites production. For *Hypericum* essential oils, the knowledge of the enzymes involved in the production of its compounds as well as their regulatory mechanisms can

possibly lead to the increase in the production of essential oils with higher amount of the desirable compounds. For instance, (*E)*-caryophyllene, one of the major constituents of essential oils of some *Hypericum* species, should be an interesting molecule to increase production due to its abovementioned pharmacological activities. Moreover, the enzyme responsible for its synthesis, *(E)*-caryophyllene synthase, has already been isolated and characterized in several species (*Artemisia annua* (Cai *et al.*, 2002), *Arabidopsis thaliana* (Chen *et al.*, 2003) and *Cucumis sativus* (Mercke *et al.*, 2004). Different approaches can be used in order to modulate its production, such as over-expression of genes involved in the production of the desirable compounds, manipulation of transcription factors controlling the expression of cascades of genes, or even the identification of tissue/organ-specific promoters (Lange *et al.*, 1999; Mahmoud *et al.*, 2002).

6- Conclusion

Hypericum is an important genus containing a vast array of secondary metabolites distributed by water, alcoholic extracts and essential oils. Although essential oil composition of several *Hypericum* species has been reported, its pharmacological studies are scarce.

Acknowledgements

This work was sponsored by EU (FSE/FEDER) and Portuguese Government (FCT) through the Project PTDC/AGR-AAM/70418/2006 and the Grant SFRH/BD/13283/2003.

7- References

Abreu, I., M. Reis, A. Marsaioli & P. Mazzafera (2004). Essential oil composition of *Hypericum brasiliense* choise. *Flavour and Fragrance Journal*(19), 80-82.

Adam, K., A. Sivropoulou, S. Kokkini, T. Lanaras & M. Arsenakis (1998). Antifungal Activities of *Origanum vulgare* subsp. *hirtum*, *Mentha spicata*, *Lavandula angustifolia*, and *Salvia fruticosa* Essential Oils against Human Pathogenic Fungi. *J. Agric. Food Chem. 46*(5), 1739-1745.

Aligiannis, N., E. Kalpoutzakis, I. B. Chinou, S. Mitakou, E. Gikas & A. Tsarbopoulos (2001). Composition and Antimicrobial Activity of the Essential Oils of Five Taxa of Sideritis from Greece. *J. Agric. Food Chem. 49*(2), 811-815.

Andrews, R., L. Parks & K. Spence (1980). Some effects of Douglas fir terpenes on certain microorganisms. *Applied and Environmental Microbiology 40*, 301-304.

Apaydin, S., U. Zeybek & I. Ince (1999). *Hypericum triquetrifolium* Turra. extract exhibits antinociceptive activity in the mouse. *J Ethnopharmacol 67*, 307-312.

Avato, P., F. Raffo, G. Guglielmi, C. Vitali & A. Rosato (2004). Extracts from St John's wort and their antimicrobial activity. *Phytotherapy Research 18*(3), 230-232.

Bakkali, F., S. Averbeck, D. Averbeck & M. Idaomar (2008). Biological effects of essential oils - A review. *Food and Chemical Toxicology 46*(2), 446-475.

Baser, K. H. C., T. Ozek, H. R. Nuriddinov & A. B. Demirci (2002). Essential oils of two *Hypericum* species from Uzbekistan. *Chemistry of Natural Compounds. 38*(1), 54-57.

Bertoli, A., F. Menichini, M. Mazzetti, G. Spinelli & I. Morelli (2003). Volatile constituents of the leaves and flowers of *Hypericum triquetrifolium* Turra. *Flavour and Fragrance Journal 18*(2), 91-94.

Bombardelli, E. & P. Morazzoni (1995). *Hypericum perforatum*. *Fitoterapia 66*, 43-68.

Bougard, F., A. Gravot, S. Milesi & E. Grontier (2001). Production of Plant Secondary Metabolites: a Historical Perspective. *Plant Science 161*, 839-851.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. *International Journal of Food Microbiology 94*, 223– 253.

Cai, Y., J. Jia, J. Crock, Z. Lin, C. XY & C. R (2002). A cDNA clone for β-caryophyllene synthase from *Artemisia annua*. *Phytochemistry 61*, 523-529.

Cakir, A., S. Kordali, H. Kilic & E. Kaya (2005). Antifungal properties of essential oil and crude extracts of *Hypericum linarioides* Bosse. *Biochemical Systematics and Ecology 33*(3), 245-256.

Cakir, A., S. Kordali, H. Zengin, S. Izumi & T. Hirata (2004). Composition and antifungal activity of essential oils isolated from *Hypericum hyssopifolium* and *Hypericum heterophyllum*. *Flavour and Fragrance Journal 19*(1), 62-68.

Cakir, A., A. Mavi, A. YildIrIm, M. E. Duru, M. Harmandar & C. Kazaz (2003). Isolation and characterization of antioxidant phenolic compounds from the aerial parts of *Hypericum hyssopifolium* L. by activity-guided fractionation. *Journal of Ethnopharmacology 87*(1), 73-83.

Camejo-Rodrigues, J., L. Ascensão, M. À. Bonet & J. Vallès (2003). An ethnobotanical study of medicinal and aromatic plants in the Natural Park of "Serra de São Mamede" (Portugal). *Journal of Ethnopharmacology 89*(2-3), 199-209.

Cardona, M., J. Marco, J. Sendra, E. Seoane & J. Ibanez (1983). Waxes and volatile oils in *Hypericum ericoides* (Guttiferae). *Lipids 18*(6), 439-442.

Chen, F., D. Tholl, J. C. D'Auria, A. Farooq, E. Pichersky & J. Gershenzon (2003). Biosynthesis and Emission of Terpenoid Volatiles from Arabidopsis Flowers. *Plant Cell 15*(2), 481-494.

Conforti, F., G. A. Statti, R. Tundis, F. Menichini & P. Houghton (2002). Antioxidant activity of methanolic extract of *Hypericum triquetrifolium* Turra aerial part. *Fitoterapia 73*(6), 479-483.

Costa, E. V., S. D. Teixeira, F. A. Marques, M. C. T. Duarte, C. Delarmelina, M. L. B. Pinheiro, J. R. Trigo & B. H. L. N. Sales Maia (2008). Chemical composition and antimicrobial activity of the essential oils of the Amazon Guatteriopsis species. *Phytochemistry 69*(9), 1895-1899.

Couladis, M., P. Baziou, P. V. Petrakis & C. Harvala (2001). Essential oil composition of *Hypericum perfoliatum* L. growing in different locations in Greece. *Flavour and Fragrance Journal 16*(3), 204-206.

Couladis, M., I. B. Chinou, O. Tzakou & P. V. Petrakis (2003). Composition and antimicrobial activity of the essential oil of *Hypericum rumeliacum* subsp. *apollinis* (Boiss. & Heldr.). *Phytotherapy Research 17*(2), 152-154.

Cowan, M. (1999). Plant Products as Antimicrobial Agents. *Clinical Microbiology Reviews 12*(4), 564-582.

Crockett, S. L., B. Demirci, K. H. C. Baser & I. A. Khan (2007). Analysis of the Volatile Constituents of Five African and Mediterranean *Hypericum* L. (Clusiaceae, Hypericoideae) Species. *Journal of Essential Oil Research 19*(4), 302-306.

Daudt, R., G. von Poser, G. Neves & S. Rates (2000). Screening for the antidepressant activity of some species of *Hypericum* from South Brazil. *Phytotherapy Research 14*(5), 344-346.

Decosterd, L., E. Hoffmann, R. Kyburz, D. Bray & K. Jostettmann (1991). A new phloroglucinol derivative from *Hypericum calycinum* with antifungal and in vitro antimalarial activity. *Planta Medica 57*, 548–551.

Demirci, B., K. H. C. Baser, S. L. Crockett & I. A. Khan (2005). Analysis of the Volatile Constituents of Asian *Hypericum* L. (Clusiaceae, Hyperidoideae) Species. *Journal of Essential Oil Research 17*(6), 659-663.

Demirci, B., T. Kıyan, F. Demirci & K. Başer (2008). Antiangiogenetic effects of *Hypericum perforatum* L. essential oil. *PSE-natural products in cancer therapy*.

Demirci, F. & K. H. C. Baser (2006). Volatiles of *Hypericum bupleuroides* Griseb. *The Journal of Essential Oil Research 18*(6), 650-651.

Ferraz, A., D. H. Faria, M. N. Benneti, A. Brondani da Rocha, G. Schwartsmann, A. Henriques & G. L. von Poser (2005). Screening for antiproliferative activity of six southern Brazilian species of *Hypericum*. *Phytomedicine 12*(1-2), 112-115.

Ferraz, A., D. H. Faria, M. N. Benneti, A. Brondani da Rocha, G. Schwartsmann, A. Henriques & G. L. von Poser (2005a). Screening for antiproliferative activity of six southern Brazilian species of *Hypericum*. *Phytomedicine 12*(1-2), 112-115.

Ferraz, A. B. F., R. P. Limberger, S. A. L. Bordignon, G. L. v. Poser & A. T. Henriques (2005). Essential oil composition of six *Hypericum* species from southern Brazil. *Flavour and Fragrance Journal 20*(3), 335-339.

Ferreira, A., C. Proença, M. L. M. Serralheiro & M. E. M. Araújo (2006). The *in vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Portugal. *Journal of Ethnopharmacology 108*(1), 31-37.

Ferretti, G., F. Maggi & B. Tirillini (2005). Essential oil composition of *Hypericum richeri* Vill. from Italy. *Flavour and Fragrance Journal 20*(3), 295-298.

Franklin, G., L. Conceição, E. Kombrink & A. Dias (2008). *Hypericum perforatum* plant cells reduce

Agrobacterium viability during co-cultivation. *Planta 227*, 1401–1408.

Franklin, G., M. Oliveira & A. C. P. Dias (2007). Production of transgenic *Hypericum perforatum* plants via particle bombardment-mediated transformation of novel organogenic cell suspension cultures. *Plant Science 172*(6), 1193-1203.

Fritz, D., C. R. Venturi, S. Cargnin, J. Schripsema, P. M. Roehe, J. A. Montanha & G. L. von Poser (2007). Herpes virus inhibitory substances from *Hypericum connatum* Lam., a plant used in southern Brazil to treat oral lesions. *Journal of Ethnopharmacology 113*(3), 517-520.

Galati, E., G. Contartese, N. Miceli, M. Taviano, V. Sdrafkakis, M. Couladis, O. Tzakou & F. Lanuzza (2008). Antiinflammatory and antioxidant activity of *Hypericum rumeliacum* Boiss. subsp.*apollinis* (Boiss. & Heldr.) Robson & Strid methanol extract. *Phytotherapy Research 22*(6), 766-771.

Griffin, S., S. Wyllie, J. Markham & D. Leach (1999). The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. *Flavour and Fragrance Journal 14*(5), 322-332.

Gudžić, B., S. Đorđević, R. Palić & G. Stojanovi (2001). Essential oils of *Hypericum olympicum* L. and *Hypericum perforatum* L. *Flavour and Fragrance Journal 16*(3), 201-203.

Gudžić, B., J. M. Nedeljković, S. Đorđević & J. J. Čomor (1997). Composition and anti-microbial activity of essential oil of Hyperici herb (*Hypericum perforatum* L.) from Vlasina Region. *Facta Universitatis 1*(4), 47-51.

Gudžic, B., A. Šmelcerović, S. Dordević, N. Mimica-Dukić & M. Ristić (2007). Essential oil composition of *Hypericum hirsutum* L. *Flavour and Fragrance Journal 22*(1), 42-43.

Guedes, A. P., L. R. Amorim, A. Vicente & M. Fernandes-Ferreira (2004). Variation of the essential oil content and composition in leaves from cultivated plants of *Hypericum androsaemum* L. *Phytochemical Analysis 15*, 146-151.

Guedes, A. P., L. R. Amorim, A. M. S. Vicente, G. Ramos & M. Fernandes-Ferreira (2003). Essential Oils from Plants and *in Vitro* Shoots of *Hypericum androsaemum* L. *J. Agric. Food Chem. 51*(5), 1399-1404.

Hartwell, J. L. (1970). Plants used against cancer. A survey. *Lloydia 33*, 288-392.

Heilmann, J., K. Winkelmann & O. Sticher (2003). Studies on the antioxidative activity of phloroglucinol derivatives isolated from *Hypericum* species. *Planta medica 69*(3), 202-206.

Hölzl, J. & M. Petersen (2003). Culture and biotechnology of *Hypericum*. *Chemical constituents of Hypericum ssp.* E. Ernst. London and New York, Taylor & Francis**:** 77-93.

Hosni, K., K. Msaâda, M. Ben Taârit, O. Ouchikh, M. Kallel & B. Marzouk (2008). Essential oil composition of *Hypericum perfoliatum* L. and *Hypericum tomentosum* L. growing wild in Tunisia. *Industrial Crops and Products 27*(3), 308-314.

Hostanska, K., S. Bommer, M. Weber, B. Krasniqi & R. Saller (2003a). Comparison of the growth-inhibitory effect of *Hypericum perforatum* L. extracts, differing in the concentration of phloroglucinols and flavonoids, on leukaemia cells. *Journal of Pharmacy and Pharmacology 55*(7), 973-980.

Ishiguro, K., M. Yamaki, M. Kashihara, S. Takagi & K. Isoi (1990). Sarothralin G: a new antimicrobial compound from *Hypericum japonicum*. *Planta Medica 56*, 274–276.

Ivanova, D., D. Gerova, T. Chervenkov & T. Yankova (2005). Polyphenols and antioxidant capacity of Bulgarian medicinal plants. *Journal of Ethnopharmacology 96*(1-2), 145-150.

Jaric, S., Z. Popovic, M. Macukanovic-Jocic, L. Djurdjevic, M. Mijatovic, B. Karadzic, M. Mitrovic & P. Pavlovic (2007). An ethnobotanical study on the usage of wild medicinal herbs from Kopaonik Mountain (Central Serbia). *Journal of Ethnopharmacology 111*(1), 160-175.

Jayasuriyab, H., A. M. Clark & J. D. McChesney (1991). New antimicrobial filicinic acid derivatives from *Hypericum drummondii*. *Journal of Natural Products 54*(5), 1314–1320.

Karim, H., M. Kamel, B. Mouna, C. Thouraya & M. Brahim (2007). Essential oil composition of *Hypericum triquetrifolium* Turra. aerial parts. *Ital J Biochem 56*(1), 40-56.

Kızıl, G., Z. Toker, H. Özen & Ç. Aytekin (2004). The Antimicrobial Activity of Essential Oils of *Hypericum scabrum*, *Hypericum scabroides* and *Hypericum triquetrifolium*. *Phytotherapy Research 18*, 339–341.

Kubo, I., S. Chaudhuri, Y. Kubo, Y. Sanchez, T. Ogura, T. Saito, H. Ishikawa & H. Haraguchi (1996). Cytotoxic and Antioxidative Sesquiterpenoids from *Heterotheca inuloides*. *Planta Medica*(5), 427-430.

Kültür, S. (2007). Medicinal plants used in KIrklareli Province (Turkey). *Journal of Ethnopharmacology 111*(2), 341-364.

Lange, B. & R. Croteau (1999). Genetic engineering of essential oil production in mint. *Current Opinion in Plant Biology 2*, 139–144.

Lila, M. (2005). Valuable Secondary Products from *In Vitro* Culture. *Plant develpoment and Biotechnology*. R. Trigiano and D. Gray, CRC Press**:** 285-289.

Mahmoud, S. & R. Croteau (2002). Strategies for transgenic manipulation of monoterpene biosynthesis in plant. *Trends Plant Science 7*, 366–373.

Maquart, F. X., F. Chastang, A. Simeon, P. Birembaut, P. Gillery & Y. Wegrowski (1999). Triterpenes from *Centella asiatica* stimulate extracellular matrix accumulation in rat experimental wounds. *European Journal of Dermatology 9*(4), 289-96.

Martin, S., E. Padilla, M. A. Ocete, J. Galvez, J. Jiménez & A. Zarzuelo (1993). Anti-Inflammatory Activity of the Essential Oil of *Bupleurum fruticescens*. *Planta Medica*(6), 533-536.

Martínez-Poveda B, Quesada AR & M. MA (2005). Hyperforin, a bio-active compound of St. John's Wort, is a new inhibitor of angiogenesis targeting several key steps of the process. *International Journal of Cancer 117*(5), 775–780.

Mathis, C. & G. Ourisson (1964a). Étude chimio-taxonomique du genre *Hypericum* : II. Identification de constituants de diverses huiles essentielles d'*Hypericum*. *Phytochemistry 3*(1), 115-131.

Mathis, C. & G. Ourisson (1964b). Étude chimio-taxonomique du genre *Hypericum* : III. Répartition des carbures saturés et des monoterpènes dans les huiles essentielles d'*Hypericum*. *Phytochemistry 3*(1), 133-141.

Mathis, C. & G. Ourisson (1964c). Etude chimio-taxonomique du genre *Hypericum*: IV. Repartition des sesquiterpenes, des alcools monoterpeniques et des aldehydes satures dans les huiles essentielles d'*Hypericum*. *Phytochemistry 3*(3), 377-378.

Mercke, P., I. F. Kappers, F. W. A. Verstappen, O. Vorst, M. Dicke & H. J. Bouwmeester (2004). Combined Transcript and Metabolite Analysis Reveals Genes Involved in Spider Mite Induced Volatile Formation in Cucumber Plants. *Plant Physiol. 135*(4), 2012-2024.

Mockutė, D., G. Bernotienė & A. Judžentienė (2003). Volatile compounds of the aerial parts of wild St. John´s wort (*Hypericum perforatum* L.) plants. *Chemija 14*(3), 108-111.

Morteza-Semnani, K. & M. Saeedi (2005). The essential oil composition of *Hypericum androsaemum* L. leaves and flowers from Iran. *Flavour and Fragrance Journal 20*(3), 332-334.

Mourey, A. & N. Canillac (2002). Anti-listeria monocytogenes activity of essential oils components of conifers. *Food Control 13*, 289–292.

Mukherjee, P., R. Verpoorte & B. Suresh (2000). Evaluation of *in vivo* wound healing activity of *Hypericum patulum* (family: Hypericaceae) leaf extract on different wound model in rats. *Journal of Ethnopharmacology 70*, 315–321.

Mukherjee, P. K., G. S. Saritha & B. Suresh (2001). Antibacterial spectrum of *Hypericum hookerianum*. *Fitoterapia 72*(5), 558-560.

Nahrstedt, A. & V. Butterweck (1997). Biologically active and other chemical constituents of the herb of *Hypericum perforatum* L. *Pharmacopsychiatry 30*, 129-134.

Nogueira, T., F. Duarte, F. Venâncio, R. Tavares, M. Lousã, C. Bicchi & P. Rubiolo (1998). Aspectos Quimiotaxonómicos do Género *Hypericum* L. em Portugal. *Silva Lusitana 6*(1), 55-61.

Nogueira, T., M. J. Marcelo-Curto, A. C. Figueiredo, J. G. Barroso, L. G. Pedro, P. Rubiolo & C. Bicchi (2008). Chemotaxonomy of *Hypericum* genus from Portugal: Geographical distribution and essential oils composition of *Hypericum perfoliatum*, *Hypericum humifusum, Hypericum linarifolium* and *Hypericum pulchrum*. *Biochemical Systematics and Ecology 36*(1), 40-50.

Öztürk, B., S. Apaydin, E. Goldeli, I. Ince & U. Zeybek (2002). *Hypericum triquetrifolium* Turra. extract exhibits antiinflammatory activity in the rat. *J Ethnopharmacol 80*, 207-209.

Öztürk, N., S. Korkmaz & Y. Öztürk (2007). Wound-healing activity of St. John's Wort (*Hypericum perforatum* L.) on chicken embryonic fibroblasts. *Journal of Ethnopharmacology 111*(1), 33-39.

Öztürk, Y., S. Aydin, R. Beis, K. Baser & H. Berberolu (1996). Effects of *Hypericum calycinum* L. Extract on the Central Nervous System in Mice. *Phytotherapy Research 10*(8), 700-702.

Panizi, L., G. Flamini, P. L. Cioni & I. Morelli (1993). Composition and antimicrobial properties of essential oils of four Mediterranean Lamiaceae. *Journal of Ethnopharmacology 39*, 167–170.

Pavlović, M., O. Tzakou, P. V. Petrakis & M. Couladis (2006). The essential oil of *Hypericum perforatum* L., *Hypericum tetrapterum* Fries and *Hypericum olympicum* L. growing in Greece. *Flavour and Fragrance Journal 21*(1), 84-87.

Petrakis, P. V., M. Couladis & V. Roussis (2005). A method for detecting the biosystematic significance of the essential oil composition: The case of five Hellenic *Hypericum* L. species. *Biochemical Systematics and Ecology 33*(9), 873-898.

Pieroni, A., C. L. Quave & R. F. Santoro (2004). Folk pharmaceutical knowledge in the territory of the Dolomiti Lucane, inland southern Italy. *Journal of Ethnopharmacology 95*(2-3), 373-384.

Pintore, G., M. Chessa, G. Boatto, R. Cerri, M. Usai & B. Tirillini (2005). Essential oil composition of *Hypericum perforatum* L. var. angustifolium DC growing wild in Sardinia (Italy). *The Journal of essential oil research 17*(5), 533-535

Pistelli, L., A. Bertoli, S. Zucconelli, I. Morelli, L. Panizzi & F. Menichini (2000). Antimicrobial activity of crude extracts and pure compounds of *Hypericum hircinum*. *Fitoterapia 71*(Supplement 1), S138-S140.

Poutaraud, A. & P. Girardin (2005). Improvement of medicinal plant quality: a *Hypericum perforatum* literature review as an example. *Plant Genetic Resources 3*(2), 178–189.

Prado, B., R. Rabanal & C. Sánchez-Mateo (2002). Evaluation of the central properties of several *Hypericum* species from the Canary Islands. *Phytotherapy Research 16*(8), 740-744.

Rabanal, R., C. Bonkanka, M. Hernández-Pérez & C. Sánchez-Mateo (2005). Analgesic and topical anti-inflammatory activity of *Hypericum canariense* L. and *Hypericum glandulosum* Ait. . *Journal of Ethnopharmacology 96*(3), 591-596.

Rabanal, R. M., A. Arias, B. Prado, M. Hernández-Pérez & C. C. Sánchez-Mateo (2002). Antimicrobial studies on three species of *Hypericum* from the Canary Islands. *Journal of Ethnopharmacology 81*(2), 287-292.

Radulovic, N., V. Stankov-Jovanovic, G. Stojanovic, A. Smelcerovic, M. Spiteller & Y. Asakawa (2007). Screening of *in vitro* antimicrobial and antioxidant activity of nine *Hypericum* species from the Balkans. *Food Chemistry 103*(1), 15-21.

Radusiene, J., A. Judzentiene & G. Bernotiene (2005). Essential oil composition and variability of *Hypericum perforatum* L. growing in Lithuania. *Biochemical Systematics and Ecology 33*(2), 113-124.

Ranččić, A., M. Soković, J. Vukojević, A. Simić, P. Marin, S. Duletić-Laušević & D. Djoković (2005). Chemical Composition and

Antimicrobial Activities of Essential Oils of *Myrrhis odorata* (L.) Scop, *Hypericum perforatum* L and *Helichrysum arenarium* (L.) Moench. *Journal of Essential Oil Research 17*(3), 341-345.

Rao, S. R. & G. A. Ravishankar (2002). Plant cell cultures: Chemical factories of secondary metabolites. *Biotechnology Advances 20*, 101–153.

Riva, G. d. l., J. González-Cabrera, R. Vázquez-Padrón & C. Ayra-Pardo (1998). *Agrobacterium tumefaciens*: a natural tool for plant transformation. *EJB Electronic Journal of Biotechnology 1*(3), 1-16.

Robson, N. (2006). Studies in the genus *Hypericum* L. (Clusiaceae). 1 Section 9. *Hypericum sensu lato* (part 3): subsection 1. *Hypericum* series 2. *Senanensia*, subsection 2. *Erecta* and section 9b. *Graveolentia*. *Systematics and Biodiversity 4*, 19-98

Rocha, L., A. Marston, O. Potterat, M. A. C. Kaplan, H. Stoeckli-Evans & K. Hostettmann (1995). Antibacterial phloroglucinols and flavonoids from *Hypericum brasiliense*. *Phytochemistry 40*(5), 1447-1452.

Sánchez-Mateo, C. C., C. X. Bonkanka, M. Hernández-Pérez & R. M. Rabanal (2006). Evaluation of the analgesic and topical anti-inflammatory effects of *Hypericum reflexum* L. fil. *Journal of Ethnopharmacology 107*(1), 1-6.

Sánchez-Mateo, C. C., B. Prado & R. M. Rabanal (2002). Antidepressant effects of the methanol extract of several *Hypericum* species from the Canary Islands. *Journal of Ethnopharmacology 79*(1), 119-127.

Santos, P. A. G., A. C. Figueiredo, J. G. Barroso, L. G. Pedro & J. J. C. Scheffer (1999). Composition of the essential oil of *Hypericum foliosum* Aiton from five Azorean islands. *Flavour and Fragrance Journal 14*(5), 283-286.

Saroglou, V., P. D. Marin, A. Rancic, M. Veljic & H. Skaltsa (2007). Composition and antimicrobial activity of the essential oil of six *Hypericum* species from Serbia. *Biochemical Systematics and Ecology 35*(3), 146-152.

Schmitt, A. C., A. P. Ravazzolo & G. L. von Poser (2001). Investigation of some *Hypericum* species native to Southern of Brazil for antiviral activity. *Journal of Ethnopharmacology 77*(2-3), 239-245.

Schwarz, D., P. Kisselev & I. Roots (2003). St. John's Wort Extracts and Some of Their Constituents Potently Inhibit Ultimate Carcinogen Formation from Benzo[a]pyrene-7,8-Dihydrodiol by Human CYP1A1. *Cancer Res 63*(22), 8062-8068.

Schwob, I., J.-M. Bessiere, V. Masotti & J. Viano (2004). Changes in essential oil composition in Saint John's wort (*Hypericum perforatum* L.) aerial parts during its phenological cycle. *Biochemical Systematics and Ecology 32*(8), 735-745.

Schwob, I., J. M. Bessiere, M. Dherbomez & J. Viano (2002). Composition and antimicrobial activity of the essential oil of *Hypericum coris*. *Fitoterapia 73*(6), 511-513.

Schwob, I., J. Viano, G. Jann-Para, J.-M. Bessiere & M. Dherbomez (2006). Composition and antimicrobial activity of the essential oil of *Hypericum hyssopifolium* ssp. hyssopifolium from southeast France. *The Journal of essential oil research 18*(4), 469-471.

Silva, B. A., F. Ferreres, J. O. Malva & A. C. P. Dias (2005). Phytochemical and antioxidant characterization of *Hypericum perforatum* alcoholic extracts. *Food Chemistry 90*(1-2), 157-167.

Sokmen, A., B. M. Jones & M. Erturk (1999). The *in vitro* antibacterial activity of Turkish medicinal plants. *Journal of Ethnopharmacology 67*, 79–86.

Spiteller, M., T. Özen, A. Smelcerovic, S. Zuehlke & N. Mimica-Dukic (2008). Phenolic constituents and the in vitro antioxidant activity of the flowers of *Hypericum venustum*. *Fitoterapia 79*(3), 191-193.

Tambe, Y., H. Tsujiuchi, G. Honda, Y. Ikeshiro & S. Tanaka (1996). Gastric Cytoprotection of the Non-Steroidal Anti-Inflammatory Sesquiterpene, beta-Caryophyllene. *Planta Medica*(5), 469-470.

Tawaha, K., F. Q. Alali, M. Gharaibeh, M. Mohammad & T. El-Elimat (2007). Antioxidant activity and total phenolic content of selected Jordanian plant species. *Food Chemistry 104*(4), 1372-1378.

Toker, Z., G. KizIl, H. Ç. Özen, M. KizIl & S. Ertekin (2006). Compositions and antimicrobial activities of the essential oils of two *Hypericum* species from Turkey. *Fitoterapia 77*(1), 57-60.

Touafek, O., A. Nacer, A. Kabouche & Z. Kabouche (2005). Analysis of the essential oil of algerian *Hypericum perfoliatum* (L). *Flavour and Fragrance Journal 20*(6), 669-670.

Trovato, A., E. Raneri, M. Kouladis, O. Tzakou, M. Taviano & E. Galati (2001). Anti-inflammatory and analgesic activity of *Hypericum empetrifolium* Willd. (Guttiferae). *Farmaco 56*, 455–457.

Upton, R. (1997). *American Herbal Pharmacopoeia and Therapeutic Compendium, St. John's Wort Monograph*. Santacruz, CA.

Uribe, S., T. Ramirez & A. Pena (1985). Effects of β-pinene on yeast membrane functions. *Journal of Bacteriology 161*, 195-200.

Weyerstahl, P., U. Splittgerber, H. Marschall & V. K. Kaul (1995). Constituents of the leaf essential oil of *Hypericum perforatum* L. from India. *Flavour and Fragrance Journal 10*(6), 365-370.

Zheng, G.-Q., P. M. Kenney & L. K. T. Lam (1992). Sesquiterpenes from Clove (*Eugenia caryophyllata*) as Potential Anticarcinogenic Agents. *J. Nat. Prod. 55*(7), 999-1003.

Chapter 3

ESSENTIAL OILS OF *HYPERICUM ANDROSAEMUM* L.

3.1- Chapter overview

In Portugal, wild *Hypericum androsaemum* L. plants are air dried and commercialized to be taken as infusion, being widely used in folk medicine. Because of the intensive harvesting, this population is in risk of disappearing, namely in the Peneda-Gerês National Park. In order to meet the needs of the consumers, this species was included in a Portuguese programme of integrated research and exploitation of medicinal plants, including the establishment of four pilot plants at different sites of the northern region of Portugal. Additionally, we have coming to perform the respective micropropagation, for helping in the restock of the species population, and the respective essential oils characterization for a better knowledge of this species and for quality control.

From the few phytochemical studies performed in plants of this species most of them concerned the composition of its phenolic extracts (Nielsen *et al.*, 1979; Seabra *et al.*, 1989; Seabra *et al.*, 1990; Kitanov *et al.*, 1998; Dias *et al.*, 1999), being scarce the ones related to essential oils production (Mathis *et al.*, 1964a; Mathis *et al.*, 1964b; Carnduff *et al.*, 1966; Nogueira *et al.*, 1998). Phytochemical analysis of *in vitro* cultures have already been reported, but only regarding the phenolic component of *calli* and cell suspensions (Dias *et al.*, 1999; Dias *et al.*, 2000).

In order to characterize the essential oils of the species and to understand the influence of different factors on its composition, we analyzed, by GC-MS and GC (i) essential oils accumulated in the aerial part of *in vivo H. androsaemum* plants, cultivated at two different places, over a year, with interval of 2 months; (ii) essential oils isolated from different organs of the plant (leaves, stems and ripened seed capsules) and; (iii) essential oils produced by *in vitro* shoots.

Our results showed that stems were the organ with the lowest essential oil yield and narrowest range of compounds while the essential oil from leaves was the most complex. The essential oil yield from *in vitro* shoots was identical to the minimum one registered in the aerial parts of the plant; while the highest yield was registered in the essential oils of ripened seed capsules. Most of the compounds of the essential oils were identified resulting in extensive lists of essential oils composition. Apart from ripened seed capsules, in which monoterpene hydrocarbons was the major group of compounds, all the studied essential oils of this species were characterized by high levels of sesquiterpene hydrocarbons. Essential oils from plants collected during the winter, in the two experimental fields, had the highest accumulation of

sesquiterpene hydrocarbons, as well as the highest number of long chain *n*-alkanes and 1-alkenes. Differences were found in the major constituents of the essential oils studied. *(E)*–Caryophyllene, *β*-gurjunene, *γ*-muurolene, *(E)*-*γ*-bisabolene and *γ*-elemene were the main compounds responsible for the variations of the sesquiterpene hydrocarbons contents over the year in the essential oils of plants of *H. androsaemum.* The essential oils of the aerial parts and leaves shared *(E)*–caryophyllene as one of their major compounds; *γ*-elemene was common to essential oils of *in vitro* shoots, stems and aerial parts, and *α*-pinene, *β*-pinene and limonene were the major ones in the ripened seed capsules.

The work herein reported shows that the complex essential oils composition from *H. androsaemum* is season- and organ-dependent, being also influenced by *in vivo* or *in vitro* growth conditions.

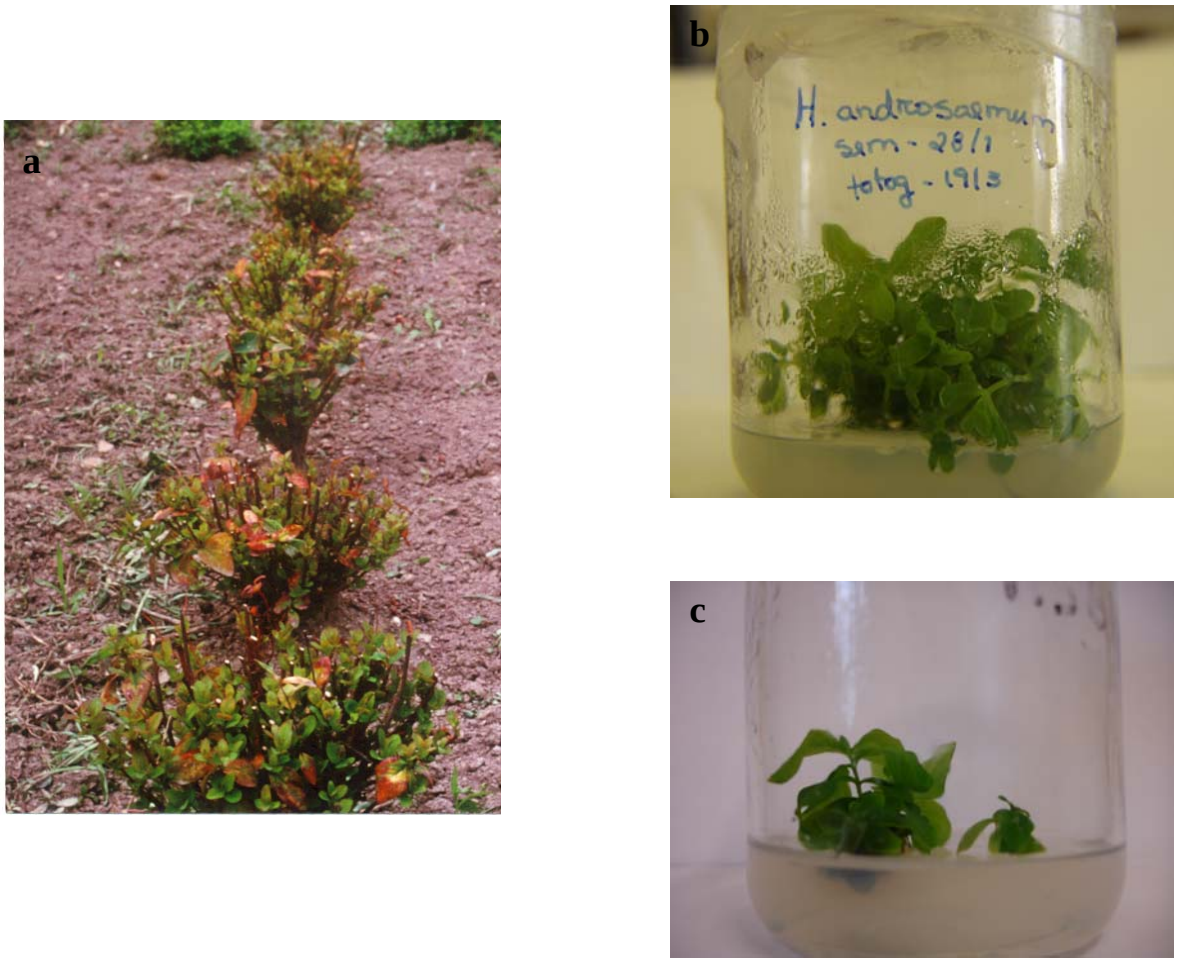


Figure 1 – **a)** Cultivated plants of *Hypericum androsaemum* L. **b)** and **c)** *In vitro* plantlets of *Hypericum androsaemum* grown on MS medium supplemented with 0.8 mg/L IAA and 0.5 mg/L KIN.

Carnduff, J., K. R. Hargreaves and A. Nechvatal (1966). "Constituents of the *Hypericum androsaemum*." *Phytochemistry* **5**(5): 1029-1029.

Dias, A., R. Seabra, P. Andrade and M. Fernandes-Ferreira (1999). "The development and evaluation of an HPLC-DAD method for the analysis of the phenolic fractions from in vivo and in vitro biomass of *Hypericum* species." *Journal of Liquid Chromatography & Related Technology* **22**(2): 215-227.

Dias, A. C. P., R. M. Seabra, P. B. Andrade, F. Ferreres and M. Fernandes-Ferreira (2000). "Xanthone biosynthesis and accumulation in calli and suspended cells of *Hypericum androsaemum*." *Plant Science* **150**(1): 93-101.

Kitanov, G. and P. Nedialkov (1998). "Mangiferin and isomangiferin in some *Hypericum* species." *Biochemical Systematics and Ecology* **26**: 647-653.

Mathis, C. and G. Ourisson (1964a). "Étude chimio-taxonomique du genre *Hypericum* : II. Identification de constituants de diverses huiles essentielles d'*Hypericum*." *Phytochemistry* **3**(1): 115-131.

Mathis, C. and G. Ourisson (1964b). "Étude chimio-taxonomique du genre *Hypericum* : III. Répartition des carbures saturés et des monoterpènes dans les huiles essentielles d'*Hypericum*." *Phytochemistry* **3**(1): 133-141.

Nielsen, H. and P. Arends (1979). "Xanthone constituents of *Hypericum androsaemum*." *Journal of Natural Products* **42**: 301-304.

Nogueira, T., F. Duarte, F. Venâncio, R. Tavares, M. Lousã, C. Bicchi and P. Rubiolo (1998). "Aspectos Quimiotaxonómicos do Género *Hypericum* L. em Portugal." *Silva Lusitana* **6**(1): 55-61.

Seabra, R. and A. Alves (1989). "Mangiferin and chlorogenic acid from *Hypericum* species." *Planta Medica* **55**: 404-405.

Seabra, R. and A. Alves (1990). "Flavonoids from *Hypericum* species." *Fitoterapia* **61**(2): 146-147.

3.2- Publications

This chapter comprises the following publications:

Guedes AP, Amorim LR, Vicente A & Fernandes-Ferreira M. 2004. Variation of the essential oil content and composition in leaves from cultivated plants of *Hypericum androsaemum* L.. *Phytochemical Analysis*, 15: 146-151.

Guedes AP, Amorim LR, Vicente A, Ramos G & Fernandes-Ferreira M. 2003. Essential oils from plants and *in vitro* shoots of *Hypericum androsaemum* L.. *Journal of Agriculture and Food Chemistry*, 51 (4): 1399-1404.

Variation of the Essential Oil Content and Composition in Leaves from Cultivated Plants of *Hypericum androsaemum* L.

Ana P. Guedes[1], Lúcia R. Amorim[1], Ana Vicente[2] and M. Fernandes-Ferreira[1]

[1]Department of Biology, University of Minho, Campus Gualtar, 4710-057 Braga, Portugal

[2]Direcção Regional de Agricultura de Entre Douro e Minho, Quinta de S. José, S. Pedro de Merelim, 4700-859 Braga, Portugal

Abstract

The amount and composition of the essential oil from leaves of *Hypericum androsaemum* L. cultivated in Arouca (Portugal) were determined in six samples harvested during 1 year at intervals of 2 months. The seasonally dependent essential oil content ranged from 0.7 mg/g biomass dry weight in September to 3.4 mg/g in February. The oil contained more than 80 compounds, 70 of which (constituting 88–93% of the total oil) were identified by GC and GC-MS. An approximation of the absolute quantification of each compound and compound class was performed using a GC method with an internal standard. The relative and the absolute content of each compound and compound class changed during the year. At the end of the winter and in the spring, the essential oil was dominated by sesquiterpene hydrocarbons and accumulated a high number of intermediate to long chain *n*-alkanes and 1-alkenes. In September, the essential oil contained the lowest levels of sesquiterpene hydrocarbons (43%) and the highest levels of 1-octene and 2-hexenal (38%). In February, the essential oil had the highest level of sesquiterpene hydrocarbons (73%) and the highest diversity of intermediate to long chain n-alkanes and 1-alkenes. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords: *Hypericum androsaemum*, essential oils, sesquiterpenes, *n*-alkanes

Introduction

Hypericum androsaemum L. has been widely used in Portugal in folk medicine as cholagogue, a hepatoprotector and a diuretic and also in kidney failure (Costa, 1987; Seabra *et al.*, 1992). In Portugal, *H. androsaemum* grows in the wild in shady sites, and especially in the

National Park of Peneda-Gerês where the native population is in danger of extinction owing to intensive harvesting. After harvesting, the wild plants are typically air dried and commercialised as an infusion. In order to meet the needs of consumers, this species was included in a Portuguese programme of integrated research and exploitation of medicinal plants which involved the establishment of four pilot plants at different sites in the northern region of Portugal.

The chemical characterisation of *H. androsaemum* is of crucial importance since the quality control of the commercialised biomass is intended to be based on the analysis of the most significant constituents of the essential oil and of the phenolic fraction of the plant. However, to our knowledge there are no conclusive data indicating which compounds are responsible for the putative medicinal properties ascribed to this species. Most of the recent phytochemical reports about *H. androsaemum* concern the composition of phenolic extracts. Phenolic acids and flavonoids (Seabra *et al.*, 1989; Seabra *et al.*, 1990; Seabra *et al.*, 1992; Dias *et al.*, 1999) as well as xanthones (Nielsen *et al.*, 1979) and xanthone *C*-glucosides (Kitanov *et al.*, 1998) have been identified in *H. androsaemum*. Phenolic acids, flavonoid compounds (Dias *et al.*, 1999) and xanthones (Dias *et al.*, 2000) were also identified in *in vitro* cultures of *callus* and suspended cells of this species. The essential oils of *H. androsaemum* has been less well studied. Nonane, α-pinene, β-pinene, myrcene, limonene, undecane (Mathis *et al.*, 1964a), geraniol and α-terpineol (Mathis *et al.*, 1964b) have been reported to be the main constituents of the oil, whilst five monoterpene and eight sesquiterpene compounds, along with nonane and undecane, were identified in the essential oil by Nogueira *et al*. (1998). α-Terpineol and hydrocarbon waxes ($C_{19}H_{40}$ $C_{21}H_{44}$ $C_{23}H_{48}$), were found in unripe seed capsules (Carnduff *et al.*, 1966). However, the available data are somewhat insufficient for the characterisation of the essential oil composition of *H. androsaemum*.

In this paper we report on the qualitative and quantitative composition of the essential oil from aerial parts of cultivated plants of *H. androsaemum* sampled six times during 1 year at intervals of about 2 months.

Experimental

Plant material

H. androsaemum plants were cultivated during March 1998 at an experimental farm of the Direcção Regional de Entre Douro e Minho (DRAEDM), in Arouca (northern Portugal), by planting strips of about 15 cm lenght obtained from wild plants growing near Ponte de Lima

(Facha, Portugal). A voucher specimen (reference H.a.-A1998) is maintained in the herbarium at ERCA/DRAEDM.

Sampling and hydrodistillation of essential oils.

In order to study the essential oil composition and its possible seasonal variation, cultivated plants were randomly pruned in July, September and November 1999, and in February, April and June 2000. Subsamples of fresh leaves (ca. 10 *g*) taken from the biomass removed during pruning were subjected to hydrodistillation with 500 mL of boiling water for 1 h in a Clevenger type apparatus using 1.0 mL of *n*-hexane, containing 5 α-cholestane (1 mg/mL), to retain the hydrodistilled components. Previous experiments had shown that 1 h was sufficient to remove all of the essential oil from 10 *g* of fresh leaves of *H. androsaemum* using this protocol.

GC analysis.

The essential oil samples were analysed by GC using a Perkin Elmer (Boston, MA, USA) Autosystem chromatograph equipped with a fused silica DB-5 (J & W Scientific, Folsom, CA, USA) column (30 m × 0.25 i.d.; 0.25 µm film thickness; 50% phenyl methylpolysiloxane as stationary phase). The oven temperature was increased at a rate of 3°C/min from 60–285°C; the injector was maintained at 300°C and the flame ionisation detector (FID) was at 320°C. Hydrogen was used as carrier gas at a flow rate of 1.49 mL/min under a column head pressure of 12.5 psi. Injections were performed in the split mode (1:13 split ratio). Three replicates of each sample were analysed and the percentage values of the listed compounds correspond to the values given in the GC report without correction factors. Percentage deviations for each sample were ≤10%, even for the most volatile components such as 1-octene and *n*-nonane.

Following the procedure reported earlier for *Salvia officinalis* (Santos-Gomes and Fernandes-Ferreira, 2001), 5α–cholestane was used as an internal standard for estimation of the specific content of each essential oil component. The use of the internal standard allowed for the differential responses of the FID and for discrimination of the essential oil components at the column inlet owing to the injector split ratio. Considering that the determination of individual correction factors is impracticable because of the large number of compounds present and the non-availability of commercial standards of many of them, compounds belonging to each of four classes of terpenoids, i.e. monoterpene hydrocarbons (MH), oxygenated monoterpenes (MO), sesquiterpene hydrocarbons (SH) and oxygenated sesquiterpenes (SO), were assumed to have

the same quantitative GC correction factor. Three replicates of mixtures with equal amounts of 5 α-cholestane and limonene (MH), camphor (MO), (*E*)-caryophyllene (SH) and (*E*,*E*)-farnesol (SO) were prepared and each was analysed in triplicate. The respective average correction values, adjusted for the known level of purity of each reference compound, namely, limonene 0.74, camphor 1.01, (*E*)-caryophyllene 0.75 and (*E*,*E*)-farnesol 1.02, were used as GC correction factors for each of the compounds in the corresponding class. A correction factor of 1 was assumed for compounds that did not belong to any of these classes such as the *n*-alkanes and 1-alkenes. Using the generalised response factors for compounds within the five classes, the derived quantitative data expressed in mg/g dry weight may be considered as an approximation of the absolute quantification. The sum of the specific contents of all individual essential oil compounds was taken as a parameter for the estimation of the total specific essential oil yield.

GC-MS analysis and identification of the compounds.

The essential oils from all samples were analysed by GC-MS using a Perkin-Elmer 8500 gas chromatograph equipped with a fused silica DB-5 column with a stationary phase similar to that described above and connected to a Finnigan MAT (San Jose, CA, USA) ion trap detector (ITD) running the manufacturer's software (version 4.1) and operating in EI mode at 70eV. Injector, interface and ion-source temperatures were 300, 260 and 220º C, respectively, whilst the oven temperature program and the injection conditions were as described above for GC. Helium was used as the carrier gas with a column head pressure of 12.5 psi. The identification of the compounds was performed with the help of MS libraries and confirmed by at least two alternative methods according to the recommendations of the International Organization of the Flavour Industry (1991). For compounds available as commercial standards (from Sigma, St. Louis, MO, USA), confirmation of identity was made by comparison of GC and GC-MS and retention times, and of the respective MS with those of reference compounds. The identities of those compounds not commercially available (indicated by an asterisk in Table 1) were confirmed using the terpene library incorporated into the computer data base by the GC-MS manufacturer, which allowed the comparison of MS from ITD and retention times on a DB-5 column, and by comparison with the published values (Adams, 1989; Adams, 2001) of the retention times and retention indices of compounds analysed under similar conditions on a DB-5 column.

Results and Discussion

The essential oil content of leaves of *H. androsaemum* plants cultivated at Arouca (Portugal) rose from a minimum of 0.7 mg/g dry weight in September 1999, to a maximum of 3.4 mg/g dry weight in February 2000 (Fig. 1), these yields are comparable with those (0.6-1.4 mg/g dry weight) reported by Mathis and Ourisson (1964c). The decrease in the essential oil content over the spring and summer period coincided with the highest growth of biomass (results not shown).

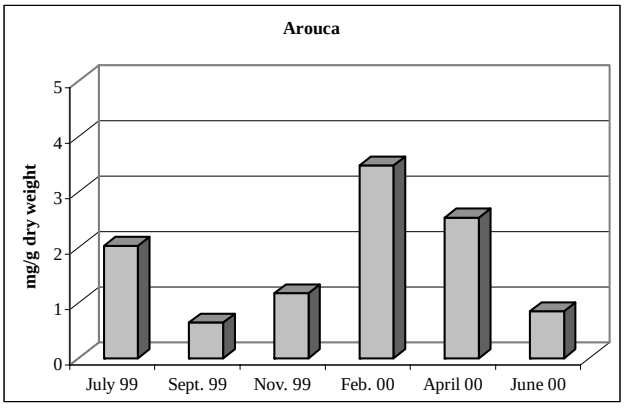


Figure 1 - Total essential oil content (mg/g dry weight) of leaves of *Hypericum androsaemum* plants cultivated at Arouca (Portugal) and harvested at different times during one year.

The composition of the essential oil obtained by hydrodistillation included more than 80 compounds, 70 of which (corresponding to 88–93% of the total oil) were identified (Table 1). The unidentified compounds included five sesquiterpene hydrocarbons and three oxygen-containing sesquiterpenes. The majority of the compounds could be assigned to six different classes: monoterpene hydrocarbons (MH), oxygen-containing monoterpenes (MO), sesquiterpene hydrocarbons (SH), oxygen-containing sesquiterpenes (SO), and *n*-alkenes/1-alkenes. The percentage of each of these classes present in the oil varied from one sample to another (Table 1), although SH constituted the main component class accounting for more than 50% of the oil content in five of the six samples (Table 1). Sesquiterpenes have already been considered to be the main components of the essential oil of this species (Nogueira *et al.*, 1998) and, despite the fact that the identities of most of them were unknown, responsible for its specific aroma (Nogueira *et al.*, 1999).

Table 1 - Composition (%) of the essential oils from plants of *Hypericum androsaemum* L. cultivated at Arouca, Portugal.

Compound	RI	July 99	Sept. 99	Nov. 99	Feb. 00	April 00	June 00
1-Octene	792	2.0	19.1	1.8	2.2	3.6	8.6
(*E*)-2-Hexenal	854	8.6	19.3	7.0	5.4	7.2	6.4
n-Hexanol	869	0.4	0.3	0.2	0.1	0.2	0.2
n-Nonane	900	9.1	2.4	4.0	0.6	5.2	6.0
α-Thujene	931	0.4	0.2	0.1	0.1	tr	0.2
α-Pinene	939	0.3	0.4	0.4	0.3	0.4	0.8
2,6-Dimethyloctane[d]	970	1.3	0.3	0.4	0.2	0.2	0.3
β-Pinene	980	2.2	2.1	1.9	1.7	2.5	3.6
Myrcene	991	0.2	0.3	0.4	0.2	0.2	0.4
Limonene	1030	1.6	2.3	2.4	1.6	2.0	4.0
1,8-Cineole	1033	0.0	tr	0.1	0.1	-	-
E-β-Ocimene	1051	0.2	0.7	1.0	0.2	0.4	0.8
γ-Terpinene	1060	tr	0.2	0.2	0.1	0.1	0.2
Terpinolene	1090	0.2	1.4	1.9	0.5	0.6	1.7
Undecane	1100	1.6	0.2	0.5	0.1	0.2	1.0
α-Thujone	1104	1.7	tr	0.6	0.4	0.2	1.0
β-Thujone	1115	-	-	0.1	0.1	-	-
Camphor	1146	tr	tr	0.1	0.1	-	-
δ-Elemene*	1337	0.2	0.3	1.4	0.3	0.4	0.3
α-Terpinyl acetate*	1350	0.1	0.3	0.2	0.1	0.1	0.2
α-Ylangene*	1374	tr	-	-	0.1	tr	tr
α-Copaene	1378	0.1	tr	0.1	0.1	0.1	tr
β-Bourbonene*	1387	0.1	tr	0.1	0.2	0.1	0.2
$C_{15}H_{24}$	1388	0.2	tr	0.1	0.4	0.2	0.2
β-Elemene*	1391	1.6	1.0	1.3	2.8	2.5	1.9
(*E*)-Caryophyllene	1419	13.7	9.0	17.0	13.5	13.4	11.4
cis-Thujopsene	1430	0.4	0.3	0.3	0.5	0.5	0.3
β-Gurjunene	1432	13.2	7.9	10.6	14.7	14.8	11.2
α-*trans*-Bergamotene*	1435	0.1	-	0.1	0.1	tr	0.1
α-Guaiene* + β-Humulene	1440 + 1439	0.1	tr	0.2	0.1	0.1	tr
$C_{15}H_{24}$	1447	3.2	2.6	3.9	2.9	3.0	2.9
α-Humulene	1454	0.1	tr	0.1	0.2	0.1	0.1
Allo-Aromadendrene	1459	tr	-	tr	0.1	tr	tr
Germacrene D isomer (?)[e]	1462	tr	-	-	0.2	-	-
β-Chamigrene	1478	tr	tr	tr	tr	tr	tr

Compound	RI	July 99	Sept. 99	Nov. 99	Feb. 00	April 00	June 00
γ-Muurolene*	1481	2.5	1.9	3.0	3.7	3.2	2.9
Germacrene D* + γ-Curcumene*	1485 + 1484	9.0	5.0	8.2	7.6	7.7	4.3
β-Selinene*	1488	0.8	0.3	0.8	0.9	0.6	0.9
α-Selinene*	1497	0.2	0.6	0.1	0.1	0.1	0.1
Germacrene B (?)[f]	1498	0.8	0.5	0.9	1.1	0.5	0.7
α-Zingiberene*	1499	0.6	0.4	0.5	0.6	0.5	0.4
$C_{15}H_{24}$	1500	0.7	tr	0.1	0.2	0.2	0.2
α-Muurolene*	1502	0.2	tr	0.1	0.1	0.1	tr
α-(*E,E*)-Farnesene*	1505	0.1	0.2	1.6	0.2	0.1	0.1
Z-γ-Bisabolene*	1515	0.1	0.2	0.2	0.5	0.2	0.6
δ-Cadinene	1523	0.4	0.3	0.5	0.4	0.2	0.4
E-γ-Bisabolene*	1530	0.9	1.1	1.3	1.8	0.9	2.1
$C_{15}H_{24}$	1534	0.4	0.5	0.8	0.5	0.4	0.8
$C_{15}H_{24}$	1536	0.9	1.1	1.4	1.8	0.9	2.2
γ-Elemene (?)[f]	1554	14.4	9.8	14.2	17.3	17.0	9.3
Caryophyllene oxide	1581	tr	0.3	0.2	0.3	0.1	0.2
$C_{15}H_{24}O$	1601	0.1	0.3	0.2	0.1	0.1	0.2
$C_{15}H_{24}O$	1618	0.2	0.4	0.4	0.7	0.5	1.2
epi-α-Cadinol*	1639	0.2	0.4	0.4	0.9	0.2	0.4
epi-α-Muurolol*	1642	0.6	1.2	1.0	0.6	0.4	0.5
δ-Cadinol*	1644	0.8	-	-	0.9	0.1	0.2
$C_{15}H_{24}O$	1646	0.8	1.3	1.3	0.9	0.7	1.2
α-Eudesmol*	1652	0.2	-	0.1	0.2	-	-
α-Cadinol*	1655	0.2	0.5	0.5	0.4	0.4	0.8
epi-α-Bisabolol*	1677	tr	tr	0.1	0.2	tr	0.2
8-Cedrane-13-al (?)[d]	1691	0.1	tr	0.1	0.3	0.1	0.2
n-Eicosane	2000	-	-	-	0.1	-	tr
1-Heneicosene	2095	-	-	-	tr	-	-
n-Heneicosane	2100	-	-	-	0.1	tr	tr
1-Docosene	2194	-	-	-	0.1	-	-
n-Docosane	2200	-	-	-	0.2	0.1	0.2
1-Tricosene	2295	-	-	-	0.1	-	tr
n-Tricosane	2300	tr	-	0.4	0.7	0.4	0.3
1-Tetracosene	2395	-	-	-	0.6	0.1	0.1
n-Tetracosane	2400	tr	-	-	0.4	0.1	0.3
1-Pentacosene	2495	-	tr	-	0.1	-	tr
n-Pentacosane	2500	tr	tr	-	1.5	1.3	1.0

Compound	RI	July 99	Sept. 99	Nov. 99	Feb. 00	April 00	June 00
1-Hexacosene	2596	-	-	-	0.2	0.1	0.1
n-Hexacosane	2600	-	-	-	0.2	0.1	0.3
n-Heptacosane	2700	tr	0.3	-	0.8	1.5	1.0
1-Octacosene	2797	-	0.9	-	0.1	tr	0.1
n-Octacosane	2800	-	-	-	0.1	0.1	-
n-Nonacosane	2900	tr	tr	-	0.2	0.6	0.9
Grouped components							
Monoterpene hydrocarbons		5.2	7.6	8.2	4.7	6.2	11.5
Oxygen-containing monoterpenes		1.7	tr	0.9	0.7	0.2	1.0
Sesquiterpene hydrocarbons		65.2	42.8	68.6	72.7	67.6	52.8
Oxygen-containing sesquiterpenes		3.3	4.3	4.2	5.5	2.8	4.9
n-Alkanes		10.7	2.9	4.9	4.9	9.6	10.8
1-Alkenes		2.0	20.1	1.8	3.3	3.9	8.9
Others		10.5	20.0	7.4	5.8	7.8	7.0
Unknowns		1.4	2.3	4.0	2.4	1.9	3.1

[a] Identifications were confirmed using authentic standards except for compounds marked with an asterisk, whose identifications were confirmed by comparison of the respective MS and retention times with those of a terpene library and by comparison with retention times and retention indices on a DB-5 column as reported by Adams (1989, 2001).
[b] RI, retention index as determined on a DB-5 column using the homologous series of *n*-alkanes.
[c] tr, trace amounts.
[d] Tentative identification based on MS only.
[e] Identification suggested by the terpene library but not confirmed by other sources.
[f] Both MS and retention times match with the corresponding compounds from the terpene library and the respective retention times coincide with the corresponding compounds described in Adams (1989). However, according to Adams (2001), the retention indexes on a DB-5 column would be higher for germacrene B and lower for γ-elemene.

With the exception of plant sample harvested in September 1999 and June 2000, the four main sesquiterpenes, namely (*E*)-caryophyllene (9.0–17.0%), β-gurjunene (7.9–14.8%), curcumene (4.3–9.0%), and the putative γ-elemene (9.3–17.3%), were responsible for more than 50% of the total essential oil. 1-Octene and 2-hexenal were also present in high amounts, especially in samples harvested in September 1999 where these two were the major components (Table 1). In samples harvested in spring and in the beginning of summer, *n*-nonane was also among the major constituents of the essential oil.

With the exception of linalool, α-(*E*,*E*)-farnesene and bicyclogermacrene, the compounds identified in the present study include those previously reported by Nogueira *et al.* (1998), and six previously identified by Mathis and Ourisson (1964a). However, geraniol and α-terpineol, previously reported by Mathis and Ourisson (1964b) as being constituents of the essential oil of this species, were not found in our samples. One of the most striking features of the essential oil was the accumulation of *n*-alkanes and 1-alkenes particularly in samples

harvested in February, April and June 2000. *n*-Nonadecane, *n*-heneicosane, and *n*-tricosane have previously been identified in unripe seed capsules of *H. androsaemum* (Carnduff *et al.*, 1966), and the presence of a complete series of *n*-alkanes from $C_{16}H_{34}$ to $C_{29}H_{60}$ in dried leaf material of this species has been reported (Brondz *et al.*, 1983). However, to our knowledge, the presence of an almost complete series of *n*-alkanes, from $C_{20}H_{42}$ to $C_{29}H_{60}$, and 1-alkenes within the same range, in the essential oil of *H. androsaemum* has not been reported before.

The variation in the percentage of total SH in the essential oil of *H. androsaemum* coincided with the variation in the specific content (mg/g) of total SH and the oil as obtained by hydrodistillation of the plant material (Fig. 2).

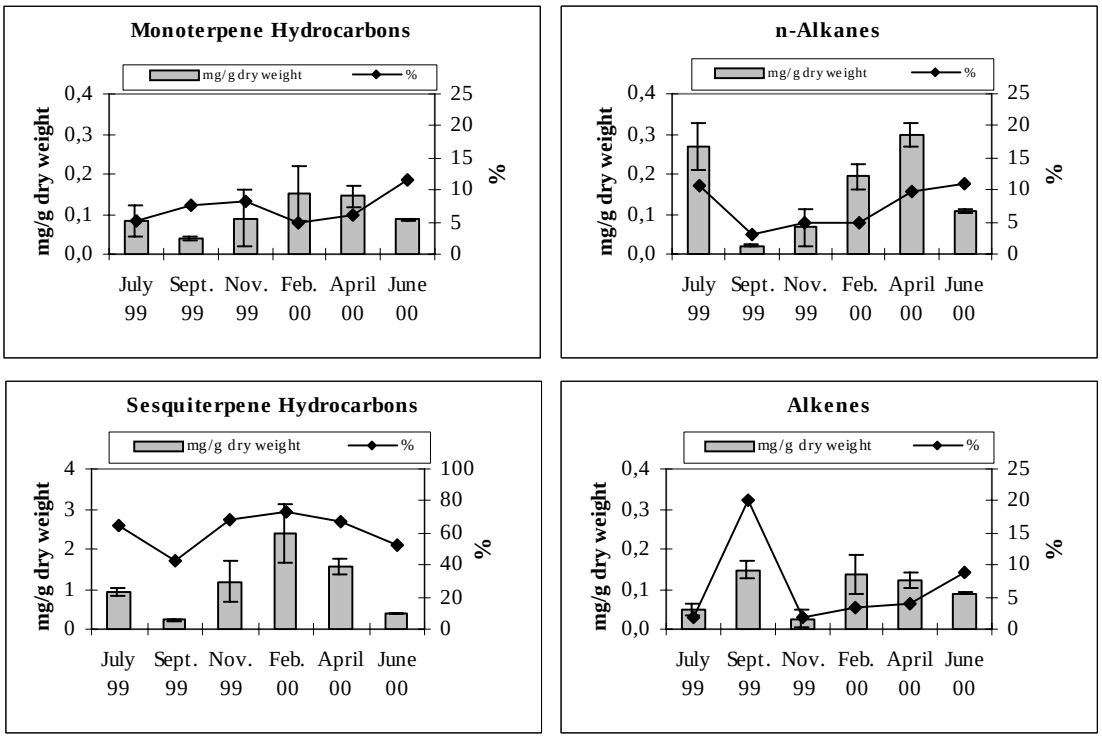


Figure 2 - Specific content (mg/g dry weight) and respective percentages of the major classes present in the essential oil isolated from leaves of *Hypericum androsaemum* plants cultivated at Arouca (Portugal) and harvested at different times during one year.

In a previous study performed on *S. officinalis* cultivated at the same experimental site in Arouca (Portugal), a similar variation in the SH content of the oil was recorded, although the maximum percentage and the maximum specific content were attained in April rather than in February (Santos-Gomes *et al.*, 2001). The percentage values of the total MH varied in a manner opposite to those of SH, particularly between February and June 2000 during which period the percentage were very similar to those of the total amount of *n*-alkanes and of 1-alkenes (Fig. 2). The variations in the specific contents of SH and MH were consistent with the variations of the

total essential oil content, while that of the *n*-alkanes diverged from February to April 2000 and that of the 1-alkenes diverged from July to September 1999 (Figs. 1 and 2). The divergence in the *n*-alkanes is mainly explained by the differential accumulation of *n*-nonane which was relatively high in April and low in February 2000 (Fig. 3), whilst the divergence in the 1-alkenes was primarily due to the high accumulation of 1-octene in September 1999 (Fig. 3).

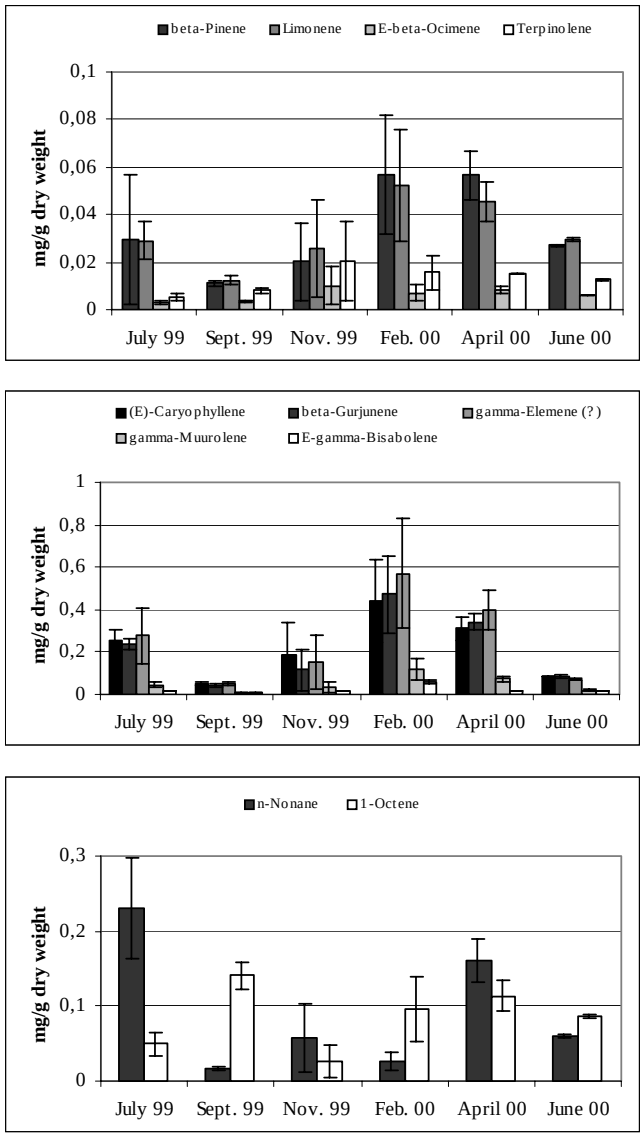


Figure 3 - Specific content (mg/g dry weight) of the main representative compounds in the classes (**A**) monoterpene hydrocarbons, (**B**) sesquiterpene hydrocarbons, and (**C**) *n*-alkanes, and 1-alkenes in the essential oil isolated from leaves of *Hypericum androsaemum* plants cultivated at Arouca (Portugal) and harvested at different times during one year.

The components mainly responsible for the variations in percentages and specific contents of the MH and the SH classes were *β*-pinene, limonene, terpinolene and (*E*)-*β*-ocimene, and (*E*)-caryophyllene, *β*-gurjunene, *γ*-muurolene, (*E*)-*γ*-bisabolene and the putative *γ*-

elemene, respectively (Fig. 3).

According to Quer (1995) plant material of *H. androsaemum* for herbal use is typically harvested in June at which time the five major components of the essential oil of plants cultivated at Arouca were, in order of decreasing amount, three sesquiterpenes, namely, (*E*)-caryophyllene, *β*-gurjunene and the putative *γ*-elemene, together with 1-octene and (*E*)-2-hexenal. With respect to the ranking of these five components, the most divergent composition was found in the essential oil from samples harvested in September where (*E*)-2-hexenal and 1-octene were the first and the second major components followed by the three SH. Additionally the essential oil isolated in September showed a very low diversity of intermediate to long chain *n*-alkanes and 1-alkenes in contrast with the oil isolated in June whose diversity with respect to these types of compounds was amongst the highest.

In conclusion, it is demonstrated that the composition of the essential oil from *H. androsaemum* changes over the year and is dominated by sesquiterpene hydrocarbons together with a high number of intermediate to long chain *n*-alkanes and 1-alkenes which accumulate at the end of winter and during spring. Therefore, the composition of the essential oil of this medicinal plant depends largely on the harvest time: in September the essential oil shows a lower level of SH (43%) and the highest level of 1-octene and 2-hexenal (38%), whilst in February the essential oil contains the highest level of SH (73%) and the highest diversity of intermediate to long chain *n*-alkanes and 1-alkenes.

Acknowledgements

This work was sponsored by the Programme PRAXIS XXI/Ad.I/Project P-1304 and by the Programme AGRO/8/ key action 8.1/Project 338.

References

Adams, R. P. (1989). *Identification of Essential Oils by Ion Trap Mass Spectroscopy.* San Diego, CA, Academic Press.

Adams, R. P. (2001). *Identification of Essential Oils Components by Gas Chromatography/Quadropole Mass Spectroscopy.* Illinois, USA, Allured Publishing Corporation, Carol Stream.

Brondz, I., T. Greibrokk and A. Aasen (1983). "*n*-Alkanes of *Hypericum perforatum*: a revision." *Phytochemistry* **22**: 295-296.

Carnduff, J., K. R. Hargreaves and A. Nechvatal (1966). "Constituents of the *Hypericum androsaemum*." *Phytochemistry* **5**(5): 1029-1029.

Costa, A. (1987). Farmacognosia. A. Costa. Lisboa, Fundação Calouste Gulbenkian. **Vol II:** 1021-1022.

Dias, A., R. Seabra, P. Andrade and M. Fernandes-Ferreira (1999). "The development and evaluation of an HPLC-DAD method for the analysis of the phenolic fractions from *in vivo* and *in vitro* biomass of *Hypericum* species." *Journal of Liquid Chromatography & Related Technology* **22**(2): 215-227.

Dias, A. C. P., R. M. Seabra, P. B. Andrade, F. Ferreres and M. Fernandes-Ferreira (2000). "Xanthone biosynthesis and accumulation in *calli* and suspended cells of *Hypericum androsaemum*." *Plant Science* **150**(1): 93-101.

Kitanov, G. and P. Nedialkov (1998). "Mangiferin and isomangiferin in some *Hypericum* species." *Biochemical Systematics and Ecology* **26**: 647-653.

Mathis, C. and G. Ourisson (1964a). "Étude chimio-taxonomique du genre *Hypericum* : II. Identification de constituants de diverses huiles essentielles d'*Hypericum*." *Phytochemistry* **3**(1): 115-131.

Mathis, C. and G. Ourisson (1964b). "Étude chimio-taxonomique du genre *Hypericum* : III. Répartition des carbures saturés et des monoterpènes dans les huiles essentielles d'*Hypericum*." *Phytochemistry* **3**(1): 133-141.

Nielsen, H. and P. Arends (1979). "Xanthone constituents of *Hypericum androsaemum*." *Journal of Natural Products* **42**: 301-304.

Nogueira, T., F. Duarte, R. Tavares, M. Curto, J. Capelo and A. Freitas (1999). "Comparative study of the aromas of *Hypericum* L. species from Portugal using olfactroscopy." *Flavour and Fragrance Journal* **14**: 195-199.

Nogueira, T., F. Duarte, F. Venâncio, R. Tavares, M. Lousã, C. Bicchi and P. Rubiolo (1998). "Aspectos Quimiotaxonómicos do Género *Hypericum* L. em Portugal." *Silva Lusitana* **6**(1): 55-61.

Santos-Gomes, P. C. and M. Fernandes-Ferreira (2001). "Organ and season-dependent variation in the essential oil composition of *Salvia officinalis* L. cultivated in two different sites." *Journal of Agricultural and Food Chemistry* **49**: 2908-2916.

Seabra, R. and A. Alves (1989). "Mangiferin and chlorogenic acid from *Hypericum* species." *Planta Medica* **55**: 404-405.

Seabra, R. and A. Alves (1990). "Flavonoids from *Hypericum* species." *Fitoterapia* **61**(2): 146-147.

Seabra, R. M., M. H. Vasconcelos, M. A. C. Costa and A. C. Alves (1992). "Phenolic compounds from *Hypericum perforatum* and *Hypericum undulatum*." *Fitoterapia* **68**: 473-474.

Essential Oils from Plants and *in Vitro* Shoots of *Hypericum androsaemum* L.

Ana P. Guedes[‡], Lúcia R. Amorim[‡], Ana Vicente[§], Gilda Ramos[⊥], and Manuel Fernandes-Ferreira[‡]

[‡]Department of Biology, University of Minho, Campus Gualtar, 4710-057 Braga, Portugal

[§]ERCA/DRAEDM, S. Pedro de Merelim, Braga, Portugal

[⊥]BIOROPE, Rua da Igreja, 4485-242 Guilhabreu, Portugal

Abstract

The essential oil yields obtained by hydrodistillation of the aerial parts of *Hypericum androsaemum* cultivated plants varied from 0.94 to 4.09 mg/g of biomass dry weight, depending of the harvest time. The respective analyses performed by gas chromatography and gas chromatography-mass spectrometry revealed more than 80 compounds, 72 of which were identified. Most of the compounds were sesquiterpene hydrocarbons, which, depending of the harvest time, corresponded to 43-78% of the total essential oil. The other compounds were distributed as monoterpene hydrocarbons, oxygen-containing sesquiterpenes, *n*-alkanes, 1-alkenes, and oxygen-containing monoterpenes, these being a minor group. In *H. androsaemum* in vitro shoots, sesquiterpene hydrocarbons represented >80% of the respective essential oil. Differences in the essential oil composition were found depending on the harvest time and origin, in vivo versus in vitro, of the plant material. The essential oil sampled in November was characterized by the highest levels of sesquiterpene hydrocarbons and a high number of *n*-alkanes and 1-alkenes, from C_{18} to C_{28}, whereas that sampled in June of the following year showed the highest levels of *n*-nonane and 1-octene as well as monoterpene hydrocarbons, the second most representative group.

Keywords: *Hypericum androsaemum*; *in vitro* shoots; essential oils; sesquiterpenes

Introduction

Hypericum androsaemum L. grows wild in shadowy sites, namely, in the northern region of Portugal where it is widely used as a medicinal herb. According to some authors this species is used in popular medicinal preparations as a cholagogue, hepatoprotector, and diuretic and in kidney failure *(1, 2)*. Usually, in northern Portugal, wild-growing *H. androsaemum* plants are harvested, air-dried, and sold by the local people to tourists, who use it to prepare the tea called "hipericão do Gerês". However, due to intensive harvesting, the local wild populations of this species are in risk of disappearing.

To meet the needs of the consumers, we established four small *H. androsaemum* experimental fields in northern Portugal where the species is being propagated. The respective chemical characterization is also one of the objectives of an integrated research and development program, which has as its goal to restock the local *H. androsaemum* populations and increase the overall knowledge on this species as well as to support its commercial and industrial exploitation.

Most of the few recent reports on the chemical characterization of this species concern the composition of phenolic extracts. Phenolic acids and flavonoids *(2-5)*; as well as xanthones *(6)* and xanthone C-glucosides *(7)* have been identified in *H. androsaemum* plants. The essential oil of this species has been less studied. Nonane, α-pinene, β-pinene, myrcene, limonene, undecane *(8)* as well as geraniol and α-terpineol *(9)* were identified as constituents of the *H. androsaemum* essential oil. The presence of α-terpineol and hydrocarbon waxes ($C_{19}H_{40}$, $C_{21}H_{44}$, and $C_{23}H_{48}$), in the *H. androsaemum* unriped seed capsules, was also reported *(10)*. Five monoterpene and eight sesquiterpene compounds as well as the *n*-alkanes nonane and undecane were identified in the essential oils of this species *(11)*. However, the available data for chemical characterization of the *H. androsaemum* essential oil are scanty. Less is known on metabolites produced by in vitro cultures of this species. Phenolic acids and flavonoid compounds *(5)* and xanthones *(12)* were identified in in vitro cultures of calli and suspended cells of this species. Up to now, however, we have found no studies on essential oils produced by in vitro cultures of *H. androsaemum*.

In recent years we have performed the micropropagation of *H. androsaemum* with the aim of helping to restock this species. Parallelly with the characterization of the essential oils of the *H. androsaemum* in vivo plants we consider that the capacity of the in vitro shoot cultures of this species in producing essential oils would deserve to be evaluated and the respective composition determined. In this paper we report the yields and composition of essential oils

accumulated in the aerial part of in vivo *H. androsaemum* plants, cultivated at Arcos de Valdevez, as well as the yield and composition of the essential oil accumulated in the respective in vitro shoots.

Material and Methods

In Vivo Cultivated Plants and Establishment of *in Vitro* shoot cultures.

H. androsaemum L. plant cultures were established in March 1998 at a DRAEDM experimental farm located at Arcos de Valdevez (northern Portugal). The cultivation was performed by planting ±15 cm long slips obtained from wild *H. androsaemum* plants growing near Ponte de Lima (Facha). A voucher specimen (herbarium voucher ref. H.a.-AV1998) is maintained in ERCA/DRAEDM.

In vitro shoot cultures of *H. androsaemum* were established on chemically defined medium containing the macronutrients of Margara N30K medium (13) and the micronutrients and organic constituents of the Murashige and Skoog (MS) medium (14) with the exception for thiamin, which was used at 0.8 mg/L. Ascorbic acid at 3 mg/L, with 30g/L sucrose, 0.5 mg/L benzylaminopurine (BAP), 0.05 mg/L α-naphthaleneacetic acid (NAA), and 0.05 mg/L gibberellin (GA_3), was added to the medium before pH adjustment at 5.7. Agar at 8 g/L was added to solidify the medium before autoclaving at 120 ºC for 20 min. Apical buds from wild plants growing in Facha were excised, surface sterilized with a solution of 2% formaldehyde in 70% ethanol, during 10 min, and used as primary explants in the establishment of the cultures. The MS medium supplemented with 0.8 mg/L indol-3-acetic acid (IAA) and 0.5 mg/L kinetin (KIN) was used in multiplication of the shoot cultures. Cultures were maintained in a growth room at 24±2ºC and 60-65% humidity with a photoperiod of 16h of light/8h of darkness. Illumination was supplied by cool white fluorescent tubes with a light intensity of 52$\mu mol \cdot m^{-2} \cdot s^{-1}$. The cultures were subcultivated on the multiplication MS medium with the interval of 2 months.

Hydrodistillation and Analysis of the Essential Oils.

For the study of the essential oil composition, some cultivated plants were randomly pruned in July and November 1999 and in June 2000, and subsamples of ~10 g of fresh biomass of the pruned branches were subjected to hydrodistillation in a Clevenger-type apparatus over 1 h, using volumes of 1.0 mL of *n*-hexane, containing 5-α-cholestane (1mg/mL), for retention of the hydrodistillate components. The humidity percentage from fresh

biomass of the samples submitted to hydrodistillation and harvested in July and November 1999 and June 2000 were 79.6, 81.6, and 74.4%, respectively. The dry weight of the plant material was determined after the respective drying at 60 ºC in a drying stove during 72 h. The same procedure was followed in the hydrodistillation of 6-month-old *in vitro* fresh shoots (91.9% of humidity) maintained by subculturing, on the multiplication MS medium, during ~2 years.

The hydrodistillates from all samples were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC- MS). GC analyses were performed using a Perkin-Elmer Autosystem gas chromatograph equipped with a fused silica DB-5 column (30m long × 0.25 i.d., 0.25 μm film thickness composed by 5% phenyl methylpolysiloxane, J&W Scientific). The temperature program was as follows: 60-285ºC at 3ºC min^{-1} for the column, 300ºC for the injector, and 300ºC for the flame ionization detector (FID). H_2 was used as carrier gas with a flow rate of 1.49 mL/min under a column head pressure of 12.5 psi. Injections were performed in a split/splitless injector with the splitter opened at a 1:13 split ratio. Three replicates of each sample were processed in the same way. Percentage values from the listed compounds correspond to the values given in the GC report without correction factors.

Following the procedure reported before for *Salvia officinalis* (15), 5α-cholestane was used as an internal standard for estimation of the specific content of each essential oil compound. This internal standard accounted for the differential responses of the FID and for the column inlet discrimination of the essential oil compounds due to the injector split ratio. Considering that the determination of individual correction factors is impractical, due to either the high number of compounds or their absence in the market, compounds of a given group (monoterpene hydrocarbons, oxygen-containing monoterpenes, sesquiterpene hydro- carbons, and oxygen-containing sesquiterpenes) were assumed to have the same GC response factor. Three replicates of mixtures at equal amounts of 5α-cholestane and limonene (monoterpene hydrocarbon), camphor (oxygen-containing monoterpene), (*E*)-caryophyllene (sesquiterpene hydrocarbon), and (*E*,*E*)-farnesol (oxygen-containing sesquiterpene) were prepared and injected three times each. The respective average correction values, corrected for the purity grade of each reference compound, were 0.741 (limonene), 1.014 (camphor), 0.747 [(*E*)-caryophyllene], and 1.018 [(*E*,*E*)-farnesol]. These values were used as GC response factors of the compounds of the corresponding group from *H. androsaemum* essential oil. A correction factor of 1 was assumed for compounds that did not belong to any of these groups, as they are the cases of *n*-alkanes and 1-alkenes. The sum of the specific contents of all individual essential oil

compounds was assumed as a parameter for the determination of the total specific essential oil yield. Given the generalization of each response factor to all compounds from the same group, all of the quantitative data expressed in micrograms of the compound per gram of biomass dry weight may be considered as a tentative of approximation to the absolute quantification.

GC-MS analyses were performed with a Perkin-Elmer 8500 gas chromatograph equipped with a fused silica DB-5 column, as that of GC, connected with a Finnigan MAT ion trap detector (ITD; software version 4.1) operating in EI mode at 70 eV. Injector, interface, and ion source temperature were 300, 260, and 220ºC, respectively. The oven temperature program and injection conditions were as above-described for GC. Helium was used as carrier gas with a column head pressure of 12.5 psi. The identification of the compounds was performed with the help of mass spectral libraries. The compounds were considered to be identified when the respective identity was confirmed by at least two methods according to recommendations of the International Organization of the Flavour Industry *(16)*. The identification of some compounds (those not denoted in **Table 1**) was confirmed by using authentic standards (all of them from the Sigma-Aldrich group). In the confirmation of the identity of compounds, not available in the market, a terpene library incorporated in the computer database by the GC-MS supplier was used, which allows the comparison of mass spectra from ITD and retention times on DB-5. The coherence of the retention times of the analyzed compounds with the retention times obtained in similar conditions with a DB-5 column and published in the literature, namely, in refs *17* and *18*, constituted an additional criterion in the confirmation of the respective identity.

Results and Discussion

Multiplication and Maintenance of *in Vitro* Shoot Cultures.

The new shoots formed from the apical buds cultivated on the establishment medium were visible 2-3 weeks after the beginning of the culture. The subculture of nodal shoot segments to the MS medium supplemented with 0.8 mg/L IAA and 0.5 mg/L KIN allowed the multiplication of the cultures by a factor of ~5, with intervals of 2 months. However, due to slow growth, shoots could be maintained in the same vessel, without subculture, during several (6-8) months. Two years after the establishment of the *H. androsaemum in vitro* cultures, either the shape or the multiplication rate of the shoots was maintained without apparent change. Micropropagation was accomplished by transferring the shoots to half-strength MS medium

without growth regulators, for their elongation, followed by auxin shock for rhizogenesis induction (results not shown).

Yields and Composition of Essential Oils Produced by *in Vivo* Cultivated Plants.

The essential oil yield obtained in the hydrodistillation of aerial parts of *H. androsaemum* cultivated plants ranged from 0.9 to 4.1 mg/g of biomass dry weight depending of the harvested time (**Figure 1**). Variations in the essential oil content of *H. androsaemum*, from 0.6 to 1.4 mg/g, had already been reported by other authors *(19)*. Drastic variations in the essential oil contens of plants from another species (*S. officinalis*), cultivated at the same site during the same time period, had already been reported *(15)*.

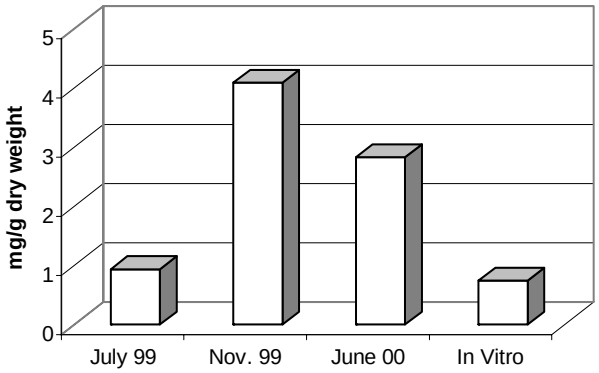


Figure 1 - Essential oil contents in in vitro shoots of *H. androsaemum* and in the respective in vivo plants, cultivated at Arcos de Valdevez and harvested in the months of July and November 1999 and June 2000.

More than 80 compounds were detected in the hydrodistillates from *H. androsaemum in vivo* plants, 72 of which were identified, with 4 of them remaining doubtful (**Table 1**). Depending on the harvest time, the identified compounds correspond to 80-90% of the total essential oil specific amount. With the exceptions of linalool, *trans*-β-farnesene, and bicyclogermacrene, the compounds previously reported by Nogueira *et al*. *(11)* were found in our samples as well as all six of the ones identified by other authors *(8)*. Geraniol and α-terpineol previously reported as constituents of the essential oil of this species *(9)*, were not found in our samples. The unidentified compounds include 7 sesquiterpene hydrocarbons and 3 oxygen-containing sesquiterpenes.

For plants harvested in July 1999 and June 2000, 69 and 71 compounds, respectively, were detected in the essential oils (**Table 1**).

Table 1 - Specific Compound Contents and Percentage Composition of the Essential Oils from In Vivo Plants Cultivated at Arcos de Valdevez, Northern Portugal, and from in vitro shoots of *Hypericum androsaemum* L.

Compound	Ret. Time (s)	*In vivo* cultivated plants						*In vitro* shoots	
		July 99		Nov. 99		June 00			
		µg/g dw	%	µg/g dw	%	µg/g dw	%	µg/g dw	%
1-Octene	142	10.7	0.9	81.4	1.6	249.0	7.3	1.0	0.2
2-Hexenal	176	31.1	2.4	275.1	5.3	271.9	7.9	2.3	0.3
n-Hexanol	185	1.4	tr	5.2	0.1	4.5	0.1	1.0	0.2
n-Nonane	214	36.5	2.9	69.8	1.3	121.8	3.6	3.8	0.3
α-Thujene	249	1.6	tr	4.3	0.1	3.3	0.1	0.5	tr
α-Pinene	258	5.6	0.5	13.4	0.3	81.3	3.2	–	–
2,6-Dimethyloctane	303	5.2	0.5	8.2	0.2	7.2	0.2	3.1	0.3
β-Pinene	314	38.2	4.2	85.2	2.2	163.5	6.4	14.8	2.1
Myrcene	334	9.2	0.9	10.9	0.3	34.4	1.3	1.2	0.2
Limonene	397	52.0	5.9	82.7	2.1	393.1	15.4	9.2	1.3
1,8-Cineol	401	1.6	tr	3.8	0.1	–	–	–	–
E-β-Ocimene	433	12.7	1.4	24.6	0.7	16.0	0.6	1.0	0.2
γ-Terpinene	453	3.0	0.5	3.8	0.1	4.7	0.2	0.5	tr
Terpinolene	512	36.3	3.9	55.0	1.4	39.0	1.5	4.2	0.6
Undecane	536	7.2	0.6	5.8	0.1	28.6	0.9	36.0	3.8
α-Thujone	549	5.8	0.5	14.1	0.3	14.3	0.5	–	–
β-Thujone	574	1.0	tr	0.6	tr	1.6	tr	–	–
Camphor	633	1.5	tr	0.7	tr	–	–	–	–
δ-Elemene	1116	3.9	0.6	15.5	0.4	5.1	0.2	0.4	tr
α-Terpinenyl acetate	1152	3.0	0.5	6.1	0.1	6.1	0.2	1.4	0.2
α-Ylangene	1201	0.6	tr	0.8	tr	0.8	tr	2.3	0.3
α-Copaene	1213	0.8	tr	3.5	0.1	2.0	0.1	–	–
β-Bourbonene	1234	0.8	tr	8.5	0.2	2.0	0.1	0.8	0.2
$C_{15}H_{24}$	1241	1.1	tr	9.1	0.2	2.7	0.1	0.4	tr
β-Elemene	1257	9.8	1.1	104.2	2.7	30.1	1.2	6.8	1.0
β-Caryophyllene	1325	115.1	12.5	585.0	15.1	243.1	9.4	36.1	5.0
Thujopsene	1344	1.7	tr	20.0	0.5	5.9	0.2	2.2	0.3
β-Gurjunene	1348	54.9	6.1	601.1	15.5	192.3	7.6	61.2	8.6
(*Z*)-*trans*-α-Bergamotene	1360	1.0	tr	2.6	0.1	1.0	tr	1.1	0.2
α-Guaiene + β-Humulene	1370	12.4	3.4	2.7	0.1	2.1	0.1	12.5	1.8
$C_{15}H_{24}$	1390	18.7	3.4	127.6	4.9	64.5	2.5	8.3	1.2
α-Himachalene	1399	0.6	tr	–	–	–	–	–	–
α-Humulene	1411	0.3	tr	5.0	0.1	1.2	0.1	4.3	0.7

Compound	Ret. Time (s)	*In vivo* cultivated plants						*In vitro* shoots	
		July 99		Nov. 99		June 00			
		µg/g dw	%	µg/g dw	%	µg/g dw	%	µg/g dw	%
Allo-Aromadendrene	1427	–	–	2.1	0.1	0.8	tr	0.8	0.2
Germacrene D isomer #1	1435	6.5	0.5	–	–	–	–	–	–
Unknown	1464	5.0	0.5	21.9	0.4	14.0	0.4	21.4	2.2
β-Chamigrene	1468	tr	tr	–	–	–	–	–	–
Germacrene D isomer #3	1470	21.9	1.9	–	–	–	–	–	–
γ-Muurolene	1479	36.1	3.8	170.1	4.4	54.4	2.1	108.8	15.3
Germacrene D + *γ*-Curcumene	1487	43.0	5.0	298.6	7.7	149.3	5.8	30.7	4.3
β-Selinene	1493	5.6	0.8	36.6	0.9	17.0	0.6	–	–
α-Selinene	1514	4.3	0.5	4.9	0.1	1.6	0.1	1.5	0.2
Germacrene B	1517	2.9	0.5	40.7	1.1	11.7	0.4	28.6	3.9
α-Zingiberene	1524	2.0	0.5	28.5	0.7	9.1	0.3	6.0	0.8
α-Longipinene	1527	1.0	tr	14.0	0.4	1.9	0.1	–	–
$C_{15}H_{24}$ (*α*-Muurolene) (?)	1540	2.4	0.5	4.6	0.1	1.7	0.1	1.4	0.2
α-Farnesene	1554	4.2	0.7	4.0	0.1	2.9	0.1	10.9	1.5
cis-*γ*-Bisabolene	1573	2.6	0.5	19.5	0.5	8.5	0.4	11.5	1.6
δ-Cadinene	1585	5.9	0.6	15.6	0.4	8.5	0.3	7.7	1.1
trans-*γ*-Bisabolene	1610	8.0	0.9	65.1	1.7	36.1	1.4	77.1	10.8
$C_{15}H_{24}$	1620	6.8	0.6	19.7	0.5	14.4	0.5	13.3	1.9
$C_{15}H_{24}$	1626	54.5	5.2	72.3	1.9	38.3	1.5	78.2	11.0
γ-Elemene	1662	73.5	8.5	699.1	17.9	212.1	8.0	69.4	9.8
Caryophyllene oxide	1722	3.3	0.5	9.5	0.2	8.2	0.2	1.0	0.2
$C_{15}H_{24}O$	1773	3.6	0.5	7.0	0.1	3.8	0.1	–	–
$C_{15}H_{24}O$	1816	6.9	0.5	38.9	0.7	22.4	0.6	4.8	0.5
tau-Cadinol	1869	8.4	0.6	17.9	0.3	15.0	0.4	2.6	0.3
tau-Muurol	1876	14.2	1.1	20.0	0.4	24.6	0.7	–	–
δ-Cadinol	1781	tr	tr	12.2	0.2		–	8.5	0.9
$C_{15}H_{24}O$	1889	16.3	1.4	41.6	0.8	47.5	0.9	–	–
α-Eudesmol	1896	8.7	0.5	–	–	1.7	tr	–	–
α-Cadinol	1904	5.4	0.5	28.4	0.5	22.3	0.6	10.6	1.2
Unknown	1918	39.4	2.9	6.9	0.1	3.8	0.1	2.4	0.2
Unknown	1922	52.5	6.9	75.0	1.4	74.4	2.1	26.5	2.7
epi-*α*-Bisabolol (?)	1989	1.7	0.5	3.8	0.1	2.5	0.1	2.3	0.2
8-Cedrane-13-al (?)	2021	2.8	0.5	8.2	0.2	4.4	0.1	–	–
n-Octadecane	2631	–	–	tr	tr	–	–	–	–
1-Nonadecene	2811	–	–	tr	tr	–	–	–	–

Compound	Ret. Time (s)	*In vivo* cultivated plants						*In vitro* shoots	
		July 99		Nov. 99		June 00			
		µg/g dw	%	µg/g dw	%	µg/g dw	%	µg/g dw	%
n-Nonadecane	2822	–	–	1.3	tr	0.9	tr	–	–
1-Heneicosene	2995	–	–	0.7	tr	–	–	–	–
n-Heneicosane	3007	–	–	2.1	0.1	1.3	tr	–	–
1-Docosene	3173	1.5	tr	–	–	–	–	–	–
n-Docosane	3183	–	–	5.6	0.1	3.3	0.1	–	–
1-Tricosene	3344	–	–	4.7	0.1	0.3	tr	–	–
n-Tricosane	3353	–	–	3.7	0.1	3.3	0.1	–	–
1-Tetracosene	3507	–	–	tr	tr	–	–	–	–
n-Tetracosane	3517	0.6	tr	18.5	0.4	6.4	0.3	–	–
1-Pentacosene	3665	–	–	2.3	0.1	–	–	–	–
n-Pentacosane	3673	–	–	2.3	0.1	2.7	0.1	–	–
Unknown	3724	–	–	2.0	0.1	–	–	–	–
n-Hexacosane	3829	–	–	17.1	0.3	3.2	0.2	1.1	0.2
Unknown	3882	1.5	tr	0.9	tr	–	–	–	–
1-Heptacosene	3973	–	–	0.9	tr	–	–	–	–
n-Heptacosane	3979	–	–	0.9	tr	–	–	–	–
n-Octacosane	4116	–	–	5.2	0.1	1.8	0.2	–	–
Unknown	4315	–	–	0.8	tr	0.9	0.1	–	–

dw – biomass dry weight; tr – trace amounts

[a] Identification of the compounds was confirmed with authentic standards. Both mass spectra and retention times of the compounds marked with an asterisk match with those of the corresponding compounds from the terpene library of the computer database, and the respective retention times match with the corresponding compounds described in the literature *(17, 18)*. dw, biomass dry weight; tr, trace amounts; (MS), tentative identification based on the mass spectra. [b] Both mass spectra and retention times match with the corresponding compounds from the terpene library of the computer database, and the respective retention times match with the corresponding compounds described in ref *17*. However, according to ref *18*, the retention times on DB-5 would be higher for germacrene B and lower for γ-elemene.

The highest number of compounds (82) was found in the essential oil from plants harvested in November 1999. The excess compounds detected in the essential oils from samples harvested in November 1999 were mainly due to the presence of an almost complete series of *n*-alkanes and 1-alkenes, from C_{18} to C_{28} (**Table 1**). To our knowledge, the presence of these *n*-alkanes and 1-alkenes in the essential oils of *H. androsaemum,* had not yet been reported. However, *n*-nonadecane, *n*-heneicosane, and *n*-tricosane had already been identified in

unripened seed capsules of *H. androsaemum (10)*. The presence of a complete series of *n*-alkanes from $C_{16}H_{34}$ to $C_{29}H_{60}$, in dried leaf material of *H. perforatum* has also been reported *(20)*.

Notwithstanding the high number of constituents, almost 50% of the total essential oil from *H. androsaemum* plants harvested in November 1999 was composed by three sesquiterpenes: *(E)*-caryophyllene (15.1%), *β*-gurjunene (15.5%), and the putative *γ*-elemene (17.9%). These compounds were among the five major constituents of the essential oils of plants harvested in July and June (**Table 1**). However, instead of a sesquiterpene, the major compound in the essential oil sample of June 2000 was a monoterpene (limonene, 15.4%). Undoubtedly, either the content or the composition of the essential oil from *H. androsaemum* plants changes with the time of harvest. Variations in essential oil composition could be induced by different physiological or environmental factors, the variation of which during the vegetative cycle may influence compound turnover. The effects of physiological factors, such as the relative development and maturation of the plant organs, and environmental factors, such as soil mineral fertilization, light intensity, climate conditions, and season, on the composition of essential oils of other plant species are well documented (*15* and references cited therein). The attack of the cultivated plants by some organisms constitutes another type of factor that contingently can influence the essential oil composition (*21* and references cited therein). Frequently *H. androsaemum* plants growing wild in several regions of northern Portugal appear to be contaminated by a rust fungus. On the studied cultivated plants, we identified two contaminant organisms: a rust fungus - *Uromyces* sp. - and an aphis - *Aphis gossypii* Glover. The populations of these contaminants appeared in April 1999 and rose during the following spring and summer months. However, any interpretation inherent to the respective effects on the essential oil composition is speculative, because no type of control of either physiological or environmental factors was made.

Most of the compounds from the essential oils of *H. androsaemum* can be distributed in five groups: monoterpene hydrocarbons, sesquiterpene hydrocarbons, oxygen-containing sesquiterpenes, *n*-alkanes, and 1-alkenes. **Figure 2** shows the specific and the relative amounts of each one of these compound groups in the studied *H. androsaemum* essential oils. Independently of the harvest time, the sesquiterpene hydrocarbons constituted the major compound group, accounting for >40-78% of the total essential oil (**Figure 2**). Sesquiterpenes had already been considered to be the main compounds from the essential oils of this species *(11)*, and despite the fact that the identity of the most of them was unknown, they were considered responsible for the specific essential oil olfactroscopic pattern of *H. androsaemum*

L. *(22)*. Oxygen-containing monoterpenes are often the main compound group in the essential oils from other species. In the essential oils here studied, this group represented no more than 0.2-1.0%.

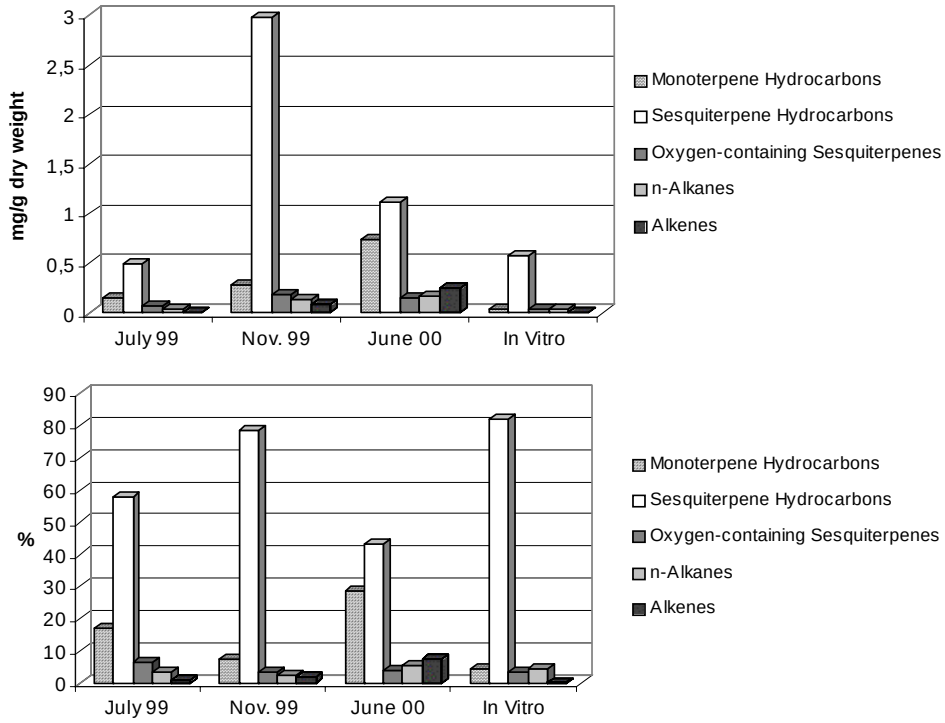


Figure 2 - Specific and relative contents of the main compound groups in the essential oils from *H. androsaemum* in vitro shoots and respective in vivo plants cultivated at Arcos de Valdevez and harvested in the months of July and November 1999 and June 2000.

The main compounds that accounted for most of the percentage variations in each group were β-pinene, limonene, α-pinene, and terpinolene, in monoterpene hydrocarbons; *(E)*-caryophyllene, β-gurjunene, γ-muurolene, *(E)*-γ-bisabolene, and the putative γ-elemene, in sesquiterpene hydrocarbons; *epi*-α-muurolol, in oxygen-containing sesquiterpenes; *n*-nonane and *n*-undecane, in *n*-alkanes; and 1-octene, in 1-alkenes (**Table 1)**.

Yield and Composition of the Essential Oil Produced by in Vitro Shoots.

The essential oil yield obtained by hydrodistillation of in vitro *H. androsaemum* shoots (0.74 mg/g of biomass dry weight) was lower than the minimum value obtained from the in vivo cultivated plants (**Figure 1**). Either the different conditions of growth or the immaturity of the in vitro shoots compared to those of in vivo plants may be responsible for the respective low content of essential oil. Analyses by GC and GC-MS revealed the presence of 52 constituents,

all of them common to the essential oils of in vivo plants (**Table 1**). Sesquiterpene hydrocarbons were the major compound group, representing >80% of the total essential oil, a value higher than that of the same group from the essential oil of in vivo plants harvested in November 1999 (**Figure 2**). The major compound of the in vitro shoots essential oil (γ-muurolene, 15.3%) was not among the five most represented constituents of the essential oils from in vivo plants. The same was true for the second and third most represented compounds (**Table 1**). On the other hand, from the series of *n*-alkanes and 1-alkenes identified in in vivo plants, only *n*-hexacosane, at 0.2%, was found. Differences in the turnover of the compounds due to the immaturity of the shoots and/or the absence of elicitor factors on shoots, namely, contaminant organisms such as the rust fungus *Uromyces* sp. and/or the aphis *A. gossypii* referred to above, would eventually explain the differences in the essential oil composition between the in vitro shoots and the in vivo plants of *H. androsaemum*. Detailed studies are needed, however, to confirm these hypotheses.

The utility of the in vitro shoot cultures in studies on the production of essential oils has not been sufficiently explored. In the case under study, the shoot cultures constitute a stage of the micropropagation process of this species, a specific goal from the practical point of view, and the search on the respective essential oils was performed with the aim to compare its composition with that of in vivo plants. However, as the development and the environment of this type of culture can be maintained under strict control, lowering the sources of variability affecting the composition of the essential oils, we consider that in vitro shoots could be advantageously used as a tool, for example, in the determination of chemotypes based on the respective essential oil composition. On the other hand, in our view, in vitro shoots or plantlets are the most suitable in vitro system models for studies on the metabolism of terpene compounds because they resemble more closely the in vivo plants.

In conclusion, the work here reported showed that composition of the essential oils from *H. androsaemum* is complex having a variable number of compounds which depending from the time of harvest and from the origin of biomass, being dominated by sesquiterpene hydrocarbons and accumulating, some times, high number of intermediate to long chain *n*-alkanes and 1-alkenes.

Acknowledgements

This work was supported by the Programme AGRO/8/key action 8.1/Project 338.

References

(1) Costa A, A. Costa, Editor. Fundação Calouste Gulbenkian: Lisboa. 1987, p. 1021-1022.

(2) Seabra RM, Vasconcelos MH, Costa MAC,Alves AC, *Fitoterapia*, 1992. 68: p. 473-474.

(3) Seabra R,Alves A, *Planta Medica*, 1989. 55: p. 404-405.

(4) Seabra R,Alves A, *Fitoterapia*, 1990. 61: p. 146-147.

(5) Dias A, Seabra R, Andrade P,Fernandes-Ferreira M, *Journal of Liquid Chromatography & Related Technology*, 1999. 22: p. 215-227.

(6) Nielsen H,Arends P, *Journal of Natural Products*, 1979. 42: p. 301-304.

(7) Kitanov G,Nedialkov P, *Biochemical Systematics and Ecology*, 1998. 26: p. 647-653.

(8) Mathis C,Ourisson G, *Phytochemistry*, 1964b. 3: p. 133-141.

(9) Mathis C,Ourisson G, *Phytochemistry*, 1964c. 3: p. 377-378.

(10) Carnduff J, Hargreaves KR,Nechvatal A, *Phytochemistry*, 1966. 5: p. 1029-1029.

(11) Nogueira T, Duarte F, Venâncio F, Tavares R, Lousã M, Bicchi C,Rubiolo P, *Silva Lusitana*, 1998. 6: p. 55-61.

(12) Dias ACP, Seabra RM, Andrade PB, Ferreres F,Fernandes-Ferreira M, *Plant Science*, 2000. 150: p. 93-101.

(13) Margara J. 1989, Paris: INRA. Institut National de la Recherche Agronomique.

(14) Murashige T,Skoog F, *Physiol. Plantarum*, 1962. 15: p. 473-497.

(15) Santos-Gomes PC,Fernandes-Ferreira M, *Journal of Agricultural and Food Chemistry*, 2001. 49: p. 2908-2916.

(16) Industry IOotF, *Z. Lebensm. Unters. Forsch*, 1991. 192: p. 530-534.

(17) Adams RP. 1989, San Diego, CA: Academic Press.

(18) Adams RP. 2001, Illinois, USA: Allured Publishing Corporation, Carol Stream.

(19) Mathis C,Ourisson G, *Phytochemistry*, 1964. 3: p. 115-131.

(20) Brondz I, Greibrokk T,Aasen A, *Phytochemistry*, 1983. 22: p. 295-296.

(21) Alves-Pereira I,Fernandes-Ferreira M, *Phytochemistry*, 1998. 48: p. 795-799.

(22) Nogueira T, Duarte F, Tavares R, Curto M, Capelo J,Freitas A, *Flavour and Fragrance Journal*, 1999. 14: p. 195-199.

3.3 Supplementary data

Essential oils profiles from leaves, stems and ripened seed capsules of *Hypericum androsaemum* L.

Several environmental and physiological factors are known to affect the production and accumulation of essential oils (EO). Therefore, apart from the EO analysis of aerial parts of cultivated plants of *Hypericum androsaemum* and *in vitro* shoots, EO isolated from leaves, stems and ripened seed capsules were also studied, in order to determine the respective EO profiles.

Aerial parts from 6-8 plants of *H. androsaemum* were randomly collected in an experimental field located in Covide, near Braga (northern Portugal) on July, and the respective leaves, stems and ripened seed capsules were separated and processed independently in the hydrodistillation and EO analysis. Subsamples of fresh leaves (10g), stems (10g) and ripened seed capsules (10g) of *H. androsaemum* were submitted to hydrodistillation with 500 ml of boiling water in a Clevenger type apparatus over 1h, using volumes of 1.0 mL of *n*-hexane, containing 5-α-cholestane (1mg/mL), for retention of the hydrodistillate components. The dry weight of the plant material was determined after drying (60ºC, 72h) in a drying stove. Further analyses of the hydrodistillates were performed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS) as previously described (Guedes *et al.*, 2003).

Different EO yields were obtained from the hydrodistillation of leaves, ripened seed capsules and stems of *H. androsaemum* plants (Figure 1). The highest one was obtained for ripened seed capsules (7.95 mg of EO per g of biomass dry weight) while the lowest one was registered in the stems (0.17 mg/g of biomass dry weight). The EO yield of leaves was 0.85 mg/g of biomass dry weight distributed in 103 compounds, 82% of which were identified.

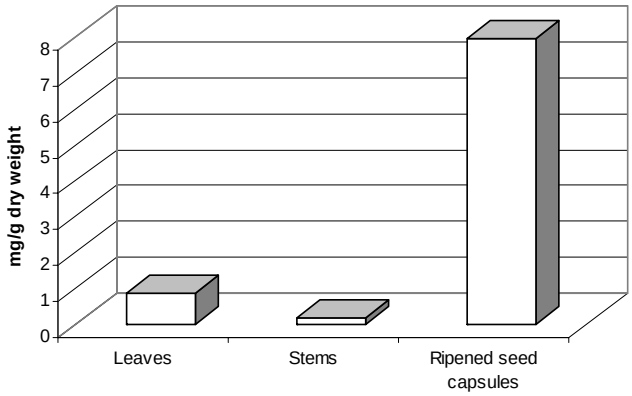


Figure 1 - Essential oil contents (mg/g dry weight) from leaves, stems and ripened seed capsules of *Hypericum androsaemum* plants growing in Covide (Portugal) and harvested in July.

From the 92 compounds detected in the EO of ripened seed capsules, 96% were identified. The EO from stems had a narrower range of compounds (63 compounds identified) compared to leaves and ripened seed capsules. Variations in the EO content of this species had already been reported. Indeed, EO yields of aerial parts of cultivated plants of *H. androsaemum* ranged from 0.9 to 4.1 mg/g of biomass dry weight, while in the leaves it ranged from 0.7 to 3.4 mg/g dry weight (Guedes *et al.*, 2003; Guedes *et al.*, 2004). Smaller variations in the EO yields of this species were observed by Mathis and Ourisson (1964) (0.6 to 1.4 mg/g of biomass dry weight). EO hydrodistillated from *H. androsaemum in vitro* shoots had a yield of 0.74 mg/g of biomass dry weight distributed in about 52 detected compounds (Guedes *et al.*, 2003). Thus, the EO content from ripened seed capsules and stems were, respectively, the highest and the lowest ones from the EO contents reported so far for *H. androsaemum*.

The composition of leaves, ripened seed capsules and stems EO of *H. androsaemum* are shown in Table 1.

Table 1- Composition of the essential oils from leaves, stems and ripened seed capsules of *Hypericum androsaemum* plants growing in Covide (Portugal) and harvested in July.

Compound	KI	Leaves		Stems		Rip seed caps	
		µg/g dry weight	%	µg/g dry weight	%	µg/g dry weight	%
1-Octene	792	5.7	0.5	3.8	2.1	5.4	0.1
(E)-2-Hexenal	854	15.6	1.6	0.3	tr	3.4	tr
n-Hexanol	869	0.2	tr	0.3	tr	3.5	tr
n-Nonane	900	6.5	0.7	44.0	23.9	9.8	0.1
α-Thujene	930					9.4	0.2
α-Pinene	939	1.4	0.2	0.9	0.5	1123.2	18.8
Camphene	950					15.3	0.3
Dimethyl-biciclo-(3,1)-hepta-2(8),3-diene	956					6.7	0.1
2,6-Dimethyl-octane	972	0.4	tr	0.6	0.5	2.2	tr
Sabinene	976					5.0	0.1
β-Pinene	979	2.8	0.4	3.2	2.3	1666.1	27.8
6-Methyl-5-Hepten-2-one	988	7.2	0.7	1.2	0.5	26.7	0.3
Myrcene	991	1.2	0.1	0.3	0.2	81.6	1.4
n-Decane	1000	2.8	0.3	0.2	tr	5.5	0.1
α-Terpinene	1017	0.5	0.1			3.2	tr
p-Cymene	1026	0.2	tr			17.3	0.3
Limonene	1030	4.2	0.5	0.9	0.7	2061.8	34.4
1,8-Cineole	1033	1.7	0.1				
Z-β-Ocimene	1040	0.1	tr	0.3	0.2	1.4	tr
Benzene acetaldehyde	1043					1.2	tr
E-β-Ocimene	1050	0.5	0.1				
γ-Terpinene	1060	0.8	0.1			4.3	0.1
trans-Linalool Oxide (furanoid)	1073	1.3	0.1			26.6	0.3
Terpinolene	1089	1.2	0.1			21.8	0.4
Linalool	1097	3.2	0.3	15.4	8.2	141.2	1.7

Compound	KI	Leaves		Stems		Rip seed caps	
		µg/g dry weight	%	µg/g dry weight	%	µg/g dry weight	%
trans-Sabinene hydrate	1098	3.9	0.4				
Nonanal	1104	1.7	0.2			3.7	tr
cis-limonene oxide	1115					2.4	tr
trans-limonene oxide	1119					12.0	0.1
cis-Verbenol	1125					3.3	tr
trans-Verbenol	1135					11.5	0.1
Camphor	1146	5.3	0.5	0.9	0.5	0.8	tr
Camphene Hydrate	1147					5.7	0.1
trans-3-Pinanone	1161					11.3	0.1
Pinocarvone	1165					21.6	0.3
cis-3-Pinanone	1175	3.5	0.3			58.9	0.7
Terpinen-4-ol	1177	18.0	1.7	1.2	0.5	70.9	0.9
α-Terpineol	1190	0.8	0.1	0.2	tr	71.0	0.9
cis-Dihydrocarvone	1193					12.8	0.2
trans-3-Dihydrocarvone	1197					26.4	0.3
Verbenone	1204					33.1	0.4
n-Decanal	1205	0.6	0.1				
trans-Carveol	1217	1.5	0.1			17.5	0.2
Carvone	1242					9.0	0.1
Linalyl acetate	1257	0.8	0.1			3.5	tr
Perilla aldehyde	1271					3.3	tr
Thymol	1290	2.4	0.2			1.4	tr
n-Tridecane	1300					0.5	tr
δ-Elemene	1337	0.3	tr				
α-Cubebene	1348	7.0	0.9			0.9	tr
α-Ylangene	1374	0.6	0.1			2.1	tr
α-Copaene	1376	2.3	0.3			2.5	tr
Geranyl acetate	1381	0.3	tr				
β-Bourbonene	1383	0.4	0.1			0.9	tr
β-Cubebene	1388	0.3	tr				
β-Elemene	1391	3.9	0.5	1.2	0.9	5.0	0.1
iso-Italicene	1395	2.3	0.3				
Italicene	1405	4.6	0.6			1.3	tr
α-Cedrene	1409	1.0	0.1				
(E)-Caryophyllene	1417	78.3	10.3	3.4	2.5	55.2	0.9
cis-Thujopsene	1430	1.3	0.2			0.1	tr
α-Guaiene	1440	25.0	3.3	6.8	5.0	22.2	0.4
Aromadendrene	1443	2.2	0.3	0.3	tr	1.9	tr
α-Himachalene	1451	4.9	0.6	0.8	0.5	11.5	0.2
α-Humulene	1453	17.4	2.3	0.8	0.5	11.9	0.2
(E)-β-Farnesene	1455	2.0	0.3				
allo-Aromadendrene	1459	55.7	7.3	5.6	4.1	60.7	1.0
α-Acoradiene	1468	0.8	0.1			0.9	tr
β-Chamigrene	1478	9.9	1.3	0.7	0.5		
γ-Muurolene	1481	31.1	4.1	5.2	3.7	10.3	0.2
Curcumene	1485	58.9	7.7	2.1	1.4	48.0	0.8
β-Selinene	1486	28.5	3.7	1.7	1.2	16.1	0.3
α-Selinene	1495	6.0	0.8	0.1	tr	4.1	0.1
(E,E)-α-Farnesene	1505	9.8	1.3			6.3	0.1
γ-Cadinene	1512	0.7	0.1	0.4	0.3	0.6	tr

Compound	KI	Leaves		Stems		Rip seed caps	
		µg/g dry weight	%	µg/g dry weight	%	µg/g dry weight	%
(Z)-γ-Bisabolene	1515	4.9	0.7	0.4	0.2	6.2	0.1
SH	1519	4.8	0.7				
Calamenene (?)	1521	7.9	1.1			1.2	tr
δ-Cadinene	1523	6.1	0.8	0.6	0.5	8.2	0.1
SH	1530	0.6	0.1				
α-Cadinene	1534	42.3	5.6	1.3	1.1	11.9	0.2
SH	1540	2.6	0.3	0.1	tr		
α-Calacorene	1542	38.6	5.1	1.5	1.1	2.1	tr
SH	1543					11.1	0.2
γ-Elemene (?)	1556	35.9	4.7	9.6	6.9	29.9	0.5
E-Nerolidol	1564	2.1	0.2			1.4	tr
Spathulenol	1574	0.5	tr			0.1	tr
cis-3-Hexenyl benzoate	1577	0.3	tr			0.2	tr
Caryophyllene Oxide	1582	33.7	3.3	3.6	2.0	100.2	1.2
Globulol	1587	4.5	0.4	0.7	0.5	2.9	tr
Viridiflorol	1594	2.3	0.3	0.8	0.5	3.5	tr
Humulene Epoxide II	1607	7.4	0.7	1.0	0.5		
β-Oplopenone	1612	4.3	0.4	1.3	0.5		
SO	1617	6.8	0.7				
10-*epi*-*γ*-Eudesmol	1621	3.5	0.3	2.9	1.6		
γ-Eudesmol	1626	9.1	0.9	0.9	0.5	0.5	tr
epi-*α*-Cubenol	1632	9.5	0.9	1.1	0.5	12.8	0.2
epi-*α*-Cadinol	1639	1.4	0.1	1.6	0.9		
epi-*α*-Muurolol	1643	11.1	1.1	1.6	0.9	3.3	tr
α-Eudesmol	1651	13.9	1.3	1.2	0.5	10.6	0.1
α-Cadinol	1654	14.1	1.3	2.1	1.1	9.6	0.1
epi-*α*-Bisabolool	1678	19.9	1.9	4.7	2.5	17.5	0.2
(Z)-*α*-*trans*-Bergamotol	1694	23.8	2.3	2.8	1.6	4.4	tr
n-Nonadecane	1900					3.1	tr
n-Eicosane	2000					0.2	tr
n-Heneicosane	2100					3.7	0.1
n-Docosane	2200	0.2	tr				
n-Tricosane	2300	0.7	0.1	0.1	tr		
n-Tetracosane	2400	0.5	tr	0.1	tr		
n-Pentacosane	2500	0.4	tr	0.7	0.5		
n-Hexacosane	2600			0.2	tr		
n-Heptacosane	2700			1.9	1.1		
n-Octacosane	2800			0.3	0.2		
n-Nonacosane	2900			1.5	0.9		
Monoterpene Hydrocarbons		11.6	1.5	5.7	3.9	5010.3	83.7
Oxygenated monoterpenes		40.4	3.8	17.7	9.3	536.0	6.5
Monoterpene Esters		1.2	0.2			3.5	tr
Sesquiterpene Hydrocarbons		498.7	65.8	42.4	30.5	334.2	5.6
Oxygenated sesquiterpenes		167.9	16.2	21.1	11.6	166.8	2.0
1-Alkenes		5.7	0.5	3.8	2.1	5.4	0.1
n-Alkanes		11.1	1.1	48.9	26.5	22.9	0.3
Others		111.5	10.9	30.3	16.1	151.6	1.8

KI- Kovats retention index on a DB-5 column; **tr**- trace amounts

The majority of the compounds detected could be grouped in monoterpene hydrocarbons (MH), oxygenated monoterpenes (MO), monoterpene esters (ME), sesquiterpene hydrocarbons (SH), oxygenated sesquiterpenes (SO), *n*-alkanes and 1-alkenes, excepting for stems in which no monoterpene ester was identified. However, as can be seen in Figure 2, the distribution of the identified compounds in the referred groups was greatly different in the three organs studied.

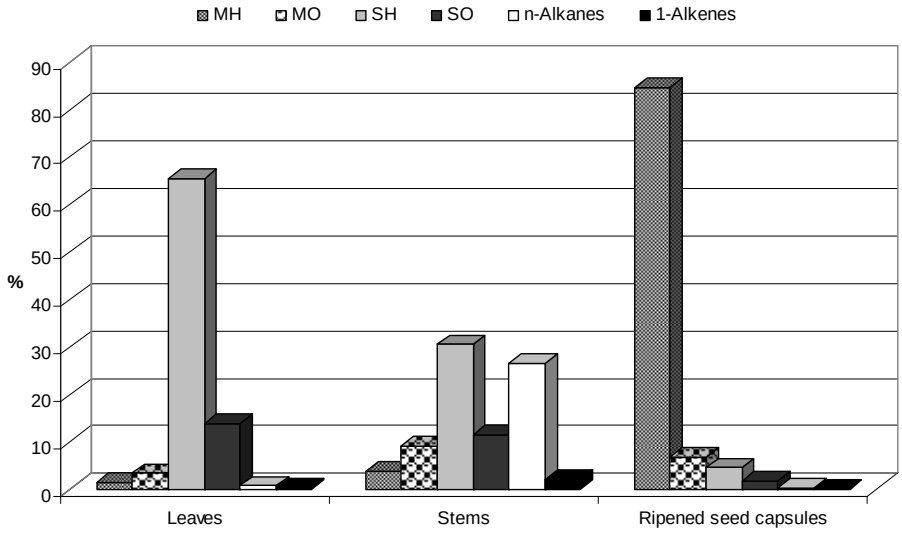


Figure 2 - Essential oil specific contents (%) of the major group of compounds from leaves, stems and ripened seed capsules of *Hypericum androsaemum* plants growing in Covide (Portugal) and harvested in July.

EO from leaves was characterized by its richness in sesquiterpenoids, with sesquiterpene hydrocarbons accounting for 66% and oxygenated sesquiterpenes, 16%. Sesquiterpenoids represented also more than 42% of the total EO of stems, with 31 compounds common to stems and leaves. Leaves and flowers EO of *H. androsaemum* from Iran were also rich in these compounds, representing almost 99% and 98% of the total EO, respectively (Morteza-Semnani *et al.*, 2005). The predominance of sesquiterpene hydrocarbons in EO of *H. androsaemum* had already been reported by several authors (Nogueira *et al.*, 1998; Guedes *et al.*, 2003; Guedes *et al.*, 2004). Monoterpene hydrocarbons (84%) made up the highest contribution in the EO of ripened seed capsules, while oxygenated monoterpenes and sesquiterpene hydrocarbons represented only ~7% and ~6%, respectively. The results show a pattern of production and/or accumulation of the EO constituents in ripened seed capsules clearly different of those of the other organs of the plant, such as stems and leaves. Thus, apart from the ripened seed capsules, the EO of *H. androsaemum* can be characterized by a high amount of sesquiterpenoids. The EO of *H. hircinum*, a species that belong to the same section

of *H. androsaemum* (Section Androsaemum, Mathis *et al.*, 1964), was also dominated by the sesquiterpene hydrocarbons (82%). However, this high level of sesquiterpene hydrocarbons can not be considered specific of the section, since EO of *H. hircinum* collected in Italy were richer in monoterpenes, being the sesquiterpenes content lower than 13% (Bertoli *et al.*, 2000). Sesquiterpenes were also abundant in EO of other species of *Hypericum* genus, such as *H. olympicum* (Pavlović *et al.*, 2006; Smelcerovic *et al.*, 2007), *H. perforatum* (Schwob *et al.*, 2002; Pavlović *et al.*, 2006), *H. richeri* (Smelcerovic *et al.*, 2007) and *H. tetrapterum* (Pavlović *et al.*, 2006).

The distribution of *n*-alkanes in the EO from leaves, stems and ripened seed capsules of *H. androsaemum* was uneven (Figure 2). In the EO from stems, this group represented 27%, while in leaves and ripened seed capsules it represented less than 1%. In EO of *H. undulatum*, we have found a similar predominance of *n*-alkanes in stems, comparing to leaves, ripened seed capsules and flowers (Guedes, unpublished data). In the EO of *H. androsaemum* stems, *n*-nonane was the compound that most contributed to the high amount of *n*-alkanes, representing 24% of the total EO. Four long chain *n*-alkanes (*n*-hexacosane, *n*-heptacosane, *n*-octacosane and *n*-nonacosane) were only detected in EO of this organ. The presence of long chain *n*-alkanes in EO of *H. androsaemum* and *H. perforatum* has been previously reported (Brondz *et al.*, 1983; Guedes *et al.*, 2003; Guedes *et al.*, 2004). The number of *n*-alkanes in the EO of *H. androsaemum* aerial parts and leaves, as well as in flowers is usually very low, sometimes being represented by only two compounds (Nogueira, 2002; Morteza-Semnani *et al.*, 2005). Linalyl acetate was the only monoterpene ester identified in the EO of ripened seed capsules of this species while in its leaves, this compound was accompanied by geranyl acetate and in the EO of its stems no monoterpene ester has been found (Mathis *et al.*, 1964; Nogueira, 2002; Guedes *et al.*, 2003; Guedes *et al.*, 2004). On the other hand, another monoterpene ester, terpeninyl acetate, was not identified in the present study but it was detected in aerial parts and *in vitro* shoots of *H. androsaemum* (Guedes *et al.*, 2003; Guedes *et al.*, 2004). 1-Alkenes were represented by only one compound common to all samples, 1-octene, whose highest amount occurred in the stems (2%). In aerial parts of plants of *H. androsaemum* harvested in later summer in Portugal, this compound represented almost 20% of the total EO, being among the 5 most represented compounds (Guedes *et al.*, 2004). Besides 1-octene, the presence of an almost complete series of 1-alkenes, from $C_{21}H_{42}$ to $C_{28}H_{56}$ in EO of fresh leaves of *H. androsaemum*, harvested between February and June 2000, was reported (Guedes *et al.*, 2004).

The EO from leaves and stems of *H. androsaemum* differed in its major constituents. All of the 5 major compounds from leaves were sesquiterpene hydrocarbons [(*E*)-

caryophyllene, curcumene, *allo*-aromadendrene, α-cadinene and α-calacorene], whereas in the stems they were 1 *n*-alkane (*n*-nonane), 1 oxygenated monoterpene (linalool), 2 sesquiterpene hydrocarbons (γ-elemene and α-guaiene) and 1 not yet identified compound. *(E)*-Caryophyllene, curcumene and *allo*-aromadendrene represented, respectively 10%, 8% and 7% of the total EO *H. androsaemum* from leaves. Although being dominant in the leaves EO, *(E)*-caryophyllene was among the less represented compounds in the stems and ripened seed capsules. Similar results were achieved in the study of the *H. undulatum* EO composition whose leaves showed the highest content of *(E)*-caryophyllene contrarily to what occurred with the stems and ripened seed capsules where this compound was less represented (Guedes, unpublished data). *(E)*-Caryophyllene had already been reported as one of the major constituents in the EO from *H. androsaemum* (Guedes *et al.*, 2003; Guedes *et al.*, 2004), *H. perforatum* (Baser *et al.*, 2002), and *H. carinatum* and *H. ternum* (Ferraz *et al.*, 2005). Even though, *allo*-aromadendrene has been present in the EO of the three organs of *H. androsaemum* analysed, it was not detected in the *H. androsaemum* EO (Nogueira *et al.*, 1998) or it was only present in trace amounts (Guedes *et al.*, 2003; Guedes *et al.*, 2004). Five of all the sesquiterpene hydrocarbons identified were exclusively detected in the leaves (δ-elemene, β-cubebene, *iso*-italicene, α-cedrene and *(E)*-β-farnesene) and excepting for δ-elemene, none of these referred compounds were identified in EO of this species in previous studies (Mathis *et al.*, 1964; Nogueira *et al.*, 1998; Guedes *et al.*, 2003; Guedes *et al.*, 2004). In EO isolated from dried leaves of *H. androsaemum* cultivated in Iran the major compounds were caryophyllene oxide and ishwarane (Morteza-Semnani *et al.*, 2005). Ishwarane was not identified in this study neither in other studies on the EO composition of *H. androsaemum* (Mathis *et al.*, 1964; Nogueira *et al.*, 1998; Guedes *et al.*, 2003; Guedes *et al.*, 2004). Caryophyllene oxide was the major oxygen-containing sesquiterpene in the three organs here studied. Nevertheless, its higher contribution to the total EO occurred in the leaves, in which it represented 3% of the total EO. Limonene was the major monoterpene hydrocarbon detected in the EO from leaves (0.5%). This compound was considered the major one in the EO isolated from aerial parts, flowers and "fruits" of *H. androsaemum*, and the second major in the leaves of the same species (Mathis *et al.*, 1964). *E*-β-Ocimene, 1,8-cineole and *trans*-sabinene hydrate were exclusively detected in leaves.

Stems were the organs in which the oxygenated monoterpenes were present in highest percentage (9%). This group was represented by linalool, camphor, terpinen-4-ol and α-terpineol. Nevertheless, the predominance of oxygenated monoterpenes was due to linalool,

which accounted for 8% of the total EO. Linalool was also identified by Nogueira and co-workers (1998) in *H. androsaemum* EO, but in lower amounts. Such accumulation of linalool in stems was coincident with the absence of their derivatives, linalool oxide and linalyl acetate. These two monoterpenoids were identified in both leaves and ripened seed capsules, even though in lower amounts. Linalool oxide is known to be produced through an oxidative modification of linalool (Pichersky *et al.*, 1994). Three monoterpene hydrocarbons accounted for more than 80% of the total EO content in the ripened seed capsules (α-pinene; β-pinene and limonene). Previous works reported also the presence of these three monoterpene hydrocarbons in cultivated plants of *H. androsaemum*, however in much lower amounts (Nogueira *et al.*, 1998; Guedes *et al.*, 2003; Guedes *et al.*, 2004) while in shoots α-pinene was not identified (Guedes *et al.*, 2003). The other monoterpene hydrocarbons, α-thujene, camphene and sabinene were exclusively detected in the EO of ripened seed capsules.

Concluding remarks

Several factors had already been reported to affect the EO composition of *H. androsaemum*, namely harvesting time and growth conditions (*in vivo* or *in vitro*) (Guedes *et al.*, 2003; Guedes *et al.*, 2004). The work here reported showed different EO composition profiles in leaves, stems and ripened seed capsules of *H. androsaemum*. Sesquiterpene hydrocarbons constituted the main fraction in EO from leaves and stems. However, in stems EO *n*-alkanes were also abundant, being *n*-nonane its major compound. The ripened seed capsules contained high levels of monoterpene hydrocarbons, mainly due to limonene, β-pinene and α-pinene contents.

References

Baser, K. H. C., T. Ozek, H. R. Nuriddinov & A. B. Demirci (2002). Essential oils of two *Hypericum* species from Uzbekistan. *Chemistry of Natural Compounds. 38*(1), 54-57.

Bertoli, A., L. Pistelli, I. Morelli, G. Spinelli & F. Menichini (2000). Constituens of *Hypericum hircinum* oils. *Journal of Essential Oil Research 12*, 617-620.

Brondz, I., T. Greibrokk & A. Aasen (1983). *n*-Alkanes of *Hypericum perforatum*: a revision. *Phytochemistry 22*, 295-296.

Ferraz, A. B. F., R. P. Limberger, S. A. L. Bordignon, G. L. v. Poser & A. T. Henriques (2005). Essential oil composition of six *Hypericum* species from southern Brazil. *Flavour and Fragrance Journal 20*(3), 335-339.

Guedes, A. P., L. R. Amorim, A. Vicente & M. Fernandes-Ferreira (2004). Variation of the essential oil content and composition in leaves from cultivated plants of *Hypericum androsaemum* L. *Phytochemical Analysis 15*, 146-151.

Guedes, A. P., L. R. Amorim, A. M. S. Vicente, G. Ramos & M. Fernandes-Ferreira (2003). Essential Oils from Plants and *in Vitro* Shoots of *Hypericum androsaemum* L. *J. Agric. Food Chem. 51*(5), 1399-1404.

Mathis, C. & G. Ourisson (1964). Étude chimio-taxonomique du genre *Hypericum* : III. Répartition des carbures saturés et des monoterpènes dans les huiles essentielles d'*Hypericum*. *Phytochemistry 3*(1), 133-141.

Morteza-Semnani, K. & M. Saeedi (2005). The essential oil composition of *Hypericum androsaemum* L. leaves and flowers from Iran. *Flavour and Fragrance Journal 20*(3), 332-334.

Nogueira, T. (2002). O género *Hypericum* L. em Portugal Continental. Contribuição para o estudo quimiotaxonómico. Instituto Superior de Agronomia. Lisboa, Universidade Técnica de Lisboa. **PhD. Thesis**.

Nogueira, T., F. Duarte, F. Venâncio, R. Tavares, M. Lousã, C. Bicchi & P. Rubiolo (1998). Aspectos Quimiotaxonómicos do Género *Hypericum* L. em Portugal. *Silva Lusitana 6*(1), 55-61.

Pavlović, M., O. Tzakou, P. V. Petrakis & M. Couladis (2006). The essential oil of *Hypericum perforatum* L., *Hypericum tetrapterum* Fries and *Hypericum olympicum* L. growing in Greece. *Flavour and Fragrance Journal 21*(1), 84-87.

Pichersky, E., R. A. Raguso, E. Lewinsohn & R. Croteau (1994). Floral Scent Production in Clarkia (Onagraceae) (I. Localization and Developmental Modulation of Monoterpene Emission and Linalool Synthase Activity). *Plant Physiol. 106*(4), 1533-1540.

Schwob, I., J.-M. Bessière & J. Viano (2002). Composition of the essential oils of *Hypericum perforatum* L. from southeastern France. *Comptes Rendus Biologies 325*(7), 781-785.

Smelcerovic, A., M. Spiteller, A. P. Ligon, Z. Smelcerovic & N. Raabe (2007). Essential oil composition of *Hypericum* L. species from Southeastern Serbia and their chemotaxonomy. *Biochemical Systematics and Ecology 35*(2), 99-113.

Chapter 4

ESSENTIAL OILS OF *HYPERICUM PERFORATUM* L.

4.1- Chapter overview

Hypericum perforatum (St. John's wort), the most studied species of the genus, has been used as a medicinal plant for centuries because of its anti-inflammatory, sedative, analgesic, diuretic, anti-malarial, and wound healing properties (Zobayed *et al.*, 2004). This species is also a natural herbal alternative used mainly for the treatment of depression in cases where standard antidepressants, such as Prozac, Paxil, and Zoloft may be prescribed. This species was also shown to have potential as a novel anticancer drug (Schempp *et al.*, 2002). Biological activities of *H. perforatum* are mainly attributed to compounds of phenolic extracts (Kitanov, 2001). Therefore, standardization of natural health of *H. perforatum*, one of the five top selling phytopharmaceuticals in North America (Zobayed *et al.*, 2004), are based mainly on the quantification of hypericin, pseudohypericin, and hyperforin (Orth *et al.*, 1999). Other set of secondary metabolites, essential oils, have already been reported for plants of this species grown in different countries, however in a less extent (Nogueira *et al.*, 1998; Pintore *et al.*, 2005; Pavlović *et al.*, 2006; Saroglou *et al.*, 2007). To date, commercial production of *H. perforatum* is generally based on field grown plant material but the quality of these products may be affected by different environmental conditions, pollutants, microorganisms, viruses, and insects which can alter the concentration of its medicinal metabolites (Zobayed 2004). Indeed, even though a significant number of studies on the essential oils composition of *H. perforatum* have been published, literature on the factors that might influence its production and accumulation is scarce.

As part of the phytochemical investigation in species of the *Hypericum* genus in our laboratory, we analyzed the chemical composition of (i) essential oils from two different cultivars of *Hypericum perforatum* (*H. perforatum* common cultivar and *H. perforatum* cv. *Topaz*) cultivated in two different sites (Arcos de Valdevez and Merelim) over a year, with 2 months of interval; (ii) essential oils isolated from flowers; and (iii) essential oils produced by *in vitro* shoot cultures established and maintained in the scope of this work.

Highly complex essential oils were obtained from *H. perforatum* plants. The highest essential oils yields of *H. perforatum* were obtained in the flowers. In the two experimental fields the maximum values of essential oils yields were obtained in plants of *H. perforatum* cv. 'Topaz'. Most of the essential oils of plants of the two cultivars were characterized by the predominance of the sesquiterpene hydrocarbons, as the major group of compounds. Only flowers of *H. perforatum* common cultivar, cultivated in Merelim did not follow this tendency,

having the monoterpene hydrocarbons as the most represented group of compounds. Over the most part of the year, an inverse correlation in the variation of the sesquiterpene hydrocarbons and monoterpene hydrocarbons was recorded in the essential oils of *H. perforatum* and *H. perforatum* 'Topaz'.

Variations in the content of the main constituents of the essential oils were registered in the two cultivars, in both experimental fields. Germacrene D, *(E)*-caryophyllene, and *β*-selinene were the compounds that most contributed to the high amount of sesquiterpene hydrocarbons in the essential oils of the vegetative aerial parts. Essential oils from flowers were characterized by its high content of 2-methyl-octane, which was the second most represented compound in some of the essential oils isolated from vegetative aerial parts of plants cultivated in the two sites.

Sesquiterpene hydrocarbons was also the major group of compounds in the essential oils of *in vitro* shoots. However, the major compound in those essential oils, *n*-nonane, was not among the most represented ones in the cultivated plants.

Differences in the composition of the essential oils over the year, herein reported, can be attributed to seasonal variation, which influences the development stage of the plants. Additionally, the present results show that other factors, such as plant organ and growth conditions (*in vivo* or *in vitro*) affect also the essential oils composition of *H. perforatum*.

a **b**

Figure 1- a) *Hypericum perforatum* flowers **b)** *In vitro* plantlets of *Hypericum perforatum* cv. Topaz grown on MS basal medium without plant growth regulators after 50 days of culture.

Kitanov, G. M. (2001). Hypericin and pseudohypericin in some *Hypericum* species. *Biochemical Systematics and Ecology 29*(2), 171-178.

Nogueira, T., F. Duarte, F. Venâncio, R. Tavares, M. Lousã, C. Bicchi & P. Rubiolo (1998). Aspectos Quimiotaxonómicos do Género *Hypericum* L. em Portugal. *Silva Lusitana 6*(1), 55-61.

Orth, H., C. Rentel & P. Schmidt (1999). Isolation, Purity Analysis and Stability of Hyperforin as a Standard Material from *Hypericum perforatum* L. *Journal of Pharmacy and Pharmacology 51*(2), 193-200.

Pavlović, M., O. Tzakou, P. V. Petrakis & M. Couladis (2006). The essential oil of *Hypericum perforatum* L., *Hypericum tetrapterum* Fries and *Hypericum olympicum* L. growing in Greece. *Flavour and Fragrance Journal 21*(1), 84-87.

Pintore, G., M. Chessa, G. Boatto, R. Cerri, M. Usai & B. Tirillini (2005). Essential oil composition of *Hypericum perforatum* L. var. angustifolium DC growing wild in Sardinia (Italy). *The Journal of essential oil research 17*(5), 533-535

Saroglou, V., P. D. Marin, A. Rancic, M. Veljic & H. Skaltsa (2007). Composition and antimicrobial activity of the essential oil of six *Hypericum* species from Serbia. *Biochemical Systematics and Ecology 35*(3), 146-152.

Schempp, C., V. Kirkin, B. Simon-Haaraus, A. Kersten, J. Kiss, C. Termeer, B. Gilb, T. Kaufmann, C. Borner, J. Sleeman & J. Simon (2002). Inhibition of tumour cell growth by hyperforin, a novel anticancer drug from St. John's wort that acts by induction of apoptosis. *Oncogene 21*(8), 1242-1250.

Zobayed, S. M. A., S. J. Murch, H. P. V. Rupasinghe & P. K. Saxena (2004). *In vitro* production and chemical characterization of St. John's wort (*Hypericum perforatum* L. cv 'New Stem'). *Plant Science 166*(2), 333-340.

4.2- Manuscripts

This chapter comprises the following manuscripts:

Guedes, AP, Vicente, AM & Fernandes-Ferreira, M. Essential Oils from vegetative aerial parts and flowers of *Hypericum perforatum.*

Guedes, AP, Vicente, AM & Fernandes-Ferreira, M. Essential Oils from Cultivated Plants and *In vitro* Shoots of *Hypericum perforatum* 'Topaz'.

Essential Oils from vegetative aerial parts and flowers of *Hypericum perforatum*

Ana P. Guedes[1], Ana Vicente[2] and M. Fernandes-Ferreira[1]

[1] CITAB- Centro de Investigação e de Tecnologias Agro-Alimentares e Biológicas, Department of Biology, University of Minho, Campus de Gualtar, 4720-057 Braga, Portugal

[2] DRAPN- Direcção Regional de Agricultura e Pescas do Norte, 4715-017 Braga, Portugal

Abstract

The composition of the essential oils (EO) from aerial parts of *H. perforatum* plants was characterized after hydrodistillation of plant branches harvested during one year at intervals of 2-3 months, in two experimental fields located in northern Portugal (Arcos de Valdevez and Merelim). In both experimental fields the highest and the lowest EO yields were registered in June and December, respectively. The analysis of the EO by GC-MS revealed the presence of more than 110 compounds, the most of which were identified, including germacrene D, 2-methyl-octane, *(E)*-caryophyllene and *β*-selinene as the major ones. Variations in the specific contents of the main compound classes, over the year were registered. The sesquiterpene hydrocarbons constituted the major group in all samples harvested over the year in the two experimental fields, whose maximum percentage content was reached in the winter decreasing afterwards. Over the most part of the year, the percentage content of this group of compounds varied in an opposite manner to that of monoterpene hydrocarbons. The EO of the vegetative aerial parts showed, over the year, a poor fraction of monoterpene hydrocarbons. Flowers harvested in June gave the highest essential oils yields from all the plant samples studied, as well as the highest amounts of 2-methyl-octane, the main EO constituent. In flowers from plants growing in Arcos de Valdevez the sesquiterpene hydrocarbons constituted the main group of compounds followed by alkanes, monoterpene hydrocarbons, oxygenated sesquiterpenes and oxygenated monoterpenes. In flowers from plants growing in Merelim the major compound group was that of monoterpene hydrocarbons, followed by alkanes and oxygenated monoterpenes.

Keywords: Hypericum perforatum, essential oils, flowers, sesquiterpene hydrocarbons, 2-methyl-octane

1- Introduction

The genus *Hypericum* belongs to the Clusiaceae family and encompasses about 460 species accommodated in 36 sections (Robson, 2006). In Portugal, there are 17 species arranged in nine sections of the genus (Nogueira *et al.*, 2008).

Hypericum perforatum (St. John's wort) is the most studied species of the genus being its extracts widely used as antiviral, wound healing, antioxidant, antimicrobial, antifungal, anxiolytic and anticonvulsant, as well as in depressive disorders (Vandenbogaerde *et al.*, 2000; Barnes *et al.*, 2001; Bilia *et al.*, 2002; Butterweck *et al.*, 2003; Avato *et al.*, 2004; Hosseinzadeh *et al.*, 2005). Infusions prepared from the flowers, leaves and fruits of various *Hypericum* species have been used as a vermifuge, diuretic, as well as a wound healing, sedative, antihysterical and antidepressive agent (Prado *et al.*, 2002; Rabanal *et al.*, 2002). Decoctions of *H. perforatum* flowering aerial parts have been used to treat liver troubles (Camejo-Rodrigues *et al.*, 2003), as well as depression and rheumatism (Nogueira *et al.*, 1998).

The aforementioned activities are mainly attributed to flavonoids, xanthones, tannins, phloroglucinols (hyperforin and adhyperforin) and naphtodianthrones (hypericin, protopseudohypericin, pseudohypericin and protohypericin) (Kitanov, 2001). The biological activities from *H. perforatum* essential oils (EO) have been less studied than those from the phenolic components. However, its antimicrobial activity has already been demonstrated against several Gram-positive and Gram-negative bacteria and fungus, as *Candida albicans* (Gudžić *et al.*, 1997; Saroglou *et al.*, 2007).

Several reports describing the EO composition of *H. perforatum* grown in different countries have shown the sesquiterpene hydrocarbons, *(E)*-caryophyllene, germacrene D, *β*-farnesene, and the alkanes, 2-methyl-octane, 2-methyl-decane and 2-methyl-dodecane as the compounds present with the highest frequency (Gudžić *et al.*, 1997; Nogueira *et al.*, 1998; Gudžić *et al.*, 2001; Baser *et al.*, 2002; Mockutė *et al.*, 2003; Pintore *et al.*, 2005; Pavlović *et al.*, 2006; Saroglou *et al.*, 2007). EO isolated from *H. perforatum* plants cultivated in Italy, Greece and Serbia were characterized by the high contents of *α*-pinene (Pintore *et al.*, 2005; Pavlović *et al.*, 2006; Saroglou *et al.*, 2007). On the other hand, EO of *H. perforatum* from Uzbekistan showed high contents of oxygenated sesquiterpenes, such as caryophyllene oxide and spathulenol (Baser *et al.*, 2002; Mockutė *et al.*, 2003). A different composition was found in the EO of *H. perforatum* from India, in which the two major compounds were ishawarane and *α*-cuprenene (Weyerstahl *et al.*, 1995). The EO yields and composition are known to be

affected by several biotic and abiotic factors (Figueiredo *et al.*, 2008). Indeed, variations in EO composition of several species of the *Hypericum* genus were previously reported, depending on genetic factors (Petrakis *et al.*, 2005), geographical distribution and environmental factors (Couladis *et al.*, 2001; Smelcerovic *et al.*, 2007), ontogeny (Schwob *et al.*, 2004; Nogueira *et al.*, 2008), and plant organ (Bertoli *et al.*, 2003).

Despite the great scientific interest in the phenolic fraction of *H. perforatum*, the high potential therapeutic properties of its volatile fraction make also imperative studies on its EO profiles. Even though a significant number of studies on the chemical characterization of *H. perforatum* have been published, literature on the variation profiles of its EO is scarce. Thus, as part of the phytochemical investigation in species of the *Hypericum* genus, we determined and report here the variation profiles of the EO of *H. perforatum* (common cultivar) growing in two different sites of the Northern of Portugal (Arcos de Valdevez and Merelim) and harvested with intervals of 2-3 months over one year, as well as the EO composition of their flowers.

2- Material and Methods

2.1- Plant material

Plants of *Hypericum perforatum* L. common cultivar were grown in two experimental farms from the Direcção Regional de Agricultura e Pescas do Norte (DRAPN), located at Arcos de Valdevez and Merelim (Northern region of Portugal) kept under the responsibility of DRAPN. Voucher specimens are maintained in an active bank in DRAPN. Aerial parts from 6-8 cultivated plants were randomly collected in accessions made in the months of January, April, June, September and December.

Flowers were separated from one subsample of plant material randomly collected in June, and processed independently in the hydrodistillation and EO analysis. As anthesis occurred from May to July, in an asynchronous way, the flower sample included flowers of different ages within that time interval.

2.2- Essential oils isolation

Subsamples of fresh vegetative aerial parts (10g) and fresh flowers (10g) were submitted to hydrodistillation with 500 ml of boiling water in a Clevenger type apparatus over 1h, using volumes of 1.0 mL of *n*-hexane, containing 5-α-cholestane (1mg/mL), for retention of the hydrodistillate components. The dry weight of the plant material was determined after

drying (60ºC, 72h) in a drying stove. Further analyses of the hydrodistillates were performed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS).

2.3- Analysis

GC-MS analysis was performed with a Perkin-Elmer 8500 gas chromatograph equipped with a fused silica DB5 column (30 m long x 0.25 ID, 0.25 μm film thickness composed by 5%-phenyl methylpolysiloxane, J & W Scientific), connected with a Finnigan MAT Ion Trap Detector (ITD; software version 4.1) operating in EI mode at 70eV. GC-MS analysis was also performed with a Thermo Trace GC Ultra gas chromatograph equipped with a fused silica TR-5 (5% phenyl methylpolysiloxane) connected to a Thermo-Finnigan Polaris Q ion trap detector, operating in EI FullScan mode in the range 40-400 m/z. Analysis conditions were the same for the two equipments. Injector, interface and ion-source temperatures were 300ºC, 260ºC and 220ºC respectively. The oven temperature program included a ramp from 60ºC (0.00 min) to 285 °C at 3°C min^{-1}. Helium (He) was used as carrier gas with a column head pressure of 12.5 psi for Perkin Elmer equipment; while for the Thermo-Finnigan, He was used as carrier gas with an even flow rate of 1.5 $mL.min^{-1}$. The injections of the samples (0.5 μl) were made in splitless mode with the split valve opening at the end of 0.1 second after the injection.

The identification of the compounds was performed according to recommendations of the International Organization of the Flavor Industry (1991). Mass spectra libraries, namely NIST and a terpene library containing mass spectra, retention times and retention indices on DB-5 and other similar columns, were used in the identification of all compounds as well as the comparison with published data, namely retention times and retention indices of EO compounds on DB-5 column (Adams, 1989; Adams, 2001). The retention indices of the EO constituents were determined relative to *n*-alkanes of a complete series, from *n*-octane to *n*-tetratriacontane, eluted in the same conditions as the EO samples, as well as co-eluted with the EO samples.

GC analyses were performed using a Perkin Elmer Autosystem gas chromatograph equipped with a fused silica DB5 column as that of GC-MS. The temperature oven was increased at a rate of 3°C min^{-1} from 60ºC to 285ºC; the injector was kept at 300ºC and the flame ionisation detector (FID) at 320ºC. Hydrogen was used as carrier gas at flow rate of 1.49 mL/min under a column head pressure of 12.5 psi. Injections were performed in a split/splitless injector with the splitter opened at the 1:13 split ratio. Three replicates of each sample were processed in the same way. Percentage values from the listed compounds correspond to the values given in the GC report without correction factors. Following the procedure reported

before (Guedes *et al.*, 2004), 5-α-cholestane was used as an internal standard for estimation of the specific content of each essential oil compound. Considering that the determination of individual correction factors is impracticable, due to either the high number of compounds or their absence in the market, compounds of a given group (monoterpene hydrocarbons, oxygen-containing monoterpenes, sesquiterpene hydrocarbons, and oxygen-containing sesquiterpenes) were assumed to have the same GC response factor. The values used as GC response factors of each compound were those previously reported for each group of compounds (Guedes *et al.*, 2004). Given the generalised response factors attributed to the compounds belonging to the same group, the derived quantitative data expressed in mg/g of biomass dry weight may be considered as an approximation of the absolute quantification. The sum of the specific contents of all individual EO compounds was taken as a parameter for the estimation of the total specific EO yield.

3- Results and Discussion

3.1- Yields and composition of essential oils produced by aerial parts of cultivated plants

The sum of the values given by the absolute quantification of all the compounds showed EO contents in flowers significantly higher than in the vegetative aerial parts, independently of the grown site (Figure 1).

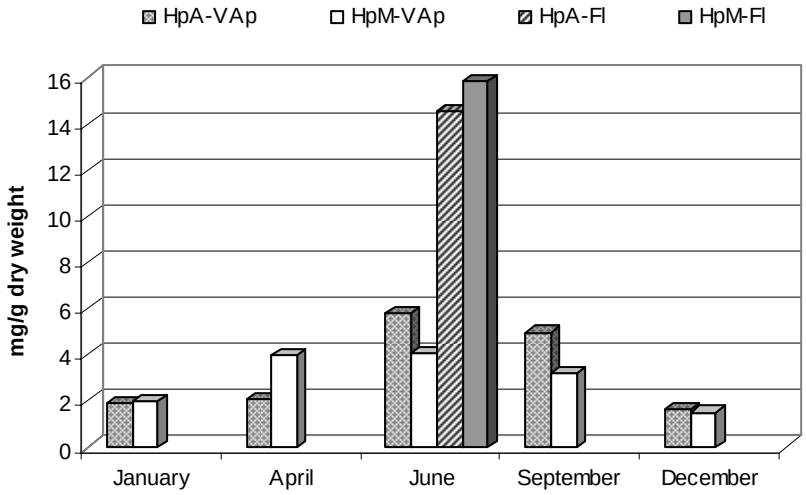


Figure 1- Total essential oil contents from aerial parts of *Hypericum perforatum* plants cultivated in Arcos de Valdevez (HpA) and Merelim (HpM) experimental fields and harvested at different times during the year. **VAp**- Vegetative aerial parts; **Fl**- Flowers.

The EO contents ranged from 1.6-5.8 mg/g of dry weight in Arcos de Valdevez (HpA-VAp) and from 1.5-4.1 mg/g of dry weight in Merelim (HpM-VAp). As can be seen, in Figure 1, the EO contents in the vegetative aerial parts started rising from their lowest levels in December (1.5-1.6 mg/g dry weight) up to their highest levels in the summer. In the winter, EO contents of *H. perforatum* were similar in HpA and HpM. However in June, as well as in September, HpA-VAp showed higher EO contents than HpM-VAp, in contrast to what occurred in April. The EO contents in the flowers were almost four fold higher than in the vegetative aerial parts (14.6 mg/g of dry weight in HpA-Fl and 15.9 mg/g of dry weight in HpM-Fl). Similar results were reported by Schowb and co-workers (2004). The observed variations in the EO contents between both *H. perforatum* plant populations grown in the two fields could be attributed to pedologic and climatic factors. In fact, low yields of EO in the winter should be related with the climacteric conditions typical of this season, such as low temperatures. Usually, in this period, plants of *H. perforatum* loose their vegetative aerial parts. In December a substantial reduction in the growth of the aerial parts of the plants was registered in the two sites (data not shown). Influence of environmental factors in the EO contents have been already reported for several species, including some of the *Hypericum* genus (Couladis *et al.*, 2001; Schwob *et al.*, 2002; Mockutė *et al.*, 2003; Mockute *et al.*, 2008). Flowers of *H. perforatum* possess translucent glands and type B cannals structures which accumulate EO (Ciccarelli *et al.*, 2001; Maffi *et al.*, 2005). The higher amounts of EO obtained in flowers might be related to the type and density of secretory structures differentiated in this organ.

About 100 compounds were identified in the EO of both populations (HpA and HpM) of *H. perforatum* (Table 1). Most of the compounds were distributed mainly by monoterpenes (hydrocarbons - MH, and oxygenated - MO), sesquiterpenes (hydrocarbons - SH, and oxygenated - SO) and alkanes (Table 1) and in both cases the EO were dominated by the hydrocarbon compounds, either sesquiterpenes or monoterpenes.

Table 1- Composition of the essential oils from aerial parts and flowers of *Hypericum perforatum* plants, cultivated in Arcos de Valdevez (HpA) and Merelim (HpM) experimental fields and harvested at different times during the year.

Compound	KI	January		April		June				September		December	
		HpA VAp (%)	HpM VAp (%)	HpA VAp (%)	HpM VAp (%)	HpA VAp (%)	HpA Fl (%)	HpM VAp (%)	HpM Fl (%)	HpA VAp (%)	HpM VAp (%)	HpA VAp (%)	HpM VAp (%)
1-Octene	793	0.5	0.3	0.2	0.2	0.5	0.1	0.3	0.1	0.3	0.3	0.4	0,4
(E)-2-Hexenal	854		0.3	0.5	0.5	1.2		1.0		0.6	0.6	0.5	0,1
2-methyl-octane	864	10.5	11.2	7.3	9.8	13.1	24.3	14.8	28.8	12.0	11.4	8.7	5,6
n-Nonane	900	2.3	3.0	6.8	2.1	2.5	1.0	1.7	1.1	3.8	3.8	2.1	1,4
α-Thujene	929	0.8	tr	0.3	0.2	1.5	0.3	1.2	0.3	0.1	0.9	0.6	0,4
α-Pinene	936	4.5	2.2	6.1	2.5	4.9	3.6	3.1	9.8	2.7	6.0	4.4	2,7
2,6-Dimethyl-octane	972	0.9	1.3	0.9	0.7	1.0	0.5	1.1	0.6	1.3	0.7	0.6	0,4
Sabinene	975	0.9	0.3	1.5	1.2	3.2	0.9	1.6	0.4	0.4	1.5	1.2	1,1
β-Pinene	978	1.4	0.3	1.4	0.9	3.3	2.7	2.1	12.2	0.5	2.3	1.4	1,3
6-Methyl-5-Hepten-2-one	988					tr		tr	tr				
Myrcene	986	0.5	0.3	0.8	0.6	1.4	0.7	0.8	0.8	0.3	1.2	0.8	0,6
n-Decane	1000					tr	tr	tr	tr	tr	tr	tr	
Hexyl acetate	1005			0.5	0.3	0.1		tr	tr	0.1	0.3		
α-Terpinene	1018			0.1	0.1	0.5	0.1	0.3	0.1	tr	0.4	0.1	tr
p-Cymene	1026					0.1	tr	0.1	tr		tr		
Limonene	1031	0.5	tr	0.3	0.3	0.8	0.4	0.5	0.5	0.1	0.6	0.3	0,4
Z-β-Ocimene	1041	1.4	0.6	1.5	1.2	0.8	1.2	0.5	0.9	0.5	1.3	1.1	1,4
E-β-Ocimene	1052	8.6	5.9	8.7	9.2	4.6	11.6	2.3	8.9	3.3	6.7	7.3	10,5
γ-Terpinene	1061	tr		0.2	0.2	0.9	0.3	0.5	0.2	0.1	0.6	0.3	0,1
Methyl decane	1069		0.3	tr	0.1	0.5	1.3	0.4	1.0	0.1	0.1	0.1	
trans-Linalool oxide (furanoid)	1073							tr					
Terpinolene	1090			0.1	0.1	0.2	0.1	0.1	0.1	tr	0.2	0.1	
Linalool	1099					0.2		0.1	tr				
n-Undecane	1100	tr	0.3	0.2	0.2	0.3	0.4	0.4	0.3	0.2	tr	0.2	tr
n-Nonanal	1104	tr	0.3	0.1	0.1	tr	tr	0.1	0.1	tr	0.1	0.2	0,1
α.Campholenal	1121	tr	tr	0.1	0.1	0.1		0.1	0.1	0.1	0.1	0.2	0,1
Camphor	1145					tr		tr	tr				
n-Nonanol	1170			0.3	0.2	0.1	0.4	0.1	0.3	0.2	0.4	0.3	1,0
Terpinen-4-ol	1176	0.5	0.5	0.2	0.2	0.6	0.3	0.2	0.2	0.1	0.5	0.3	tr
α-Terpineol	1189	tr	0.2	tr	tr	0.1	0.1	tr	0.2	tr	0.1		
n-Decanal	1205	0.5	0.8	1.1	0.9	0.2		0.1	tr	0.4	0.7	0.7	1,7
trans-Carveol	1217					tr							
Methyl dodecane	1265					tr	0.2	tr	0.2				
n-Decanol	1273			0.1	0.2	tr		tr		0.1	0.1	0.2	0,3
n-Tridecane	1300					tr	tr	tr	0.1				
SH	1327	tr	tr	0.1	0.1	0.1	0.1	0.1		0.1	0.1	0.1	tr
δ-Elemene	1337	1.1	1.0	0.9	0.9	0.5	0.4	0.7	0.2	0.8	0.7	0.8	1,1
α-Cubebene	1348			0.1	tr	tr	0.1	tr	0.1	0.1	0.1		

Compound	KI	January		April		June				September		December	
		HpA VAp (%)	HpM VAp (%)	HpA VAp (%)	HpM VAp (%)	HpA VAp (%)	HpA Fl (%)	HpM VAp (%)	HpM Fl (%)	HpA VAp (%)	HpM VAp (%)	HpA VAp (%)	HpM VAp (%)
α–Ylangene	1370			tr	0.1	tr	tr	tr	0.1	tr	tr		
α-Copaene	1374		tr	0.1		0.3	0.3	0.1	0.1	tr	0.1	0.1	tr
β-Bourbonene	1382	0.5	0.3	0.1	0.1	0.2	0.1	0.3	0.1	0.1	0.2	0.1	0,4
β-Cubebene	1388		0.5						0.1		tr		
β-Elemene	1390	0.9	0.3	0.2	0.5	0.6	0.3	0.6	0.1	0.8	0.7	0.8	0,8
Dodecanal	1406	tr				tr		tr	tr	tr			
α-Cedrene	1412	0.3	0.3	0.7	0.3	0.4	0.3	0.5	0.2	0.7	0.6	0.3	0,4
SH	1415			0.1						0.1	tr		
(E)-Caryophyllene	1417	7.3	6.2	5.5	7.7	10.8	17.8	12.5	10.2	11.3	10.2	5.5	5,2
β-Gurjunene	1427	0.5	0.6	0.3	0.4	0.2	0.3	0.3	0.1	0.4	0.3	0.3	0,6
α-Guaiene	1439					0.1	0.1	0.1	0.1	tr	tr		
Aromadendrene	1440			0.2	0.1	0.7	0.2	0.4	0.1	tr	0.1	0.3	0,4
(Z)-*β*-Farnesene	1442	0.2	0.3		0.2	0.1	0.1	0.2	0.1	0.2	0.2		
SH	1446			tr		tr	0.1	tr	tr	0.1	tr		
α-Himachalene	1450	0.9	0.7	0.7	0.8	0.9	0.9	1.0	0.5	1.0	0.9	0.8	0,8
α-Humulene	1453			tr				tr			tr		
E-*β*-Farnesene	1455					tr	tr	0.2	tr	tr			
allo-aromadendrene	1459	0.5	1.0	1.1	0.8	0.4	2.7	0.4	1.2	1.6	0.7	0.5	0,4
α-acoradiene	1468			0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.2	tr	
Dodecanol	1474						tr						
γ-Gurjunene	1475		4.9	0.1	5.2	3.5	0.2	3.3		3.5	2.4	9.1	9,9
γ-Muurolene	1478	tr	tr	0.1	tr	tr	tr	tr	tr	tr	tr		
Germacrene D	1481	41.7	39.5	29.6	35.7	14.1	11.0	21.8	9.0	30.2	21.3	32.4	32,7
Curcumene	1483			1.3		tr		0.1					
SH	1487			tr	tr	tr	0.1	0.1	0.1	tr	tr		
β-Selinene	1488	7.3	6.0	5.8	6.0	3.2	3.1	4.6	1.8	5.3	4.9	5.3	5,9
α-Selinene	1494			0.8	0.2						0.3		
α-Muurolene	1497	0.3	0.3	0.2	0.1	0.1	0.2	0.2	0.1	0.4	0.2	0.2	0,1
n-Pentadecane	1500			tr	0.1	0.1		0.1		0.2	0.1	0.1	tr
(E,E)-*α*-Farnesene	1503		0.1	0.1	0.1	tr	0.1	0.1	tr	0.1	0.1	tr	
γ-Cadinene	1508	0.5	0.5	0.7	0.5	0.4	0.9	0.6	0.3	1.0	0.6	0.3	0,4
Z-*γ*-Bisabolene	1510			0.3	0.2	0.2	0.3	0.3	0.2	0.2	0.2		
δ-Cadinene	1521	0.9	0.6	1.2	0.8	0.6	0.9	0.9	0.5	0.9	1.0	0.8	0,8
SH	1527					tr		tr			tr		
E-*γ*-Bisabolene	1530			0.1	0.1	tr	0.1	0.1	tr	0.1	tr		
α-Cadinene	1534			0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	tr	
SH	1540					tr		tr					
α-Calacorene	1543			0.1	tr	tr	tr	tr	tr	0.1	0.1		
SO (?)	1549			0.1		0.4	tr	0.3	0.1		0.1		
γ-Elemene (?)	1551					0.1		tr		tr			
E-Nerolidol	1564	0.1	0.5	0.8	0.8	0.8	0.6	0.6	0.4	0.8	1.0	0.9	1,1
Spathulenol	1574	0.5			0.6	0.2	0.2	0.2	0.1		0.1	0.5	

Compound	KI	January		April		June				September		December	
		HpA VAp (%)	HpM VAp (%)	HpA VAp (%)	HpM VAp (%)	HpA VAp (%)	HpA Fl (%)	HpM VAp (%)	HpM Fl (%)	HpA VAp (%)	HpM VAp (%)	HpA VAp (%)	HpM VAp (%)
cis-3-Hexenyl benzoate	1577		0.3	0.6	0.1	2.9	0.1	2.0	0.2	0.4	0.4		0,8
Caryophyllene Oxide	1580	0.3	0.3	0.2	0.3	5.0	0.9	3.8	2.1	0.3	0.1	0.5	0,4
Globulol	1585				0.1	0.1	tr	tr	tr	0.1	0.1	tr	tr
Viridiflorol	1592	tr	0.1	0.2	0.3	0.1	0.1	0.1	0.1	0.2	0.4	0.3	tr
Guaiol	1593			0.2		0.1	0.1	tr	0.1	0.1	tr		
β-Copaen-4-*α*-ol	1597	tr	0.2	1.2	0.3	0.1	0.1	0.1	0.1	0.2	0.4	0.3	0,3
Humulene Epoxide II	1603					0.2		0.2				tr	
β-Oplopenone	1610					0.1	tr	0.1	tr	0.1	tr		
SO	1612		tr		0.2	0.1		0.1		0.2	0.3	0.3	tr
10-*epi*–*γ*-Eudesmol	1619	tr	0.1	0.2	0.1	0.2	0.2	0.2	0.1	0.2	0.2	0.3	0,1
γ-Eudesmol	1624		0.3	0.1	0.1	tr	0.1	0.1	tr	tr	0.1	0.1	tr
epi-*α*-Cubenol	1633	1.5	1.8	0.1	0.5	0.4	0.1	0.3	0.1	1.0	0.9	1.9	2,8
epi-*α*–Cadinol	1640		0.6	1.0	0.8	1.7	2.5	0.5	1.2	0.7	1.0	1.0	0,8
epi-*α*-Muurolol	1642		0.2	0.3	0.3	0.2	0.1	1.0	0.1	0.3	0.4	0.3	0,4
SO	1646					0.1		0.1					
α-Cadinol	1654	0.9	1.2	1.6	1.2	0.7	0.7	0.8	0.4	1.2	1.4	1.8	1,7
β-Bisabolol	1673			0.1	tr	0.2	tr	0.1	tr	tr	0.8	0.2	
epi-*α*-Bisabolol	1677			0.1		1.9	0.7	1.9	0.2	1.6	0.5	0.6	
n-Tetradecanol	1681		0.6	0.2	0.4	0.3		0.3		0.4			
Benzyl benzoate	1738					tr	tr	tr					
n-Hexadecanol	1881					0.2	0.1	0.3	tr	0.1	0.1		
Nonadecane	1900						0.1		0.2				
n-Eicosane	2000						tr	tr	0.1				
n-Heneicosane	2100						0.2	tr	0.3	tr	tr		
Nonadecanal	2111			0.3	0.1	0.3	0.1	1.0	tr	4.1	2.6	0.1	
n-Docosane	2200			tr			tr	tr	tr	tr	tr	0.3	
n-Tricosane	2300	0.5	0.5	0.5	0.2	tr	0.1	0.1	0.1	0.1	0.1		0,4
n-Tetracosane	2400		tr	0.1	tr	tr	tr	tr	tr	tr	tr	0.1	tr
n-Pentacosane	2500	0.1	0.7	0.7	0.2	0.1	0.1	0.1	tr	0.3	0.2	0.4	0,4
n-Hexacosane	2600						tr		tr	tr	tr		
n-Heptacosane	2700		tr	0.1	tr	0.1	0.1	0.1	0.1	0.2	0.2	0.1	tr
n-Octacosane	2800						tr		tr	tr	tr		
n-Nonacosane	2900			tr		0.1	0.3	0.2	0.2	0.1	0.3		
Monoterpene Hydrocarbons		18.5	9.7	21.2	16.5	22.2	21.9	13.1	34.2	8.0	21.6	17.5	18.5
Oxygenated Monoterpenes		0.5	0.7	0.2	0.2	0.9	0.3	0.3	0.4	0.1	0.6	0.3	0.0
Sesquiterpene Hydrocarbons		62.6	63.2	50.8	61.0	37.9	40.7	49.6	25.5	59.3	46.3	57.6	60.2
Oxygenated Sesquiterpenes		3.3	5.4	6.2	5.5	12.6	6.3	10.4	5.1	6.8	7.7	8.9	7.8
Alkanes		14.2	17.4	16.6	13.0	17.9	28.8	18.5	33.0	18.4	16.9	12.7	8.3
Others		0.9	3.6	5.0	3.8	8.6	2.0	8.0	1.8	7.5	6.8	3.0	5.2

VAp- Vegetative aerial parts; **Fl-** Flowers; **KI-** Kovats retention index on a DB-5 column; **tr-** trace amounts

Regardless the cultivation site and time of harvest, sesquiterpene hydrocarbons constituted the major group of compounds in the EO of the vegetative aerial parts of plants of this species. However, different variation profiles of their specific contents were registered over the year depending of the experimental field where the plants were growing (Figures 2a and 2b).

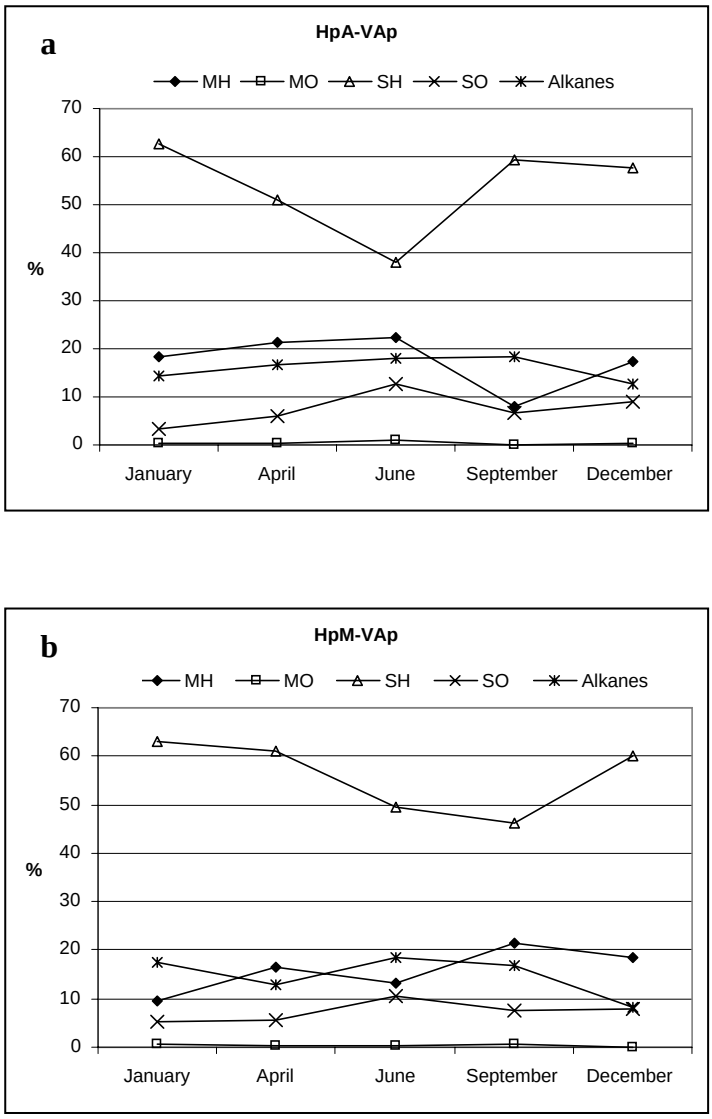


Figure 2- Relative contents of the major groups of compounds of the essential oils from vegetative aerial parts of *Hypericum perforatum* plants cultivated in **(a)** Arcos de Valdevez (HpA-VAp), and **(b)** Merelim (HpM-VAp) experimental fields and harvested at different times over the year. **MH**- Monoterpene Hydrocarbons; **MO**- Oxygenated Monoterpenes; **SH**- Sesquiterpene Hydrocarbons; **SO**- Oxygenated Sesquiterpenes.

As it can be seen in Figure 2a, a drastic decrease in the EO sesquiterpene hydrocarbons content was registered in HpA-VAp from January (62.6%) to June (37.9%). In HpM-VAp sesquiterpene hydrocarbons content ranged from 63.2%, in January, to 46.3%, in September

(Figure 2b). An increase in the content of sesquiterpene hydrocarbons was then observed from June to September, in HpA-VAp and from September to December, in HpM-VAp. An inverse variation in the relative content of the monoterpene hydrocarbons group occurred in HpA-VAp, which decreased from 22.2%, in June, to 8.0%, in September. In both sites the variation in the contents of the total monoterpene hydrocarbons was inversely correlated with that of the sesquiterpene hydrocarbons, with exception for HpM-VAp between April and June. Biosynthesis of the compounds from these groups follows different pathways in different cell compartments, with monoterpenes synthesised in the plastids and sesquiterpenes synthesised in the cytosol (Davis *et al.*, 2000). Variations of these two groups of compounds can reflect different overall development of both subcellular compartments in the different times of the year. Alkanes were the second most represented group of compounds in HpA-VAp samples harvested in September and in HpM-VAp samples harvested in January and June.

Different compositions of the *H. perforatum* EO have been reported. Our results are consistent with those reported for plants grown in Italy (Pintore *et al.*, 2005), France (Schwob *et al.*, 2002), Serbia (Gudžić *et al.*, 1997; Gudžić *et al.*, 2001) and Lithuania (Mockutė *et al.*, 2003), whose total sesquiterpene hydrocarbons constituted also the major group of compounds. However, plants of *H. perforatum* growing in some different regions of Lithuania were characterized by their high levels of oxygenated sesquiterpenes (Radusiene *et al.*, 2005). On the other hand, some authors have found low contents of oxygenated monoterpenes in the EO of *H. perforatum* (Mockutė *et al.*, 2003; Pintore *et al.*, 2005; Radusiene *et al.*, 2005; Pavlović *et al.*, 2006; Smelcerovic *et al.*, 2007).

Germacrene D, the major compound in *H. perforatum* EO, was the one that most contributed for the variation of the sesquiterpene hydrocarbons group in HpA-VAp and HpM-VAp, excepting between September and December, in the case of HpA-VAp. *(E)*-Caryophyllene and *β*-selinene were the second and third major sesquiterpene hydrocarbons in both HpA-VAp and HpM-VAp EO. However, the variation of these two compounds did not follow the pattern of the variation of the sesquiterpene hydrocarbons group (Table 1). The HpA and HpM EO composition herein reported was similar to the EO composition of *H. perforatum* plants growing in Southern France, in what respect the high sesquiterpene specific amounts, and especially those of germacrene D (Schwob *et al.*, 2002). Germacrene D and *(E)*-caryophyllene were also two of the major components in EO of *H. perforatum* plants growing in Lithuania (Mockutė *et al.*, 2003). However, in the EO of plants from this species growing in the South-East of France the most represented sesquiterpene hydrocarbons were *(E)*-

caryophyllene, γ-muurolene, (*E*)-β-farnesene and β-funebrene (Schwob *et al.*, 2004), the last one of which was not identified in our *H. perforatum* plants.

2-Methyl-octane was the second major constituent of the EO in HpA-VAp and HpM-VAp. In HpA-VAp it ranged from 7.3% in April to 13.1% in June. In HpM-VAp this compound reached its maximum in June (14.8%) decreasing then until 5.6%, in December. High levels of this alkane were also found in the EO of *H. perforatum* by other authors (Nogueira *et al.*, 1998; Gudžić *et al.*, 2001; Pintore *et al.*, 2005; Pavlović *et al.*, 2006). In this work, the alkanes group includes 2-methyl-octane together with other branched and *n*-alkanes. In plant samples harvested in September in both experimental fields, a complete series of *n*-alkanes from C_{21} to C_{29} was identified. The presence of an almost complete series of *n*-alkanes, from $C_{18}H_{38}$ to $C_{28}H_{58}$ was also found in the EO of another species of this genus, *Hypericum androsaemum* (Guedes *et al.*, 2003). In dried leaf of *H. perforatum* a higher series of *n*-alkanes, from $C_{16}H_{34}$ to $C_{29}H_{60}$, had already been reported (Brondz *et al.*, 1983).

Little variations in the contents of the oxygenated sesquiterpenes were registered in plants grown in both fields although a slight increase had occurred from April to June, when the respective maxima were reached (HpA- 12.6% and HpM- 10.4%). In contrast, the oxygenated sesquiterpenes group was the dominant fraction in the EO of vegetative parts of *H. perforatum* plants grown in France (Schwob *et al.*, 2004). Maximum levels of oxygenated sesquiterpenes coincided with maximum amounts of caryophyllene oxide (HpA-VAp, 5.0% and HpM-VAp, 3.8%). From April to June, not only the increase in caryophyllene oxide contents but also the increase of *epi*-α-bisabolol (1.9%), contributed to the increase of the percentage of the total oxygenated sesquiterpenes in the EO of both HpA-VAp and HpM-VAp. In previous studies, caryophyllene oxide was reported as one of the most important volatile constituents of the EO of *H. perforatum* plants growing in France (Schwob *et al.*, 2004) and Lithuania (Mockutė *et al.*, 2003). The main constituents of monoterpene hydrocarbons were α-pinene and *E*-β-ocimene. The accentuated decrease in the contents of the total monoterpene hydrocarbons, from June to September, in HpA-VAp, could be explained by the decrease in specific contents of these two compounds as well as those of sabinene and β-pinene. In HpM, the most represented monoterpene hydrocarbons were *E*-β-ocimene and α-pinene, which ranged from 2.3% to 10.5% and 2.2% to 6.0%, over the year, respectively.

3.2- Essential oils from H. perforatum *flowers*

The contents of the main groups of compounds detected in EO of *H. perforatum* flowers harvested in June are shown in Figure 3 (HpA-Fl and HpM-Fl).

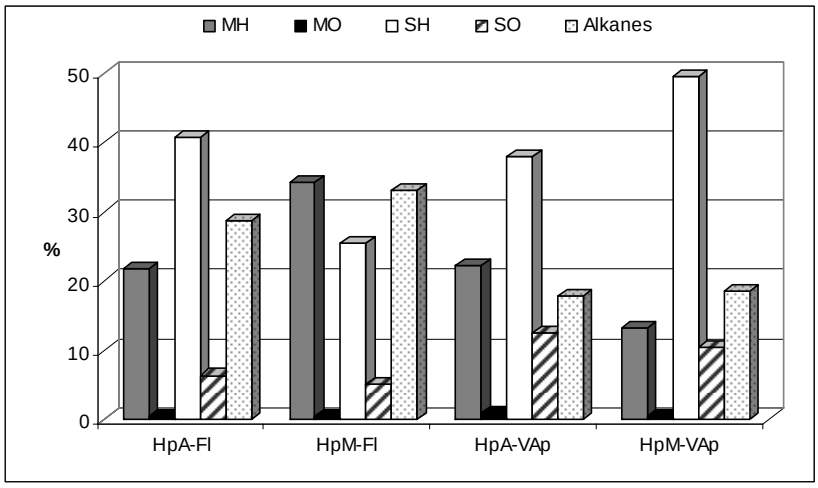


Figure 3- Relative contents of major groups of compounds of the essential oils from flowers and vegetative aerial parts of *Hypericum perforatum* plants cultivated in Arcos de Valdevez (HpA) and Merelim (HpM) experimental fields and harvested in June. **Fl**- Flowers; **VAp**- Vegetative aerial parts; **MH**- Monoterpene Hydrocarbons; **MO**- Oxygenated Monoterpenes; **SH**- Sesquiterpene Hydrocarbons; **SO**- Oxygenated Sesquiterpenes

Sesquiterpene hydrocarbons constituted the major group of compounds in HpA-Fl (40.7%) while in HpM-Fl the major group was that of monoterpene hydrocarbons (34.2%). Alkanes constituted the second most represented group (HpA-Fl – 28.8% and HpM-Fl – 33.0%). In both cases (HpA and HpM), the percentage contents of alkanes were higher in flowers than in the vegetative aerial parts (Figure 3). The percentage content of the monoterpene hydrocarbons group in HpM-Fl was twice higher than that of HpM-VAp in inverse correlation with oxygenated sesquiterpenes group that was around twice lower than those of HpM-VAp. The differences in the composition between the EO from HpM-Fl and those of the corresponding vegetative aerial parts (HpM-VAp) were greater than those shown between HpA-FL and HpA-VAp (Figure 3). In flowers EO from *H. perforatum* plants growing in France, sesquiterpene hydrocarbons were the dominant fraction (Schwob *et al.*, 2004). On the other hand, in EO from flowers from *H. perforatum* growing in Lithuania, oxygenated sesquiterpenes constituted the main group of compounds (Radusiene *et al.*, 2005). The group of the oxygenated sesquiterpenes represented no more than 6.3% in HpA-Fl and 5.1% in HpM-Fl.

The most represented compound in EO from HpA-Fl and HpM-Fl was 2-methyl-octane, representing 24.3% and 28.8%, respectively, almost twice higher than in the EO of the respective vegetative aerial parts. In contrast to our results, this alkane was present in low amounts in flowers from *H. perforatum* plants growing in Lithuania (Radusiene *et al.*, 2005) and was not detected in flowers from *H. perforatum* growing in France (Schwob *et al.*, 2004). In EO from both HpA-Fl and HpM-Fl, a complete series of *n*-alkanes from C_{19} to C_{29} was detected. From this series, C_{19}, C_{26} and C_{28} were not detected in the EO from HpM-VAp, while HpA-VAp only shared C_{23}, C_{24}, C_{25}, C_{27}, C_{29} with the EO from its respective flowers. *n*-Eicosane was the only *n*-alkane from this series common to that of *H. perforatum* EO studied by Radusiene and co-workers (2005) whereas *n*-heptadecane, not detected in our work, was the only one found in flowers from *H. perforatum* growing in France (Schwob *et al.*, 2004). *(E)*-Caryophyllene (17.8% in HpA-Fl and 10.2% in HpM-Fl) and germacrene D (11.0% in HpA-Fl and 9.0% in HpM-Fl) were the two main constituents of the sesquiterpene hydrocarbons group and two of the most represented in the total EO of *H. perforatum* flowers. High levels of *(E)*-caryophyllene in EO from flowers of this species had already been reported (Schwob *et al.*, 2004; Radusiene *et al.*, 2005). On the other hand, regarding germacrene D, our results are somewhat contradictory with those reported by other authors, who detected minor amounts of this compound in EO from flowers of *H. perforatum* (Schwob *et al.*, 2004; Radusiene *et al.*, 2005).

E-β-Ocimene was the major monoterpene hydrocarbon in the EO of HpA-Fl (11.6%) and the third one in the EO of HpM-Fl (8.9%). The most represented monoterpene hydrocarbon in the EO of HpM-Fl was β-pinene (12.2%) followed by α-pinene (9.8%). α-Pinene accounted for only 3.6% of the total EO of HpA-Fl. Low amounts of α-pinene were previously reported in EO from flowers of *H. perforatum* (Schwob *et al.*, 2004; Radusiene *et al.*, 2005) while β-pinene was not identified in EO from *H. perforatum* growing in Lithuania (Radusiene *et al.*, 2005).

In conclusion, sesquiterpene hydrocarbons constituted the major group of compounds in vegetative aerial parts of *H. perforatum* plants, being also the dominant group in EO of flowers from *H. perforatum* plants cultivated in Arcos de Valdevez. The EO of flowers from plants growing in Merelim, had however, monoterpene hydrocarbons and alkanes as the dominant groups. The EO of vegetative aerial parts of *H. perforatum* were characterized by a high content of germacrene D, the most represented compound over the year. 2-Methyl-octane was the main constituent in the EO of *H. perforatum* flowers in both cultivation sites, and the second one in its respective vegetative aerial parts.

Acknowledgements

This work was sponsored by EU (FSE/FEDER) and Portuguese Government (FCT) through the Project PTDC/AGR-AAM/70418/2006 and the Grant SFRH/BD/13283/2003.

References

Adams, R. P. (1989). *Identification of Essential Oils by Ion Trap Mass Spectroscopy*. San Diego, CA, Academic Press.

Adams, R. P. (2001). *Identification of Essential Oils Components by Gas Chromatography/Quadropole Mass Spectroscopy*. Illinois, USA, Allured Publishing Corporation, Carol Stream.

Avato, P., F. Raffo, G. Guglielmi, C. Vitali & A. Rosato (2004). Extracts from St John's wort and their antimicrobial activity. *Phytotherapy Research 18*(3), 230-232.

Barnes, J., L. A. Anderson & D. J. Phillipson (2001). St John's wort (*Hypericum perforatum* L.): a review of its chemistry, pharmacology and clinical properties *Journal of Pharmacy and Pharmacology 53*(5), 583-600.

Baser, K. H. C., T. Ozek, H. R. Nuriddinov & A. B. Demirci (2002). Essential oils of two *Hypericum* species from Uzbekistan. *Chemistry of Natural Compounds. 38*(1), 54-57.

Bertoli, A., F. Menichini, M. Mazzetti, G. Spinelli & I. Morelli (2003). Volatile constituents of the leaves and flowers of *Hypericum triquetrifolium* Turra. *Flavour and Fragrance Journal 18*(2), 91-94.

Bilia, A. R., S. Gallori & F. F. Vincieri (2002). St. John's wort and depression: Efficacy, safety and tolerability-an update. *Life Sciences 70*(26), 3077-3096.

Brondz, I., T. Greibrokk & A. Aasen (1983). *n*-Alkanes of *Hypericum perforatum*: a revision. *Phytochemistry 22*, 295-296.

Butterweck, V., V. Christoffel, A. Nahrstedt, F. Petereit, B. Spengler & H. Winterhoff (2003). Step by step removal of hyperforin and hypericin: activity profile of different *Hypericum* preparations in behavioral models. *Life Sciences 73*(5), 627-639.

Camejo-Rodrigues, J., L. Ascensão, M. À. Bonet & J. Vallès (2003). An ethnobotanical study of medicinal and aromatic plants in the Natural Park of "Serra de São Mamede" (Portugal). *Journal of Ethnopharmacology 89*(2-3), 199-209.

Ciccarelli, D., A. C. Andreucci & A. M. Pagni (2001). Translucent Glands and Secretory Canals in *Hypericum perforatum* L. (Hypericaceae): Morphological, Anatomical and Histochemical Studies During the Course of Ontogenesis. *Annals of Botany 88*(4, Part 1), 637-644.

Couladis, M., P. Baziou, P. V. Petrakis & C. Harvala (2001). Essential oil composition of *Hypericum perfoliatum* L. growing in different locations in Greece. *Flavour and Fragrance Journal 16*(3), 204-206.

Davis, E. M. & R. Croteau (2000). Cyclization Enzymes in the Biosynthesis of Monoterpenes, Sesquiterpenes and Diterpenes. *Topics in Current Chemistry 209*, 54-95.

Figueiredo, A., J. Barroso, L. Pedro & J. Scheffer (2008). Factors affecting secondary metabolite production in plants: volatile components and essential oils. *Flavour and Fragrance Journal 23*, 213–226.

Gudžić, B., S. Đorđević, R. Palić & G. Stojanovi (2001). Essential oils of *Hypericum olympicum* L. and *Hypericum perforatum* L. *Flavour and Fragrance Journal 16*(3), 201-203.

Gudžić, B., J. M. Nedeljković, S. Đorđević & J. J. Čomor (1997). Composition and anti-microbial activity of essential oil of Hyperici herb (*Hypericum perforatum* L.) from Vlasina Region. *Facta Universitatis 1*(4), 47-51.

Guedes, A. P., L. R. Amorim, A. Vicente & M. Fernandes-Ferreira (2004). Variation of the essential oil content and composition in leaves from cultivated plants of *Hypericum androsaemum* L. *Phytochemical Analysis 15*, 146-151.

Guedes, A. P., L. R. Amorim, A. M. S. Vicente, G. Ramos & M. Fernandes-Ferreira (2003). Essential Oils from Plants and *in Vitro* Shoots of *Hypericum androsaemum* L. *J. Agric. Food Chem. 51*(5), 1399-1404.

Hosseinzadeh, H., G.-R. Karimi & M. Rakhshanizadeh (2005). Anticonvulsant effect of *Hypericum perforatum*: role of nitric oxide. *Journal of Ethnopharmacology 98*(1-2), 207-208.

Kitanov, G. M. (2001). Hypericin and pseudohypericin in some *Hypericum* species. *Biochemical Systematics and Ecology 29*(2), 171-178.

Maffi, L., L. Camoni, R. Fornasiero & A. Bianchi (2005). Morphology and development of secretory structures in *Hypericum perforatum* and *H. richeri*. *Nordic Journal of Botany 23*(4), 453-461.

Mockute, D., G. Bernotiene & A. Judzentiene (2008). The Essential Oils with Dominant Germacrene D of *Hypericum perforatum* L. Growing Wild in Lithuania. *Journal of Essential Oil Research 20*(2), 128-131.

Mockutė, D., G. Bernotienė & A. Judžentienė (2003). Volatile compounds of the aerial parts of wild

St. John´s wort (*Hypericum perforatum* L.) plants. *Chemija 14*(3), 108-111.

Nogueira, T., F. Duarte, F. Venâncio, R. Tavares, M. Lousã, C. Bicchi & P. Rubiolo (1998). Aspectos Quimiotaxonómicos do Género *Hypericum* L. em Portugal. *Silva Lusitana 6*(1), 55-61.

Nogueira, T., M. J. Marcelo-Curto, A. C. Figueiredo, J. G. Barroso, L. G. Pedro, P. Rubiolo & C. Bicchi (2008). Chemotaxonomy of *Hypericum* genus from Portugal: Geographical distribution and essential oils composition of *Hypericum perfoliatum*, *Hypericum humifusum, Hypericum linarifolium* and *Hypericum pulchrum*. *Biochemical Systematics and Ecology 36*(1), 40-50.

Pavlović, M., O. Tzakou, P. V. Petrakis & M. Couladis (2006). The essential oil of *Hypericum perforatum* L., *Hypericum tetrapterum* Fries and *Hypericum olympicum* L. growing in Greece. *Flavour and Fragrance Journal 21*(1), 84-87.

Petrakis, P. V., M. Couladis & V. Roussis (2005). A method for detecting the biosystematic significance of the essential oil composition: The case of five Hellenic *Hypericum* L. species. *Biochemical Systematics and Ecology 33*(9), 873-898.

Pintore, G., M. Chessa, G. Boatto, R. Cerri, M. Usai & B. Tirillini (2005). Essential oil composition of *Hypericum perforatum* L. var. angustifolium DC growing wild in Sardinia (Italy). *The Journal of essential oil research 17*(5), 533-535

Prado, B., R. Rabanal & C. Sánchez-Mateo (2002). Evaluation of the central properties of several *Hypericum* species from the Canary Islands. *Phytotherapy Research 16*(8), 740-744.

Rabanal, R. M., A. Arias, B. Prado, M. Hernández-Pérez & C. C. Sánchez-Mateo (2002). Antimicrobial studies on three species of *Hypericum* from the Canary Islands. *Journal of Ethnopharmacology 81*(2), 287-292.

Radusiene, J., A. Judzentiene & G. Bernotiene (2005). Essential oil composition and variability of *Hypericum perforatum* L. growing in Lithuania. *Biochemical Systematics and Ecology 33*(2), 113-124.

Robson, N. (2006). Studies in the genus *Hypericum* L. (Clusiaceae). 1 Section 9. *Hypericum sensu lato* (part 3): subsection 1. *Hypericum* series 2. *Senanensia*, subsection 2. *Erecta* and section 9b. *Graveolentia*. *Systematics and Biodiversity 4*, 19-98

Saroglou, V., P. D. Marin, A. Rancic, M. Veljic & H. Skaltsa (2007). Composition and antimicrobial activity of the essential oil of six *Hypericum* species from Serbia. *Biochemical Systematics and Ecology 35*(3), 146-152.

Schwob, I., J.-M. Bessiere, V. Masotti & J. Viano (2004). Changes in essential oil composition in Saint John's wort (*Hypericum perforatum* L.) aerial parts during its phenological cycle. *Biochemical Systematics and Ecology 32*(8), 735-745.

Schwob, I., J.-M. Bessière & J. Viano (2002). Composition of the essential oils of *Hypericum perforatum* L. from southeastern France. *Comptes Rendus Biologies 325*(7), 781-785.

Smelcerovic, A., M. Spiteller, A. P. Ligon, Z. Smelcerovic & N. Raabe (2007). Essential oil composition of *Hypericum* L. species from Southeastern Serbia and their chemotaxonomy. *Biochemical Systematics and Ecology 35*(2), 99-113.

Vandenbogaerde, A., P. Zanoli, G. Puia, C. Truzzi, A. Kamuhabwa, P. De Witte, W. Merlevede & M. Baraldi (2000). Evidence That Total Extract of *Hypericum perforatum* Affects Exploratory Behavior and Exerts Anxiolytic Effects in Rats. *Pharmacology Biochemistry and Behavior 65*(4), 627-633.

Weyerstahl, P., U. Splittgerber, H. Marschall & V. K. Kaul (1995). Constituents of the leaf essential oil of *Hypericum perforatum* L. from India. *Flavour and Fragrance Journal 10*(6), 365-370.

Essential Oils from Cultivated Plants and *In vitro* Shoots of *Hypericum perforatum* 'Topaz'

Ana P. Guedes[1], Ana Vicente[2] and M. Fernandes-Ferreira[1]

[1] CITAB- Centro de Investigação e de Tecnologias Agro-Alimentares e Biológicas, Department of Biology, University of Minho, Campus de Gualtar, 4720-057 Braga, Portugal

[2] DRAPN- Direcção Regional de Agricultura e Pescas do Norte, 4715-017 Braga, Portugal

Abstract

The seasonal variation of the essential oils (EO) from vegetative aerial parts of *Hypericum perforatum* 'Topaz' growing at two different sites in Northern of Portugal were determined, as well as the composition of the EO from the respective flowers and *in vitro* shoots. The EO contents in flowers from plants harvested in June were four times higher than those of the respective vegetative aerial parts. The sesquiterpene hydrocarbons (SH) constituted the main fraction of the EO independently of the growing site, season or growth conditions (*in vivo* or *in vitro*). The EO content gradually decreased from January to September in the vegetative aerial parts of plants grown in Arcos de Valdevez. A more drastic reduction was observed between April and June in the vegetative aerial parts of plants growing in Merelim. The decrease of sesquiterpene hydrocarbons (SH) in both fields was compensated with the increase of the other groups of compounds, namely monoterpene hydrocarbons (MH), oxygenated sesquiterpenes (SO) and alkanes. The compounds that mostly accounted for the EO seasonal variations were α-pinene and *E*-β-ocimene (MH); germacrene D, *(E)*-caryophyllene and β-selinene (SH); caryophyllene oxide (SO); and 2-methyl-octane (alkanes). In flowers EO, the most represented compounds were 2-methyl-octane followed by *(E)*-caryophyllene. *In vitro* shoots maintained on Murashige & Skoog basal medium without any hormonal supplementation had an EO content of 2.8 mg/g of biomass dry weight, with sesquiterpene hydrocarbons as major group (40.3%) and *n*-nonane as major compound (24.2%).

Keywords: *Hypericum perforatum*, cultivated plants, *in vitro* shoots, essential oils, sesquiterpenes

1- Introduction

Hypericum perforatum L., the most studied species of the genus has been, since ancient times, used in traditional medicine for the treatment of skin wounds, eczema and burns. Since the last years of the XX century it has been widely used in the treatment of mild depression due to its antidepressant activities (Barnes *et al.*, 2001; Sánchez-Mateo *et al.*, 2002). Extracts of *H. perforatum* have revealed other biological activities such as antimicrobial, anti-inflammatory, and analgesic (Barnes *et al.*, 2001; Medina *et al.*, 2006) being also considered a potential source of novel anticancer compounds (Schempp *et al.*, 2002).

Nowadays, the demand of secondary metabolites believed to be responsible for some of the properties of the plants is becoming acute because of their non-availability, as a consequence of misuse, overexploitation, adverse climatic conditions, and political instability in cropping areas (Canter *et al.*, 2005). On the other hand some authors have shown that the composition of the *H. perforatum* essential oils (EO) are strongly dependent of the geographical distribution (Couladis *et al.*, 2001), and of their phenological cycle (Schwob *et al.*, 2004). An alternative seems to be *in vitro* micropropagation. This vegetative propagation technique allows the production of a large number of genetically uniform and pathogen-free plants in a limited time and space in which environment and nutritional factors can be easily controlled (Rout *et al.*, 2000). Besides their importance in facilitating plant propagation, *in vitro* techniques can also afford system models to study the production, accumulation, and metabolism of important metabolites. Protocols of *in vitro* micropropagation of *H. perforatum* have already been reported through shoot regeneration from leaves (Pretto *et al.*, 2000), hypocotyls (Murch *et al.*, 2000), shoot cuttings (Cellarova *et al.*, 1995), stamens (Kirakosyan *et al.*, 2000) and roots (Zobayed *et al.*, 2003). Phenolic acids and flavonoid compounds (Dias *et al.*, 1998; Dias *et al.*, 1999), as well as xanthones (Dias *et al.*, 2001) were identified in *in vitro* cultures of *calli* and suspended cells of this species. However, to our knowledge, the capacity of *H. perforatum in vitro* shoots in the production and accumulation of EO compounds has not been reported yet. In this paper we report the yields and composition of EO accumulated in the vegetative aerial parts and flowers of *H. perforatum* 'Topaz' plants cultivated in two different sites of the Northern region of Portugal [Arcos de Valdevez (HpTA) and Merelim (HpTM)], as well as those produced by *in vitro* shoots of the same Topaz cultivar.

2- Material and Methods

2.1- Essential oils

Plants of *Hypericum perforatum* L. 'Topaz' were cultivated in two experimental farms of the Direcção Regional de Agricultura e Pescas do Norte (DRAPN), located at Arcos de Valdevez and Merelim (northern region of Portugal). Voucher specimens are maintained in an active bank in DRAPN. To study the EO composition, 6-8 cultivated plants were randomly collected in January, April, June, September and December and their aerial parts were subjected to hydrodistillation and analysis following the methodology used in the study of the EO from *H. perforatum* common cultivar (pages 118-119).

2.2- In vitro *shoot cultures*

Seeds obtained from wild plants of *H. perforatum* cv. Topaz were dipped in ethanol (70%) for 2 minutes, before surface sterilization by immersion in 5% sodium hypochlorite solution for 15 minutes. To remove traces of chlorine, the seeds were washed three times with sterile distilled water and then placed on Murashige and Skoog (Murashige *et al.*, 1962) basal medium (MS) supplemented with 2% sucrose, without any growth regulators. The medium was solidified with 0.8% agar and its pH was adjusted to 5.7 prior to autoclaving at 15 psi for 20 min at 121ºC. Sterile seeds, plated in Petri dishes, were incubated at 25±2ºC. Around 200 sterile seeds were incubated under a photoperiod 16h light/8h dark or in the darkness each. Germination percentage was measured after 20 days of culture. The seeds showing radicle emergence were recorded as germinated. Nodal segments (~10mm) obtained from 4 week-old aseptic seedlings were used as primary explants in the establishment of the shoot cultures. Each plant tissue culture vessel containing 25 mL of hormone free MS basal medium solidified with 0.8% agar and covered with Magenta B-cap was inoculated with three primary explants. Cultures were maintained in a growth room at 25±2ºC with a photoperiod of 16 h light/8 h dark. Illumination was supplied by cool white fluorescent tubes with a light intensity of 52µmol $m^{-2}s^{-1}$. Shoots were subcultured to the same medium conditions with intervals of about 8 weeks. At the end of 6^{th} subculture period, shoots from about 10 flasks were submitted to hydrodistillation for EO recovery.

3- Results and Discussion

3.1- In vitro *shoot cultures*

The germination of the first seeds of *H. perforatum* 'Topaz' started 5 days after their contact with the MS medium devoid of growth regulators under 16h light photoperiod. The seeds kept in darkness on the same medium conditions, started to germinate later (Table 1). MS basal medium had already been used for germination of *H. perforatum* seeds by other authors (Smith *et al.*, 2002; Walker *et al.*, 2002) as well as half strength MS medium (Pasqua *et al.*, 2003) while other authors have preferred the water solidified with agar (Murch *et al.*, 2000; Murch *et al.*, 2002; Zobayed *et al.*, 2004).

Table 1- Germination rates of seeds from *H. perforatum* cv. Topaz on MS basal medium free of plant growth regulators

Surface-sterilized method	**Germination (%)**		**Days**	
Ethanol 70% (2 min)	Darkness	65	Darkness	10-12
Sodium Hypochlorite 5% (15 min)				
3 washes with sterile distilled water	16h light photoperiod	80	16h light photoperiod	5-7

The light is required for seed germination of some seeds (Baskin *et al.*, 2004), which seems to be the case of *H. perforatum* 'Topaz', whose seeds in presence of light germinated faster and in higher percentage (80%) than in darkness (65%). Positive effects of light on seeds germination was observed earlier in *H. perforatum* (Campbell, 1985; Willis *et al.*, 1997; Faron *et al.*, 2004; Çirak *et al.*, 2007). It was found that 18h light photoperiod was the most effective to meet light requirement for germination of *H. perforatum* seeds (Çirak *et al.*, 2004). Light is required to stimulate seed germination of several species which, according to some authors, is related with seed germination in their natural habitat (Milberg *et al.*, 2000; Baskin *et al.*, 2004). Indeed, seeds of some species have an initial light requirement for germination, while seeds of other ones seem to acquire the light requirement only after burial in the soil. Seedlings from large seeds can emerge successfully from greater depth than light can penetrate. The small ones are incapable to breach ground layers and to reach the surface given the small amount of reserve material that they possess. Consequently, small seeds are expected to have a light requirement for germination, while germination of large seeds might be expected to be indifferent to light. This hypothesis was confirmed by Milberg and co-workers (2000), who

tested the relationship between seed mass and light requirement among 54 species. The authors observed that light as a germination cue becomes less important in species with relatively large seeds. Once *H. perforatum* has very small seeds, this requirement for light to increase germination, might explain the results herein reported.

Nodal and internodal segments, from 4 weeks old seedlings when transferred to MS medium without any hormonal supplementation showed different responses. From the two primary explants, nodal segments showed to be the best one for the establishment of shoot cultures (100% of shoot development), while most of the internodal segments oxidised without plantlets' formation. The formation of shoots from the axillary meristems in the nodal segments appeared visible 1-2 weeks after the beginning of the culture. Thus, micropropagation was accomplished and maintained by routine transfer of nodal segments to hormone free MS basal medium. *H. perforatum in vitro* cultures established by this way maintained the shape of the shoots without apparent change.

3.2- Essential oils yield from in vivo *plants and* in vitro *shoots*

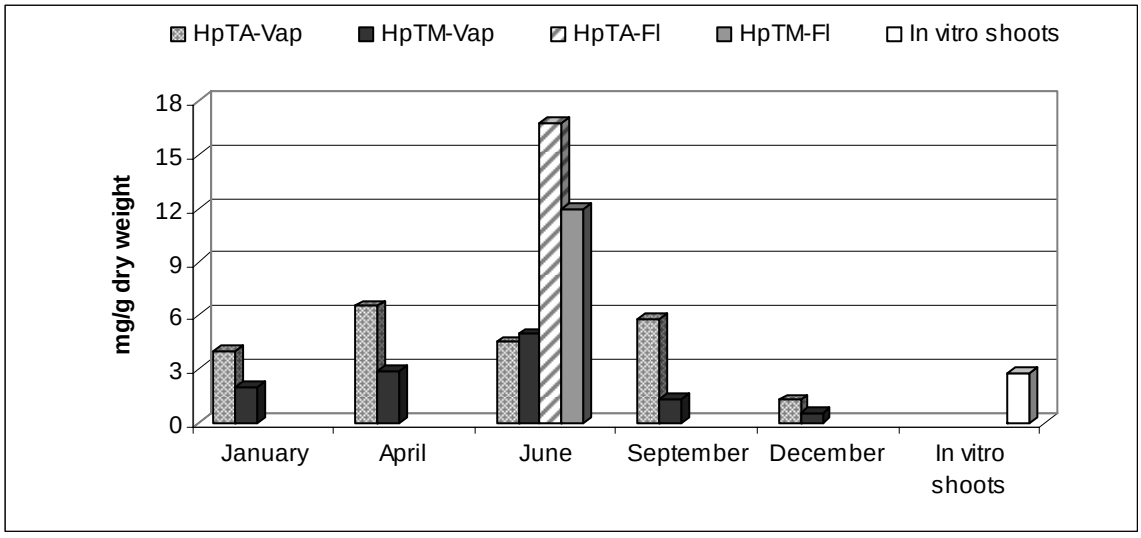


Figure 1- Total essential oil contents from vegetative aerial parts (VAp) and flowers (Fl) of *in vivo* plants of *Hypericum perforatum* 'Topaz' (HpT) grown in Arcos de Valdevez (HpTA-VAp and HpTA-FL) and Merelim (HpTM-VAp and HpTM-Fl) experimental fields and from *in vitro* shoots maintained on MS basal medium.

The EO yields obtained by hydrodistillation of vegetative aerial parts of this species cultivated in Arcos de Valdevez (HpTA-VAp) varied from 1.3 mg/g of biomass dry weight in December to 6.5 mg/g of biomass dry weight in April. In the vegetative aerial parts of plants grown in Merelim (HpTM-VAp) the lowest yield was registered in December (0.6 mg/g of

biomass dry weight) and the highest in June (5.0 mg/g of biomass dry weight). In the plants grown in both fields the EO contents was lower in the winter, under conditions of lower temperatures and short days. The EO yield of flowers was 16.8 mg/g of biomass dry weight in HpTA-Fl and 12.0 mg/g of biomass dry weight, in HpTM-Fl, that is around four and three fold higher than those obtained from the respective vegetative aerial parts. The EO yield recorded for *in vitro* shoots of *H. perforatum* 'Topaz' (2.8 mg/g of biomass dry weight) was similar to those recorded for the vegetative aerial parts harvested in April from plants grown in Merelim (Figure 1). In both cases the vegetative growth was at their higher rates. Lower essential oil yields of *in vitro* cultures are usually associated to the faster growth characteristic of this type of cultures.

3.3- Essential oils composition

The EO revealed a complex composition with 57 to 96 identified compounds depending of the sampled material (Table 2). EO from plants harvested in June showed the highest number of compounds, 91 and 96 of which were identified in HpTA-VAp and HpTM-VAp, respectively. In HpTA-Fl and HpTM-Fl 95 and 98 compounds were identified, respectively. The lowest EO yields and lowest number of compounds were recorded in December, in both experimental fields. From the 70 compounds identified in the EO of *in vitro* shoots, only two *n*-alkanes (*n*-heptadecane and *n*-octadecane), the sesquiterpene hydrocarbon, germacrene D isomer, and the oxygenated sesquiterpene, *δ*-cadinol, were not detected in the essential oils from cultivated plants (aerial parts or flowers).

Table 2 - Composition of the essential oils from vegetative aerial parts and flowers of plants of *Hypericum perforatum* 'Topaz' grown in Arcos de Valdevez (HpTA-VAp and HPTA-Fl) and Merelim (HpTM-VAp and HpTM-Fl) experimental fields and *in vitro* shoots maintained on MS basal medium.

Compound	KI	January		April		June				September		December		*In vitro* shoots (%)
		Hp TA VAp (%)	Hp TM VAp (%)	Hp TA VAp (%)	Hp TM VAp (%)	Hp TA VAp (%)	Hp TA Fl (%)	Hp TM VAp (%)	Hp TM Fl (%)	Hp TA VAp (%)	Hp TM VAp (%)	Hp TA VAp (%)	Hp TM VAp (%)	
1-Octene	793	0.3	0.3	0.1	0.2	0.3	0.1	0.4	0.1	0.3	0.2	0.5	0.6	6.9
(E)-2-Hexenal	853		0.2	0.2	0.4	0.8		1.3		0.2	0.6		tr	0.1
2-methyl-octane	864	6.2	3.8	10.2	7.1	9.0	23.9	12.8	22.2	8.2	3.8	4.4	1.3	
n-Nonane	900	5.9	3.4	1.9	5.9	5.2	3.2	6.7	3.2	7.8	6.0	5.1	1.3	24.2
α-Thujene	929	0.2	0.2	0.3	0.3	0.9	0.2	2.0	0.3	0.6	0.2	0.5	tr	

Compound	KI	January		April		June				September		December		*In vitro* shoots (%)
		Hp TA VAp (%)	Hp TM VAp (%)	Hp TA VAp (%)	Hp TM VAp (%)	Hp TA VAp (%)	Hp TA Fl (%)	Hp TM VAp (%)	Hp TM Fl (%)	Hp TA VAp (%)	Hp TM VAp (%)	Hp TA VAp (%)	Hp TM VAp (%)	
α-Pinene	936	6.2	5.8	2.0	4.7	3.1	9.5	4.9	9.2	5.5	4.0	9.5	4.4	9.2
2,6-Dimethyl-octane	972	1.3	1.0	0.9	1.0	1.7	2.3	1.8	2.2	0.9	0.7	0.4	tr	1.4
Sabinene	975	0.7	0.7	1.4	1.3	1.8	0.3	2.4	0.6	1.8	0.5	2.3	2.3	tr
β-Pinene	978	0.9	1.0	1.0	1.1	2.2	2.0	3.2	3.3	1.9	1.0	2.2	1.5	0.8
6-Methyl-5-Hepten-2-one	988					tr	tr	0.1	tr					
Myrcene	986	0.5	0.4	0.7	0.6	1.1	0.6	1.5	0.8	1.3	0.5	1.2	0.6	0.3
n-Decane	1000			tr	tr	tr	tr	0.1	tr	tr				tr
Hexyl acetate	1005	0.1		0.2	0.1	0.1	tr	0.1	0.1	0.3	0.4			
α-Terpinene	1018	0.1	0.1	0.2	0.1	0.3	0.1	0.5	0.1	0.3	tr	0.1	tr	
p-Cymene	1026	0.1		tr		0.1	tr	0.2	tr	tr				
Limonene	1031	0.2	0.3	0.4	0.3	0.6	0.2	0.9	0.4	0.7	0.2	0.5	0.6	tr
Z-β-Ocimene	1041	0.7	0.9	0.9	1.4	1.2	0.9	1.0	1.1	1.8	0.9	2.2	3.0	0.3
E-β-Ocimene	1052	3.1	4.1	6.9	7.5	5.1	9.9	3.8	10.1	7.9	5.2	9.2	14.4	1.6
γ-Terpinene	1061	0.1	0.2	0.3	0.2	0.6	0.2	1.0	0.3	0.6	0.2	0.3	tr	
Methyl decane	1069	tr	0.2	0.1	tr	0.3	1.4	0.3	1.2	0.1	tr			0.3
Terpinolene	1090	tr	0.2	0.1	0.1	0.1	tr	0.2	0.1	0.2	tr	0.1		
Linalool	1099		0.2	0.2		tr		0.1	tr					
n-Undecane	1100	0.4	0.1	0.2	0.2	0.7	0.7	0.8	0.6	0.3	0.3	0.1	tr	3.8
n-Nonanal	1104	0.1	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.3	tr	0.5		
α-Campholenal	1121	0.1	0.3	0.2	0.1	0.1	0.1	0.1	0.1	0.2	tr	0.1	tr	
allo-Ocimene	1126							0.1	0.1				tr	tr
Camphor	1145			0.1		tr	tr	tr	tr					
n-Nonanol	1170	0.3		0.1	0.4	0.1	0.2	0.1	0.2	0.2	0.5	0.5	2.1	1.1
Terpinen-4-ol	1176		0.8	0.3	0.1	0.2	0.1	0.3	0.2	0.5	0.2	0.1	0.6	
α-Terpineol	1189		0.3	0.1	tr	tr	0.1	0.1	0.1	tr	tr			
n-Decanal	1205	0.1	0.9	0.4	1.1	0.1	tr	0.1	tr	0.8	1.6	1.1	6.1	1.4
trans-Carveol	1217							tr						
Methyl dodecane	1265	tr				tr	0.3							
Decanol	1273	tr		0.1	0.2	tr		tr	0.2	0.1	0.4		2.1	0.8
Tridecane	1300	tr		tr		0.1	0.1	0.1	0.1	tr				
SH	1327	0.2	0.3	0.1	0.1	0.1	tr	0.1	tr	tr	tr	0.2	tr	
δ-Elemene	1337	1.0	1.1	1.0	0.7	0.7	0.2	0.4	0.2	0.8	0.7	0.9	0.6	tr
α-Cubebene	1348			0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1			tr
α–Ylangene	1370	0.1		tr	tr	0.1	0.1	0.1	0.1	0.1	tr			tr
α-Copaene	1374	0.4	0.7	0.1	0.3	0.3	0.3	0.2	0.3	0.2	0.2	0.1	tr	tr
β-Bourbonene	1382	0.2	0.3	tr	0.1	0.1	tr	0.2	tr	0.1	0.2	0.1	tr	0.3
β-Cubebene	1388	0.4	0.3				tr	0.1	tr		0.1			
β-Elemene	1390	0.2	0.3	0.4	0.2	0.1	tr	0.1	0.1	0.2	0.2	0.8	tr	0.3

Compound	KI	January		April		June				September		December		*In vitro* shoots (%)
		Hp TA VAp (%)	Hp TM VAp (%)	Hp TA VAp (%)	Hp TM VAp (%)	Hp TA VAp (%)	Hp TA Fl (%)	Hp TM VAp (%)	Hp TM Fl (%)	Hp TA VAp (%)	Hp TM VAp (%)	Hp TA VAp (%)	Hp TM VAp (%)	
Dodecanal	1406					tr	tr	tr	tr					
α-Cedrene	1412	1.2	1.0	0.3	1.0	0.8	0.4	0.9	0.3	1.1	1.6	0.1	0.6	1.2
SH	1415	0.4	0.4	0.1	0.1						0.1			
(E)-Caryophyllene	1417	7.4	6.4	6.8	8.1	13.8	16.6	12.2	15.6	9.4	10.9	5.6	4.4	7.7
β-Gurjunene	1427	0.6	0.8	0.4	0.4	0.4	0.1	0.2	0.1	0.4	0.3	0.4	0.2	0.3
α-Guaiene	1439	0.1		0.1	0.1	0.1	0.1	0.1	0.1	0.1				0.3
Aromadendrene	1440	0.3	0.3	0.1	0.3	0.3	0.1	0.1	tr	0.2				0.3
Z-*β*-Farnesene	1442			0.2	tr	tr	tr	0.2	tr	tr	0.3	0.3	tr	
SH2	1446					0.1	0.2	0.1	0.1	0.1	0.1			
α-Himachalene	1450	0.9	1.1	0.9	0.7	0.9	0.6	0.9	0.6	0.9	0.8	0.8	0.6	
α-Humulene	1453			0.1	tr	tr	tr	0.1		0.1	tr			0.5
E-*β*-Farnesene	1455					0.3			tr					
allo-Aromadendrene	1459	1.7	1.5	1.0	1.4	1.0	2.1	0.7	1.6	1.2	1.5	1.0	0.6	0.3
Germacrene D Isomer	1462													3.0
SH	1465													0.3
α-Acoradiene	1468	0.3	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.3	0.4	0.1	tr	
n-Dodecanol	1474						tr		tr					0.3
γ-Gurjunene	1475		0.3	tr	0.1		tr		tr		tr			
γ-Muurolene	1478	0.3	0.7	5.1	0.3		0.5	tr	0.4	tr	0.2	0.3	9.1	0.7
Germacrene D	1481	38.4	35.7	37.6	32.5	27.5	11.9	10.0	12.8	21.4	26.8	29.0	17.5	16.5
Curcumene	1483	2.6	2.9	0.1	1.8	tr	0.1	tr		tr	5.4	3.1	0.6	
SH	1487	0.1	0.3	0.1	0.1	0.1		tr	0.1	0.1				
β-Selinene	1488	5.8	6.0	6.2	4.7	5.6	1.8	2.3	1.8	6.8	4.4	5.4	5.7	1.6
α-Selinene	1494			0.2				0.2			0.8		0.2	0.8
α-Muurolene	1497		0.3	0.2	1.3	0.2		0.1	tr	0.3		0.4		0.5
n-Pentadecane	1500			0.1		tr	tr	tr		tr				
(E,E)-*α*-Farnesene	1503	0.4	0.3	0.1	0.1	0.1	0.1	tr	0.1	0.1	tr			0.1
γ-Cadinene	1508	0.9	1.2	0.7	0.8	1.3	1.0	0.4	1.0	0.9	0.8	0.3	0.2	3.8
Z-*γ*-Bisabolene	1510	0.2		0.2	0.3	0.3	0.3	0.2	0.2	0.3	0.1	0.1	0.6	0.3
SH	1516													0.3
δ-Cadinene	1521	1.7	1.6	0.9	1.2	0.9	0.6	0.6	0.5	1.6	1.0	1.2	1.9	0.8
SH	1527			0.1		tr		tr						
E-*γ*-Bisabolene	1530	0.2		0.1	0.1	tr	tr	tr	tr	0.1	tr			0.2
α-Cadinene	1534	0.2		0.1	0.1	0.1	0.1	0.1	0.1	0.1	tr			0.1
SH	1540				0.1	0.1	tr	tr						
α-Calacorene	1543			0.1		0.1	tr	0.1	tr	0.1	0.2	0.1	0.6	0.2
SO?	1549				0.1			0.6	0.1	tr				0.1
γ-Elemene (?)	1551	0.1		tr		tr		0.1		tr				

Compound	KI	January		April		June				September		December		*In vitro* shoots (%)
		Hp TA VAp (%)	Hp TM VAp (%)	Hp TA VAp (%)	Hp TM VAp (%)	Hp TA VAp (%)	Hp TA Fl (%)	Hp TM VAp (%)	Hp TM Fl (%)	Hp TA VAp (%)	Hp TM VAp (%)	Hp TA VAp (%)	Hp TM VAp (%)	
E-Nerolidol	1564	0.1	0.4	0.7	0.7	0.7	0.5	0.9	0.5	0.9	0.8	0.7	1.7	tr
Spathulenol	1574	0.2		0.1	0.5	0.2	0.1	0.2	0.1	0.2		0.7		
cis-3-Hexenyl benzoate	1577	0.3	0.9	0.5	0.1	0.9	0.1	3.7	0.1	0.3	0.5	0.4	1.3	
Caryophyllene Oxide	1580	0.3	0.3	0.3	0.3	0.8	0.5	7.6	0.9	0.1	0.3		0.2	
Globulol	1585	0.2		0.1	0.1	tr	tr	tr	tr	0.5	tr	tr	tr	
Viridiflorol	1592	0.1	0.3	0.3	0.3	0.1	0.1	0.1	0.1	0.1	0.2	0.1	tr	
Guaiol	1593	0.1		0.1	0.1	tr	tr	0.1	0.2	0.2	tr			0.1
β-Copaen-4-*α*-ol	1597	0.7	1.2	0.3	1.0	0.6	0.2	0.7	0.1		0.8	1.2	3.2	0.1
Humulene Epoxide II	1603			0.1		tr	tr	0.3		tr				
β-Oplopenone	1610			tr		tr	tr	0.1	tr					tr
SO	1612	0.1	0.3	0.2	0.3	0.1		0.1						0.3
10-*epi*−*γ*-Eudesmol	1619		0.3	0.1	0.1	tr	0.1	0.1	0.1	0.9	0.2			
γ-Eudesmol	1624	0.1	0.3	0.1	0.1	tr	tr	0.1	tr	0.1		0.1	tr	
epi-*α*-Cubenol	1633		0.1	0.2	0.9	0.2	0.1	0.2	0.1	tr	0.1	1.1	1.9	tr
epi-*α*–Cadinol	1640	0.9	0.8	0.8	0.3	0.1	1.6	0.3	1.5	1.3	0.8		0.6	0.9
epi-*α*-Muurolol	1642	0.4	0.6	0.3	0.1	0.1	tr	0.3	0.1	0.5	0.4	0.4	2.7	0.3
δ-Cadinol	1644													0.3
SO	1646					tr				0.2				
α-Cadinol	1654	1.0	1.4	1.2	1.3	0.6	0.3	0.5	0.4	1.4	1.3	1.6		0.3
β-Bisabolol	1673	tr		0.1	0.1	0.3	0.5	0.2	tr	0.1	0.4	0.1	tr	1.8
epi-*α*-Bisabolol	1677	tr		0.6	0.1	0.2	0.2	0.4	0.4	0.4	1.1	0.3	0.6	1.1
n-Tetradecanol	1681		0.3	0.3	0.2	0.1		0.3			0.2		0.2	tr
Benzyl benzoate	1738					tr	tr	tr	tr					
n-Heptadecane	1700													0.1
n-Octadecane	1800													tr
n-Hexadecanol	1881					tr	tr	0.1	tr	0.1	tr			0.4
n-Nonadecane	1900						0.1		0.1					
n-Eicosane	2000						tr		tr					
n-Heneicosane	2100			tr	tr	tr	0.2	tr	0.1					
Nonadecanal	2111			0.2	0.9	2.4	0.1	1.4	0.2	0.6	6.4			0.6
n-Docosane	2200			tr	tr		tr	tr	tr	tr			tr	
n-Tricosane	2300	0.2	0.5	0.2	0.4	tr	0.1	tr	tr	0.1	0.2	0.4	0.6	tr
n-Tetracosane	2400		0.1	0.1	0.1	tr	tr	tr	tr	tr	tr	0.1	tr	tr
n-Pentacosane	2500	0.3	0.4	0.3	0.5	0.1	tr	0.1	tr	0.2	0.3	0.4	0.6	0.3
n-Hexacosane	2600								tr					
n-Heptacosane	2700		0.3	0.1	0.2	tr	0.1	tr	0.1	0.1	0.2	0.1	0.2	0.4
n-Octacosane	2800								tr					tr
n-Nonacosane	2900	tr				0.1	0.1	0.1	0.2	0.2	0.3			0.5

Compound	KI	January		April		June				September		December		*In vitro* shoots (%)
		Hp TA VAp (%)	Hp TM VAp (%)	Hp TA VAp (%)	Hp TM VAp (%)	Hp TA VAp (%)	Hp TA Fl (%)	Hp TM VAp (%)	Hp TM Fl (%)	Hp TA VAp (%)	Hp TM VAp (%)	Hp TA VAp (%)	Hp TM VAp (%)	
Monoterpene Hydrocarbons		12.8	13.9	14.2	17.5	17.0	24.0	21.7	26.4	22.7	12.7	28.0	26.8	12.3
Oxygenated monoterpenes			1.3	0.6	0.1	0.3	0.2	0.6	0.3	0.5	0.2	0.1	0.6	
Sesquiterpene Hydrocarbons		67.0	64.1	63.0	57.0	55.7	37.7	31.2	36.7	46.8	57.2	50.3	43.8	40.3
Oxygenated sesquiterpenes		4.3	6.1	5.4	6.1	4.2	4.2	16.4	4.4	6.9	6.9	6.3	12.3	5.1
Alkanes		14.3	9.9	14.0	15.3	17.3	32.5	22.8	30.2	17.8	11.8	11.1	4.0	30.5
Others		1.6	4.6	2.8	4.0	5.6	1.4	7.3	1.9	5.3	11.2	4.3	12.5	11.9

VAp- Vegetative aerial parts; **Fl-** Flowers

KI- Kovats retention index on a DB-5 column; **tr-** trace amounts

The relative contents of the main groups of compounds from the EO of vegetative aerial parts harvested in the two sites varied over the year. However, as shown in Figures 2a and 2b, the variation profiles were different. Independently, of the harvest time, sesquiterpene hydrocarbons constituted the major group of compounds in both experimental fields.

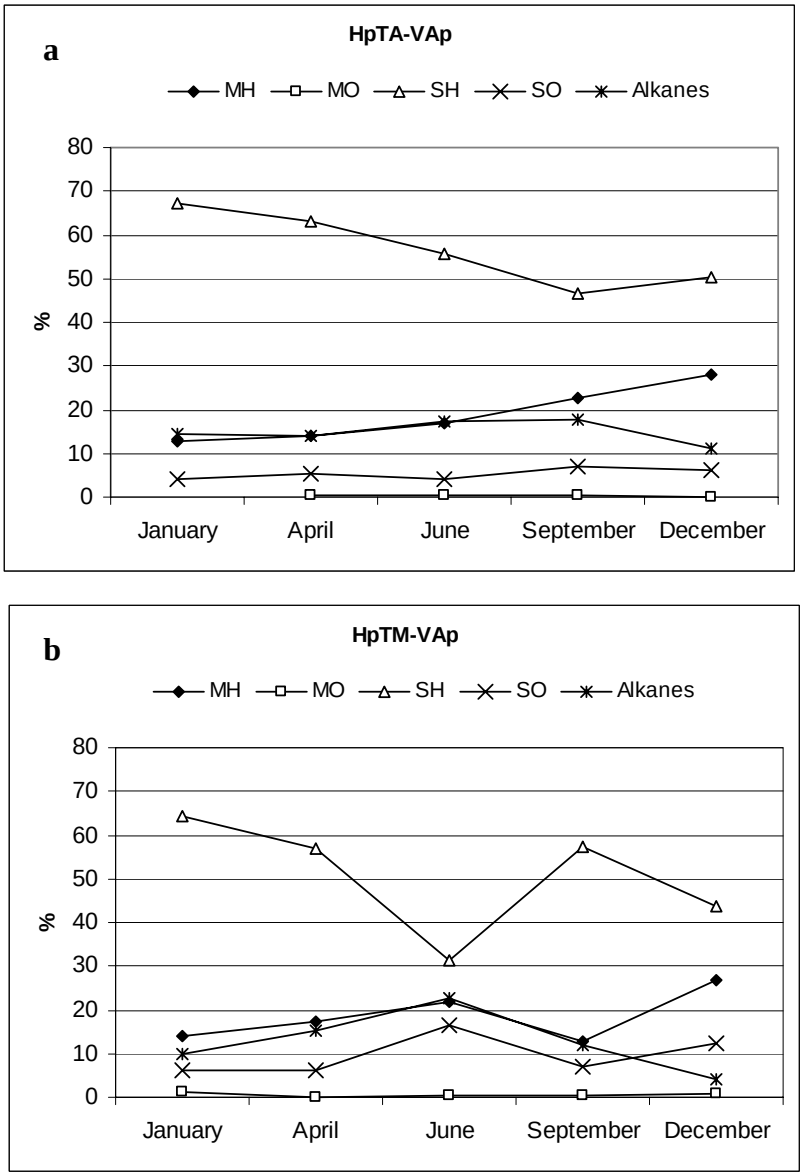


Figure 2- Contents of major groups of compounds in vegetative aerial parts of plants of *Hypericum perforatum* 'Topaz' grown in **(a)** Arcos de Valdevez (HpA-VAp), and **(b)** Merelim (HpM-VAp) experimental fields and harvested at different times over the year. **MH**- Monoterpene Hydrocarbons; **MO**- Oxygenated Monoterpenes; **SH**- Sesquiterpene Hydrocarbons; **SO**- Oxygenated Sesquiterpenes

Sesquiterpene hydrocarbons (SH) ranged from 46.8% to 67% and from 31.2% to 64.1% in the EO of HpTA-VAp and HpTM-VAp, respectively. In the HpTA-VAp EO there was a gradual decrease in the SH content from January (67.0%) to September (46.8%), increasing then ~4% until December. A drastic SH reduction was observed in the HpTM-VAp EO between April (57.0%) and June (31.2%), followed by an increase in September (57.2%). In Merelim, the variation profile of the SH content was inversely correlated with the variation profiles of the monoterpene hydrocarbons (MH) and oxygenated sesquiterpenes (SO) (Figure 2b). In this experimental field, the content of alkanes rose from January to June, decreasing

thereafter up to December. However, in Arcos de Valdevez the variation profile of SH was inversely correlated with the profile of the alkanes over all the year and inversely correlated with the profiles of MH only from January to September. In this case, the MH content increased almost evenly from around 12.8% in January to 28% in December, while SO variation was not significant. The compounds that most contributed to the variations in the contents of alkanes and SO were, 2-methyl-octane and caryophyllene oxide, respectively, whereas in the SH it was mainly germacrene D, and in a lesser extent, *β*-selinene. In HpTA-VAp EO, with exception of September, germacrene D, *(E)*-caryophyllene and *β*-selinene, were responsible for more than 40% of the total EO. In HpTM-VAp EO, germacrene D decreased from 32.5% in April to 10% in June and in the same period the percentage content of *(E)*-caryophyllene, the second major sesquiterpene hydrocarbon rose from 8.1% to 12.2%. The same pattern of variation was recorded in HpTA-VAp for these two sesquiterpene hydrocarbons. Recently, Mockute and co-workers (2008) reported the predominance of germacrene D in EO of *H. perforatum* wild plants collected at full flowering time (Mockute *et al.*, 2008). Germacrene D was also identified as the main constituent in EO of *H. perforatum* plants grown wild in Portugal (Nogueira *et al.*, 1998), southeast France (Schwob *et al.*, 2002) and north of Greece (Chatzopoulou *et al.*, 2006). *(E)*-Caryophyllene, was the most represented compound in EO of this species from different locations in Serbia (Gudžić *et al.*, 1997; Gudžić *et al.*, 2001) and Uzbekistan (Baser *et al.*, 2002).

In HpTM-VAp EO, the content of the MH group decreased between June and September, increasing afterwards in December. This group was the second most represented in the EO of HpTA-VAp and HpTM-VAp harvested in April, September and December, mainly due to the percentage contributions of *α*-pinene and *E*-*β*-ocimene. *H. perforatum* plants growing in Serbia (Saroglou *et al.*, 2007), Greece (Pavlović *et al.*, 2006) and Italy (Maggi *et al.*, 2008) had *α*-pinene, as the main EO constituent. In HpTA-VAp, SO varied from 4.2% in June to 6.9% September, while MO were present in very low amounts (0.1%-0.6%). No MO compound was detected in January. Higher amounts of SO were found in plants cultivated in Merelim (6.1% to 16.4% of the total EO).

Pathways involved in the biosynthesis of MH and SH seem to be inversely related and affected by the stages of plant development. Monoterpenes were reported to be produced in the plastids via the mevalonate-independent pathway while sesquiterpenes production was shown to be held in the cytosol via acetate-mevalonate pathway (Davis *et al.*, 2000). Thus, the variation profiles of SH and MH over the year may reflect different overall stages of

maturation of those subcellular compartments with an increasing of efficiency of plastid terpene (namely MH) pathways and decreasing of the efficiency of cytosolic terpene (namely SH) pathways during the spring and summer.

As can be seen in Table 2 and Figure 3, differences of EO composition were observed between flowers and the respective vegetative aerial parts harvested in June. Several compounds were restricted to the EO of vegetative aerial parts and other restricted to the EO from flowers. *n*-Dodecanol, *n*-nonadecane and *n*-eicosane were compounds only found in EO of flowers.

Figure 3 shows the contents of the main groups of compounds in EO isolated from the vegetative aerial parts and flowers of *H. perforatum* 'Topaz' harvested in June in Arcos de Valdevez (HpTA-VAp and HpTA-Fl) and Merelim (HpTM-VAp and HpTM-Fl), as well as in EO from *in vitro* shoots of the same cultivar. In flowers of *H. perforatum* 'Topaz', sesquiterpene hydrocarbons constituted also the main group of compounds, accounting for more than 35% of the EO, followed by alkanes with 33% and 30% in HpTA-Fl and HpTM-Fl, respectively. The alkane contents in the flowers EO were higher than those in vegetative aerial parts due mainly to the amount of 2-methyl-octane, which represented 24%, in HpTA-Fl and 22% in HpTM-Fl.

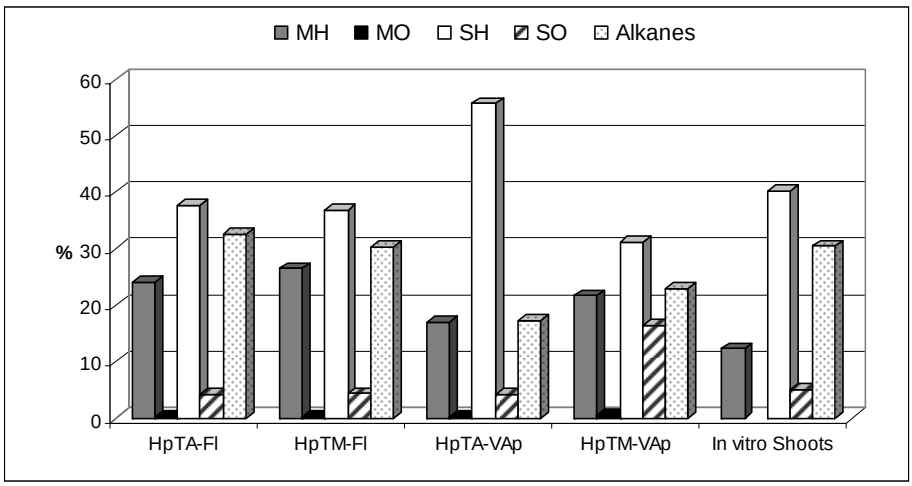


Figure 3- Content of the major group of compounds in flowers and vegetative aerial parts of *in vivo* plants of *Hypericum perforatum* 'Topaz' grown in Arcos de Valdevez (HpTA-Fl and HpTA-VAp) and Merelim (HpTM-Fl and HpTM-VAp) experimental fields, harvested in June, and *in vitro* shoots maintained on MS basal medium. **MH**- Monoterpene Hydrocarbons; **MO**- Oxygenated Monoterpenes; **SH**- Sesquiterpene Hydrocarbons; **SO**- Oxygenated Sesquiterpenes.

The SH content in the EO of flowers from plants grown in Merelim, was higher than in EO of the respective vegetative aerial parts. *(E)*-caryophyllene was the most represented SH in EO of flowers in both sites, accounting for 16.6%, in HpTA-Fl, and 15.6%, in HpTM-Fl. Germacrene D represented 11.9% and 12.8% of the total EO of HpTA-Fl and HpTM-Fl, respectively, while α-pinene and *E*-β-ocimene were the compounds that most contributed to the high percentage of the MH. The most complete series of *n*-alkanes from C_{19} to C_{29} was detected in HpTM-Fl. In HpTA-Fl, *n*-hexacosane and *n*-octacosane were not detected. Some of the *n*-alkanes of this series were not identified in the EO from the vegetative aerial parts of *in vivo* plants and *in vitro* shoots. On can say that the EO from plants of *H. perforatum* 'Topaz' grown in the two sites of the Northern of Portugal are characterized by high levels of hydrocarbon compounds and low levels of oxygenated constituents. Other authors had already mentioned the hydrocarbon compounds, namely the sesquiterpene ones, as the most represented compounds in the EO of this species (Schwob *et al.*, 2002; Schwob *et al.*, 2004; Chatzopoulou *et al.*, 2006; Saroglou *et al.*, 2007).

The SH (40%) predominated also over the MH (12%) in EO of *in vitro* shoots of *H. perforatum* 'Topaz'. Likewise it occurred with EO of HpTA-VAp, in January, no MO was found in EO of *in vitro* shoots. On the other hand, EO from *in vitro* shoots was less complex, than those found in *in vivo* plants, what may be associated with the lack of a high degree of differentiation of structures implied in the EO accumulation. In *H. perforatum* plants, the EO is accumulated in both translucent glands and type B cannals structures (Ciccarelli *et al.*, 2001; Maffi *et al.*, 2005). These authors found both type of structures in leaves and flowers of *H. perforatum* plants but in stems only type B cannals were detected. In *in vitro* culture conditions these structures might not achieve high levels of differentiation and/or maturation since shoots are at a very early stage of growth. Indeed, histological studies performed in *Salvia officinalis* showed that the number of glands seemed to be related to the physiological stage of growth, increasing with the age, for both plants in Nature and *in vitro* shoots. In the EO of *in vitro* shoots of this species, the authors found also a narrow range of compounds and a pattern of metabolites that discloses a very early part of the biosynthetic pathway (Avato *et al.*, 2005).

Excepting for HpTA-Fl the EO from shoots had the highest content of alkanes (Table 2). The most represented compound of this group was *n*-nonane, which accounted for 24.2% of the shoots EO, a value significantly higher than those found either in flowers or vegetative aerial parts of the *in vivo* plants. 2-Methyl-octane, the most represented compound in flowers and one of the major in the vegetative aerial parts was not detected in the shoots. Petroleum ether extracts of *H. perforatum* revealed the predominance of odd carbon *n*-alkanes, namely *n*-

nonacosane, as well as *n*-heptacosane, and *n*-hentriacontane (Stojanovic *et al.*, 2003). Our results are coherent with these ones, as a predominance of odd carbon *n*-alkanes in the range of C_{25}-C_{29} was recorded in the EO of both *in vivo* and *in vitro* shoots of *H. perforatum* 'Topaz' here studied. The 2nd and the 3rd most represented compounds in the shoots were germacrene D (16.5%) and α-pinene (9.2%), respectively, while *(E)*-caryophyllene was found in the 4th position with 7.7% of the total EO. 1-Octene was less represented in the EO from cultivated plants but was the 5th most represented compound in the shoots (6.9%).

As final remarks, the study here reported demonstrated that micropropagation of *H. perforatum* 'Topaz' can be easily obtained at a high frequency by *in vitro* seed germination followed by nodal subculturing. Considering the EO produced by this cultivar, regardless the time of harvest and the *in vivo* or *in vitro* biomass growth conditions, the SH constituted the major group of compounds. However, the most represented compounds in the EO were different depending of the organ and growth conditions. In the EO of *in vivo* plants the major compounds were germacrene D, *(E)*-caryophyllene and β-selinene in vegetative aerial parts of the plants and 2-methyl-octane, *(E)*-caryophyllene and germacrene D in flowers, while in the EO of *in vitro* shoots *n*-nonane, germacrene D and α-pinene were the major ones. The study here reported showed also that the absolute content of the EO as well as the relative contents of their main compound groups vary according to season and organ as well as the biomass growth conditions.

Acknowledgements

This work was sponsored by EU (FSE/FEDER) and Portuguese Government (FCT) through the Project PTDC/AGR-AAM/70418/2006 and the Grant SFRH/BD/13283/2003.

References

Avato, P., I. M. Fortunato, C. Ruta & R. D'Elia (2005). Glandular hairs and essential oils in micropropagated plants of *Salvia officinalis* L. *Plant Science 169*(1), 29-36.

Barnes, J., L. A. Anderson & D. J. Phillipson (2001). St John's wort (*Hypericum perforatum* L.): a review of its chemistry, pharmacology and clinical properties *Journal of Pharmacy and Pharmacology 53*(5), 583-600.

Baser, K. H. C., T. Ozek, H. R. Nuriddinov & A. B. Demirci (2002). Essential oils of two *Hypericum* species from Uzbekistan. *Chemistry of Natural Compounds. 38*(1), 54-57.

Baskin, J. M. & C. C. Baskin (2004). A classification system for seed dormancy. *Seed Science Research 14*(01), 1-16.

Campbell, M. H. (1985). Germination, emergence and seedling growth of *Hypericum perforatum* L. *Weed Research 25*(4), 259-266.

Canter, P., H. Thomas & E. Ernst (2005). Bringing medicinal plants into cultivation: opportunities and challenges for biotechnology. *Trends in Biotechnology 23*(4), 180-185.

Cellarova, E., K. Kimáková, Z. Daxnerová & P. Mártonfi (1995). *Hypericum perforatum* (St. John's Wort): *In vitro* culture and the production of hypericin and other secondary metabolites. *Biotechnology in Agriculture and Forestry*. Y. P. S. Bajaj. Berlin Heidelberg, Springer-Verlag. **33:** 261-275.

Chatzopoulou, P. S., T. V. Koutsos & S. T. Katsiotis (2006). Chemical Composition of the Essential Oils from Cultivated and Wild Grown St. John's Wort (*Hypericum perforatum*). *Journal of Essential Oil Research 18*(6), 643-646.

Ciccarelli, D., A. C. Andreucci & A. M. Pagni (2001). Translucent Glands and Secretory Canals in *Hypericum perforatum* L. (Hypericaceae): Morphological, Anatomical and Histochemical Studies During the Course of Ontogenesis. *Annals of Botany 88*(4, Part 1), 637-644.

Çirak, C., A. Ayan, K. Kevseroglu & Ö. Çaliskan (2004). Germination rate of St. John's wort (*Hypericum perforatum* L.) seeds exposed to different light intensities and illumination periods. *Journal of Biological Sciences*.

Çirak, C., K. Kevseroglu & A. K. Ayan (2007). Breaking of seed dormancy in a Turkish endemic *Hypericum* species: *Hypericum avicullariifolium* subsp. *depilatum* var. *depilatum* by light and some pre-soaking treatments. *Journal of Arid Environments 68*(1), 159-164.

Couladis, M., P. Baziou, P. V. Petrakis & C. Harvala (2001). Essential oil composition of *Hypericum perfoliatum* L. growing in different locations in Greece. *Flavour and Fragrance Journal 16*(3), 204-206.

Davis, E. M. & R. Croteau (2000). Cyclization Enzymes in the Biosynthesis of Monoterpenes, Sesquiterpenes and Diterpenes. *Topics in Current Chemistry 209*, 54-95.

Dias, A., R. Seabra, P. Andrade & M. Fernandes-Ferreira (1999). The development and evaluation of an HPLC-DAD method for the analysis of the phenolic fractions from *in vivo* and *in vitro* biomass of *Hypericum* species. *Journal of Liquid Chromatography & Related Technology 22*(2), 215-227.

Dias, A. C. P., R. M. Seabra, P. B. Andrade, F. Ferreres & M. F. Ferreira (2001). Xanthone production in calli and suspended cells of *Hypericum perforatum*. *Journal of Plant Physiology 158*(7), 821-827.

Dias, A. C. P., F. A. Tomás-Barberán, M. Fernandes-Ferreira & F. Ferreres (1998). Unusual flavonoids produced by callus of *Hypericum perforatum*. *Phytochemistry 48*(7), 1165-1168.

Faron, M., M. Perecin, A. Lago, O. Bovi & N. Maia (2004). Temperatura, Nitrato de Potássio e Fotoperíodo na germinação de sementes de *Hypericum perforatum* L. e *H. brasiliense* Choisy. *Bragantia 63*(2), 193-199.

Gudžić, B., S. Đorđević, R. Palić & G. Stojanovi (2001). Essential oils of *Hypericum olympicum* L. and *Hypericum perforatum* L. *Flavour and Fragrance Journal 16*(3), 201-203.

Gudžić, B., J. M. Nedeljković, S. Đorđević & J. J. Čomor (1997). Composition and anti-microbial activity of essential oil of Hyperici herb (*Hypericum perforatum* L.) from Vlasina Region. *Facta Universitatis 1*(4), 47-51.

Kirakosyan, A., H. Hayashi, K. Inoue, A. Charchoglyan & H. Vardapetyan (2000). Stimulation of the production of hypericins by mannan in *Hypericum perforatum* shoot cultures. *Phytochemistry 53*(3), 345-348.

Maffi, L., L. Camoni, R. B. Fornasiero & A. Bianchi (2005). Morphology and development of secretory structures in *Hypericum perforatum* and *H. richeri*. *Nordic Journal of Botany 23*(4), 453-461.

Maggi, F. & G. Ferretti (2008). Essential Oil Comparison of *Hypericum perforatum* L. subsp. *perforatum* and subsp. *veronense* (Schrank) Ces. from Central Italy. *Journal of Essential Oil Research 20*(6), 492-494.

Medina, M. A., B. Martínez-Poveda, M. I. Amores-Sánchez & A. R. Quesada (2006). Hyperforin: More than an antidepressant bioactive compound? *Life Sciences 79*(2), 105-111.

Milberg, P., L. Andersson & K. Thompson (2000). Large-seeded species are less dependent on light for germination than small-seeded ones. *Seed Science Research 10*, 99–104.

Mockute, D., G. Bernotiene & A. Judzentiene (2008). The Essential Oils with Dominant Germacrene D of *Hypericum perforatum* L. Growing Wild in

Lithuania. *Journal of Essential Oil Research 20*(2), 128-131.

Murashige, T. & F. Skoog (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plantarum 15*, 473-497.

Murch, S. J., K. L. Choffe, J. M. R. Victor, T. Y. Slimmon, S. KrishnaRaj & P. K. Saxena (2000). Thidiazuron-induced plant regeneration from hypocotyl cultures of St. John's wort (*Hypericum perforatum*. cv 'Anthos'). *Plant Cell Reports 19*(6), 576-581.

Murch, S. J., H. P. V. Rupasinghe & P. K. Saxena (2002). An *in vitro* and hydroponic growing system for hypericin, pseudohypericin, and hyperforin production of St. John's Wort (*Hypericum perforatum* CV New Stem). *Planta Medica 68*, 1108-1112.

Nogueira, T., F. Duarte, F. Venâncio, R. Tavares, M. Lousã, C. Bicchi & P. Rubiolo (1998). Aspectos Quimiotaxonómicos do Género *Hypericum* L. em Portugal. *Silva Lusitana 6*(1), 55-61.

Pasqua, G., P. Avato, B. Monacelli, A. R. Santamaria & M. P. Argentieri (2003). Metabolites in cell suspension cultures, *calli*, and *in vitro* regenerated organs of *Hypericum perforatum* cv. Topas. *Plant Science 165*(5), 977-982.

Pavlović, M., O. Tzakou, P. V. Petrakis & M. Couladis (2006). The essential oil of *Hypericum perforatum* L., *Hypericum tetrapterum* Fries and *Hypericum olympicum* L. growing in Greece. *Flavour and Fragrance Journal 21*(1), 84-87.

Pretto, F. R. & E. R. Santarém (2000). Callus formation and plant regeneration from *Hypericum perforatum* leaves. *Plant Cell, Tissue and Organ Culture 62*(2), 107-113.

Rout, G. R., S. Samantaray & P. Das (2000). *In vitro* manipulation and propagation of medicinal plants. *Biotechnology Advances 18*(2), 91-120.

Sánchez-Mateo, C. C., B. Prado & R. M. Rabanal (2002). Antidepressant effects of the methanol extract of several *Hypericum* species from the Canary Islands. *Journal of Ethnopharmacology 79*(1), 119-127.

Saroglou, V., P. D. Marin, A. Rancic, M. Veljic & H. Skaltsa (2007). Composition and antimicrobial activity of the essential oil of six *Hypericum* species from Serbia. *Biochemical Systematics and Ecology 35*(3), 146-152.

Schempp, C., V. Kirkin, B. Simon-Haaraus, A. Kersten, J. Kiss, C. Termeer, B. Gilb, T. Kaufmann, C. Borner, J. Sleeman & J. Simon (2002). Inhibition of tumour cell growth by hyperforin, a novel anticancer drug from St. John's wort that acts by induction of apoptosis. *Oncogene 21*(8), 1242-1250.

Schwob, I., J.-M. Bessiere, V. Masotti & J. Viano (2004). Changes in essential oil composition in Saint John's wort (*Hypericum perforatum* L.) aerial parts during its phenological cycle. *Biochemical Systematics and Ecology 32*(8), 735-745.

Schwob, I., J.-M. Bessière & J. Viano (2002). Composition of the essential oils of *Hypericum perforatum* L. from southeastern France. *Comptes Rendus Biologies 325*(7), 781-785.

Schwob, I., J. M. Bessiere, M. Dherbomez & J. Viano (2002). Composition and antimicrobial activity of the essential oil of *Hypericum coris*. *Fitoterapia 73*(6), 511-513.

Smith, M., H. Kobayashi, M. Gawienowski & D. Briskin (2002). An *in vitro* approach to investigate medicinal chemical synthesis by three herbal plants. *Plant Cell, Tissue and Organ Culture 70*, 105-111.

Stojanovic, G., R. Palic, C. H. Tarr, C. M. Reddy & O. Marinkovic (2003). *n*-alkanes and fatty acids of *Hypericum perforatum*, *Hypericum maculatum* and *Hypericum olympicum*. *Biochemical Systematics and Ecology 31*(2), 223-226.

Walker, T. S., H. Pal Bais & J. M. Vivanco (2002). Jasmonic acid-induced hypericin production in cell suspension cultures of *Hypericum perforatum* L. (St. John's wort). *Phytochemistry 60*(3), 289-293.

Willis, A. J., R. H. Groves & J. E. Ash (1997). Seed Ecology of *Hypericum gramineum*, an Australian Forb. *Australian Journal of Botany 45*(6), 1009-1022.

Zobayed, S. M. A., S. J. Murch, H. P. V. Rupasinghe & P. K. Saxena (2004). *In vitro* production and chemical characterization of St. John's wort (*Hypericum perforatum* L. cv 'New Stem'). *Plant Science 166*(2), 333-340.

Zobayed, S. M. A. & P. K. Saxena (2003). In vitro-grown roots: a superior explant for prolific shoot regeneration of St. John's wort (*Hypericum perforatum* L. cv 'New Stem') in a temporary immersion bioreactor. *Plant Science 165*(3), 463-470.

4.3 Supplementary data

An approximation of the absolute quantification of all the essential oils (EO) constituents from aerial parts and flowers of *Hypericum perforatum* plants (common variety), cultivated in Arcos de Valdevez (HpA) and Merelim (HpM) experimental fields over a year is shown in Table S1.

Table S1- Specific content (μg/g of biomass dry weight) of essential oils from vegetative aerial parts (VAp) and flowers (Fl) of *Hypericum perforatum* plants, cultivated in Arcos de Valdevez (HpA) and Merelim (HpM) experimental fields and harvested at different times during the year

Compound	KI	January		April		June				September		December	
		HpA VAp (μg/g dw)	HpM VAp (μg/g dw)	HpA VAp (μg/g dw)	HpM VAp (μg/g dw)	HpA VAp (μg/g dw)	HpA Fl (μg/g dw)	HpM VAp (μg/g dw)	HpM Fl (μg/g dw)	HpA VAp (μg/g dw)	HpM VAp (μg/g dw)	HpA VAp (μg/g dw)	HpM VAp (μg/g dw)
1-Octene	793	8.1	9.3	5.9	8.3	31.0	12.9	12.7	17.8	20.1	11.6	8.3	8.1
(E)-2-Hexenal	854		5.2	12.9	28.0	85.7		46.2		34.0	24.3	10.2	4.5
2-methyl-octane	864	244.0	271.1	184.2	483.1	898.4	3531.0	714.8	4578.9	728.0	440.5	170.9	107.2
n-Nonane	900	48.4	72.0	171.9	102.3	174.6	142.3	84.2	180.1	234.3	145.9	41.9	27.4
α-Thujene	929	12.0	2.3	5.4	7.0	76.6	39.1	41.5	44.8	6.6	25.0	8.1	5.6
α-Pinene	936	81.5	38.4	114.6	91.6	247.0	519.2	109.6	1562.0	120.7	170.2	64.2	35.9
2,6-Dimethyl-octane	972	16.3	29.9	22.2	35.3	67.2	70.6	50.9	99.1	78.5	26.2	13.3	6.8
Sabinene	975	14.9	5.1	29.2	44.6	160.4	129.3	57.4	65.2	15.9	42.3	16.9	15.1
β-Pinene	978	23.2	7.9	25.8	30.5	167.3	396.9	76.9	1941.5	24.7	64.6	19.5	15.3
6-Methyl-5-Hepten-2-one	988					1.7		1.0	2.0				
Myrcene	986	10.2	6.2	15.3	21.7	72.7	101.3	30.1	123.4	14.0	33.0	10.6	8.3
n-Decane	1000					2.1	2.5	0.2	2.8	0.8	0.6	0.5	
Hexyl acetate	1005			10.8	15.0	4.5		1.7	3.5	4.0	11.4		
α-Terpinene	1018			2.3	4.0	24.3	21.5	9.7	17.7	1.6	9.8	2.1	1.4
p-Cymene	1026					6.8	1.7	4.6	3.7		0.9		
Limonene	1031	4.6	1.4	6.7	10.4	41.3	51.3	17.2	79.2	6.0	17.5	5.0	4.0
Z-*β*-Ocimene	1041	21.9	13.7	28.0	45.4	39.3	178.6	17.4	142.9	23.7	36.7	16.4	19.7
E-*β*-Ocimene	1052	152.5	104.3	164.0	339.2	236.4	1685.4	83.3	1405.4	152.8	193.1	107.7	145.2
γ-Terpinene	1061	3.0		4.2	6.7	46.4	38.8	19.5	34.7	5.0	17.9	3.8	2.8
Methyl decane	1069		4.3	0.7	3.5	36.3	196.9	21.1	152.7	7.2	2.6	1.8	
trans-Linalool Oxide	1073							0.3					
Terpinolene	1090			1.5	2.2	11.2	12.3	4.7	16.0	1.5	5.2	1.1	
Linalool	1099					19.5		2.7	1.8				
n-Undecane	1100	1.3	9.0	3.7	9.9	15.9	57.7	18.9	40.1	12.2	2.8	3.0	1.9
n-Nonanal	1104	4.1	6.5	1.6	3.5	1.2	5.3	2.9	8.4	2.9	3.9	3.3	3.3
α-Campholenal	1121	4.2	3.2	2.6	6.8	3.2		3.3	6.7	4.0	4.8	3.4	3.8
Camphor	1145					2.1		0.4	1.6				
n-Nonanol	1170			7.1	11.2	8.6	64.3	3.8	43.6	12.0	14.2	6.8	20.1
Terpinen-4-ol	1176	7.9	12.2	5.6	12.0	38.9	39.1	11.4	28.5	6.5	21.2	4.5	3.4
α-Terpineol	1189	3.1	4.0	1.1	1.8	3.9	8.1	1.6	26.6	1.1	3.3		
n-Decanal	1205	11.3	18.7	26.4	43.1	10.8		4.5	3.2	27.6	28.7	13.8	31.3
trans-Carveol	1217					1.7							
Methyl dodecane	1265					2.6	26.2	1.3	29.3				
n-Decanol	1273			3.9	8.0	0.7		0.3		4.5	4.8	2.8	4.2
n-Tridecane	1300					1.7	5.1	1.0	7.6				
SH	1327	1.9	1.6	1.5	3.2	4.5	7.0	3.0		3.5	2.1	1.1	1.5

Compound	KI	January		April		June				September		December	
		HpA VAp (µg/g dw)	HpM VAp (µg/g dw)	HpA VAp (µg/g dw)	HpM VAp (µg/g dw)	HpA VAp (µg/g dw)	HpA Fl (µg/g dw)	HpM VAp (µg/g dw)	HpM Fl (µg/g dw)	HpA VAp (µg/g dw)	HpM VAp (µg/g dw)	HpA VAp (µg/g dw)	HpM VAp (µg/g dw)
δ-Elemene	1337	19.8	17.3	16.2	34.2	25.3	57.8	24.2	37.4	36.2	21.1	12.1	16.2
α-Cubebene	1348			1.8	0.9	1.6	10.0	1.7	8.0	2.3	1.5		
α–Ylangene	1370			0.5	3.7	0.7	6.8	1.0	20.9	0.9	0.5		
α-Copaene	1374		2.2	2.4		12.8	52.2	4.8	11.4	1.2	2.9	1.4	2.3
β-Bourbonene	1382	5.5	6.8	1.3	2.9	9.0	13.6	12.0	16.5	4.9	4.6	2.1	3.8
β-Cubebene	1388		9.9						21.1		1.2		
β-Elemene	1390	13.7	3.6	3.4	18.3	31.6	38.7	20.5	7.4	36.0	20.1	10.8	10.2
Dodecanal	1406	3.3				0.9		0.5	2.6	2.9			
α-Cedrene	1412	3.9	4.1	12.4	11.5	21.2	46.9	18.1	30.0	33.4	17.8	4.0	4.6
SH	1415			2.1						3.7	1.3		
(E)-Caryophyllene	1417	125.8	113.0	105.5	280.7	553.1	2594.0	451.3	1609.9	516.1	296.7	81.6	72.5
β-Gurjunene	1427	9.4	9.5	6.7	13.8	10.9	37.5	12.2	23.2	18.1	9.3	5.2	6.8
α-Guaiene	1439					4.1	19.9	2.2	19.2	1.0	0.8		
Aromadendrene	1440			3.3	5.8	36.3	31.3	14.4	8.4	0.8	1.9	3.5	4.6
Z-β-Farnesene	1442	3.8	4.9		4.5	6.4	19.8	5.7	11.4	11.9	5.0		
SH	1446			0.9		1.2	8.0	0.8	4.8	2.3	0.7		
α-Himachalene	1450	17.0	14.7	13.3	29.8	43.3	127.6	34.8	81.2	42.8	25.1	10.7	11.1
α-Humulene	1453			0.6				0.8			0.7		
E-β-Farnesene	1455					1.0	2.0	5.6	1.7	1.0			
allo-Aromadendrene	1459	10.7	18.7	20.3	27.9	19.3	399.1	14.8	195.3	73.6	21.0	6.5	6.9
α-acoradiene	1468			2.4	2.2	6.9	11.6	7.5	30.4	8.2	4.2	0.8	
Dodecanol	1474						5.4						
γ-Gurjunene	1475		86.5	2.4	188.3	180.7	25.0	117.3		152.5	69.3	135.0	132.0
γ-Muurolene	1478	3.0	2.8	1.1	1.5	0.4	1.6	0.6	2.6	1.0	0.7		
Germacrene D	1481	733.2	720.2	555.7	1314.2	722.8	1597.9	790.0	1420.5	1324.3	621.6	479.2	463.9
Curcumene	1483			25.0		2.0		3.1					
SH	1487			0.6	1.0	1.9	7.2	2.0	9.3	1.5	1.3		
β-Selinene	1488	127.3	108.5	108.7	221.4	161.7	451.8	165.4	281.4	242.0	141.8	78.6	81.3
α-Selinene	1494			15.3	5.7						9.6		
α-Muurolene	1497	4.6	5.1	3.6	4.8	6.9	23.2	7.5	14.4	18.6	5.0	2.9	2.7
n-Pentadecane	1500			0.3	3.5	3.6		2.5		9.8	2.1	1.3	1.4
(E,E)-α-Farnesene	1503		2.4	1.3	1.8	1.4	12.0	1.8	6.3	3.1	1.8	0.7	
γ-Cadinene	1508	9.3	9.3	13.2	18.1	19.3	127.2	19.9	52.0	43.9	17.6	3.9	3.4
cis-γ-Bisabolene	1510			6.1	7.1	10.2	50.3	9.8	31.6	8.3	5.1		
δ-Cadinene	1521	17.5	11.0	22.7	29.4	31.0	127.9	30.7	79.0	42.3	30.2	12.1	13.0
SH	1527					1.0		0.5			1.1		
E-γ-Bisabolene	1530			1.0	2.6	1.8	8.8	1.8	4.9	2.2	1.1		
α-Cadinene	1534			1.2	1.7	3.3	15.1	3.1	9.2	2.3	1.8	0.3	
SH	1540					0.2		0.5					
α-Calacorene	1543			1.2	1.2	2.0	2.8	1.5	1.4	2.2	1.6		
SO?	1549			1.5		28.1	5.8	14.8	10.8		1.0		
γ-Elemene (?)	1551					2.7		1.5		1.8			
E-Nerolidol	1564	4.1	12.6	19.6	38.1	55.4	93.8	28.1	59.0	48.0	40.9	18.9	20.5
Spathulenol	1574	11.9			29.5	17.1	22.7	7.9	24.0		2.5	10.7	
cis-3-Hexenyl benzoate	1577		10.0	13.4	2.4	193.1	20.3	98.6	34.6	23.0	14.6		16.1
Caryophyllene Oxide	1580	10.3	10.6	5.5	14.3	347.5	130.9	185.4	325.3	18.2	4.7	9.7	7.4
Globulol	1585				3.7	3.0	6.3	2.4	3.8	3.6	3.2	1.2	1.1
Viridiflorol	1592	5.2	3.8	3.8	15.7	4.9	18.5	6.2	11.7	10.9	13.5	4.5	2.6
Guaiol	1593			3.6		5.3	11.3	2.0	10.1	3.6	1.7		

Compound	KI	January		April		June				September		December	
		HpA VAp (µg/g dw)	HpM VAp (µg/g dw)	HpA VAp (µg/g dw)	HpM VAp (µg/g dw)	HpA VAp (µg/g dw)	HpA Fl (µg/g dw)	HpM VAp (µg/g dw)	HpM Fl (µg/g dw)	HpA VAp (µg/g dw)	HpM VAp (µg/g dw)	HpA VAp (µg/g dw)	HpM VAp (µg/g dw)
β-Copaen-4-*α*-ol	1597	4.2	4.2	30.4	12.9	9.2	9.5	5.3	20.9	14.2	15.2	4.3	4.1
Humulene Epoxide II	1603					14.3		8.6				0.8	
β-Oplopenone	1610					7.6	2.7	3.2	5.3	3.1	1.8		
SO	1612		0.5		11.6	6.7		5.7		10.3	10.9	4.5	2.4
10-*epi*–*γ*-Eudesmol	1619	4.4	3.9	5.8	6.8	11.8	22.3	10.8	12.5	13.4	8.9	5.7	3.2
γ-Eudesmol	1624		5.0	2.3	2.7	3.2	8.2	2.7	5.9	3.0	2.9	3.0	2.1
epi-*α*-Cubenol	1633	37.2	45.2	1.7	23.8	26.3	14.0	14.1	13.9	59.8	36.0	39.1	51.6
epi-*α*–Cadinol	1640		18.2	26.7	39.6	114.9	368.9	24.5	184.1	40.7	38.4	20.4	16.1
epi-*α*-Muurolol	1642		5.2	7.6	11.1	14.2	9.6	49.1	9.5	19.0	15.6	7.5	5.8
SO	1646					8.1		2.9					
α-Cadinol	1654	25.5	29.9	42.0	61.6	49.7	96.0	38.8	63.1	68.8	54.0	37.8	30.5
β-Bisabolol	1673			1.4	1.1	10.9	5.8	6.2	5.6	2.9	31.9	0.5	
epi-*α*-Bisabolool	1677			2.4		131.7	105.9	94.8	32.4	93.6	20.6	14.3	
n-Tetradecanol	1681		14.0	5.0	16.3	21.8		13.0					
Benzyl benzoate	1738					1.4	2.0	1.3					
n-Hexadecanol	1881					14.6	9.7	14.9	2.6	7.4	2.8		
n-Nonadecane	1900						11.3		30.2				
n-Eicosane	2000						5.8	0.1	8.0				
n-Heneicosane	2100						34.8	0.4	48.3	0.1	0.1		
Nonadecanal	2111			8.3	2.9	21.8	19.8	49.4	5.7	231.2	97.5	2.0	
n-Docosane	2200			1.1			3.2	0.3	4.5	0.1	0.4	7.3	
n-Tricosane	2300	20.2	12.5	12.1	6.9	3.1	14.4	2.4	14.0	7.5	4.0		7.5
n-Tetracosane	2400		2.2	3.0	0.8	0.8	1.5	1.0	1.0	2.2	1.3	1.9	1.1
n-Pentacosane	2500	7.2	16.7	17.4	4.7	6.0	10.5	5.6	4.7	15.4	8.0	8.0	7.1
n-Hexacosane	2600						0.8		0.3	0.6	0.7		
n-Heptacosane	2700		1.0	2.7	0.7	3.6	14.1	3.9	9.3	7.3	6.4	2.8	1.7
n-Octacosane	2800						2.6		2.2	0.1	0.5		
n-Nonacosane	2900			1.2		5.4	39.5	9.5	26.5	5.0	8.7		
Monoterpene Hydrocarbons		325.0	179.3	397.0	603.3	1129.6	3175.5	471.9	5436.4	372.4	616.2	255.5	253.5
Oxygenated Monoterpenes		11.0	16.2	6.7	13.8	66.1	47.2	16.5	58.4	7.6	24.5	4.5	3.4
Sesquiterpene Hydrocarbons		1087.7	1152.2	953.9	2238.0	1938.4	5934.5	1792.5	4050.7	2643.9	1348.2	852.6	838.1
Oxygenated Sesquiterpenes		101.6	139.0	154.2	272.5	870.1	932.1	513.6	797.8	413.3	303.6	183.2	147.5
Alkanes		336.0	418.7	420.5	640.8	1216.6	4172.7	897.8	5239.5	1109.1	650.3	252.6	160.7
Others		42.8	85.8	121.4	183.9	582.9	286.6	385.5	281.8	435.5	263.0	61.3	104.0

VAp- Vegetative aerial parts; **Fl-** Flowers; **KI-** Kovats retention index on a DB-5 column

The highest accumulation of sesquiterpene hydrocarbons occurred in September, in HpA-VAp (2.6 mg/g of biomass dry weight), and in April, in HpM-VAp (2.2 mg/g of biomass dry weight). The contents of sesquiterpene hydrocarbons, monoterpene hydrocarbons and *n*-alkanes in flowers were higher than in the respective vegetative aerial parts independently of the site of the plant growth. Proportionally, the flowers of the plants from Arcos de Valdevez were richer in SH (5.9 mg/g of biomass dry weight) than the flowers from HpM population and these ones

were richer in MH (5.4 mg/g of biomass dry weight) and alkanes (5.2 mg/g of biomass dry weight), than the former ones.

Table S2 shows the approximation of the absolute quantification of all the EO constituents from vegetative aerial parts and flowers of *in vivo* plants of *Hypericum perforatum* 'Topaz' growing in Arcos de Valdevez (HpTA-VAp and HPTA-Fl) and Merelim (HpTM-VAp and HpTM-Fl) experimental fields and *in vitro* shoots maintained in MS basal medium.

Table S2- Specific content (µg/g of biomass dry weight) of essential oils from vegetative aerial parts (VAp) and flowers (Fl) of *in vivo* plants of *Hypericum perforatum* 'Topaz' grown in Arcos de Valdevez (HpTA-VAp and HPTA-Fl) and Merelim (HpTM-VAp and HpTM-Fl) experimental fields and *in vitro* shoots maintained on MS basal medium.

Compound	KI	January		April		June				September		December		*In Vitro* shoots
		Hp TA VAp	Hp TM VAp	Hp TA VAp	Hp TM VAp	Hp TA VAp	Hp TA Fl	Hp TM VAp	Hp TM Fl	Hp TA VAp	Hp TM VAp	Hp TA VAp	Hp TM VAp	
1-Octene	793	15.9	6.3	4.9	6.2	14.8	19.7	22.9	17.5	20.4	4.2	9.4	2.8	188.5
(E)-2-Hexanal	853		3.7	13.8	14.0	44.6		75.1		16.2	8.9		1.9	3.7
2-methyl-octane	864	316.6	92.7	832.1	255.9	494.1	4021.4	736.7	2660.1	582.5	62.8	73.0	7.1	
n-Nonane	900	297.1	84.7	151.7	211.5	286.3	530.4	383.9	388.7	553.5	98.0	82.3	7.2	661.1
α-Thujene	929	11.5	5.6	17.6	6.3	36.0	26.7	87.6	33.4	34.1	1.4	6.4	1.3	
α-Pinene	936	234.9	107.3	120.9	125.8	129.1	1594.3	207.9	1105.9	288.3	48.4	114.0	21.7	251.8
2,6-Dimethyl-octane	972	67.2	23.5	72.0	35.7	92.1	385.0	103.7	267.2	60.5	10.6	6.9	1.0	37.8
Sabinene	975	25.8	12.6	87.3	34.9	72.4	57.5	103.9	71.5	96.0	5.5	27.2	10.9	0.9
β-Pinene	978	34.2	19.0	60.0	29.3	89.1	339.1	136.7	396.3	100.8	12.3	25.6	7.7	22.7
6-Methyl-5-Hepten-2-one	988					0.4	1.4	4.0	1.6					
Myrcene	986	21.0	8.4	41.1	17.1	44.6	108.5	63.8	100.6	66.7	5.6	13.3	4.0	10.5
n-Decane	1000			0.4	0.3	1.6	2.8	4.3	4.9	2.6				2.1
Hexyl acetate	1005	4.0		17.5	3.0	3.2	4.6	4.0	10.9	21.8	6.1			
α-Terpinene	1018	3.2	2.1	8.6	2.2	12.1	13.9	20.7	17.1	18.3	0.7	1.6	0.4	
p-Cymene	1026	2.9		0.5		2.4	1.8	8.8	1.8	1.4				
Limonene	1031	9.2	5.6	22.0	7.3	25.6	39.9	37.7	43.5	35.7	2.1	6.4	2.1	2.2
Z-β-Ocimene	1041	26.5	17.2	56.4	38.6	48.7	157.5	43.1	134.8	94.6	11.9	25.6	14.1	6.0
E-β-Ocimene	1052	116.3	75.8	417.9	199.5	208.1	1670.5	161.5	1206.7	416.0	63.3	110.7	69.9	46.9
γ-Terpinene	1061	4.3	3.0	15.2	4.1	23.5	25.7	41.6	31.6	33.5	1.7	3.0	0.9	
Methyl decane	1069	0.8	5.6	11.6	1.4	14.1	233.5	20.0	139.6	4.5	0.8			7.2
Terpinolene	1090	3.3	3.1	4.8	1.5	5.8	6.3	9.5	10.8	9.5	0.6	1.0		
Linalool	1099		4.4	11.2		2.0		6.0	1.4					
n-Undecane	1100	17.0	1.6	16.3	6.9	39.8	112.9	46.7	70.6	19.1	4.5	1.7	1.1	102.3
n-Nonanal	1104	6.3	8.7	8.5	1.8	3.2	9.4	6.1	7.7	19.8	0.8	8.8		
α-Campholenal	1121	10.6	6.1	11.3	4.5	5.6	9.6	3.9	7.7	10.5	1.3	3.1	1.6	
allo-ocimene	1126							3.0	6.7				0.8	1.4
Camphor	1145			6.1		0.5	0.7	1.8	1.5					
n-Nonanol	1170	13.1		8.4	13.6	5.0	37.8	6.9	22.3	10.4	7.6	7.6	14.4	31.5

Compound	KI	January		April		June				September		December		*In Vitro* shoots
		Hp TA VAp	Hp TM VAp	Hp TA VAp	Hp TM VAp	Hp TA VAp	Hp TA Fl	Hp TM VAp	Hp TM Fl	Hp TA VAp	Hp TM VAp	Hp TA VAp	Hp TM VAp	
Terpinen-4-ol	1176		17.7	24.4	4.8	10.9	20.2	20.5	25.6	34.0	3.3	3.2	2.7	
α-Terpineol	1189		4.8	4.6	0.9	1.9	9.4	2.6	9.5	3.6	1.2			
n-Decanal	1205	10.7	21.0	30.6	40.8	3.9	3.4	8.9	3.3	52.5	26.7	17.0	39.6	39.2
trans-Carveol	1217							1.9						
Methyl dodecane	1265	3.5				1.3	50.8							
n-Decanol	1273	3.6		7.3	6.7	0.2		1.9	30.1	8.6	5.4		14.9	22.6
n-Tridecane	1300	3.6		1.2		3.6	17.3	4.2	11.4	0.9				
SH	1327	5.8	3.7	5.4	1.8	3.8	3.2	3.4	3.6	2.5	0.9	2.0	0.4	
δ-Elemene	1337	39.6	21.4	58.8	19.6	28.3	27.4	16.1	22.1	42.7	8.5	10.6	4.5	2.8
α-Cubebene	1348			4.2	2.4	4.3	29.1	4.8	18.4	5.1	1.3			3.1
α-Ylangene	1370	5.7		0.8	1.1	2.6	16.4	3.4	11.1	2.9	0.5			2.7
α-Copaene	1374	13.3	12.8	4.0	6.5	10.7	54.5	8.0	39.8	8.3	2.0	1.7	1.1	2.1
β-Bourbonene	1382	9.3	8.2	0.4	3.1	5.4	6.9	6.7	5.6	4.9	1.5	1.6	0.8	6.0
β-Cubebene	1388	10.6	4.4				2.1	4.7	4.1		1.3			
β-Elemene	1390	7.3	4.3	24.3	4.4	5.0	6.2	6.4	6.1	10.0	2.4	9.7	1.0	6.4
Dodecanal	1406					0.2	1.9	0.7	2.2					
α-Cedrene	1412	42.3	17.4	18.5	25.3	34.3	73.8	36.8	40.9	58.6	19.6	1.8	3.9	32.6
SH	1415	12.2	8.1	1.8	3.2						1.7			
(E)-Caryophyllene	1417	277.6	119.1	410.3	218.8	568.2	2777.8	526.1	1866.9	497.5	132.4	66.4	21.2	212.8
β-Gurjunene	1427	24.5	13.9	27.3	10.0	15.6	23.1	8.5	15.8	21.8	3.6	4.2	1.6	4.6
α-Guaiene	1439	4.9		3.8	1.2	3.0	12.8	2.8	10.4	3.3				7.2
Aromadendrene	1440	11.3	7.0	5.5	6.6	10.7	8.1	4.2	5.6	13.5				6.3
Z-*β*-Farnesene	1442			12.9	0.7	1.7	6.2	7.5	4.8	2.3	3.9	2.7	1.4	
SH2	1446					2.3	28.3	2.8	17.6	3.6	1.3			
α-Himachalene	1450	34.0	20.1	53.1	19.5	37.2	104.0	37.0	76.5	46.4	10.2	8.5	3.2	
α-Humulene	1453			1.9	0.8	1.5	3.5	2.1		2.7	0.7			11.0
E-*β*-Farnesene	1455					13.5			2.1					
allo-Aromadendrene	1459	64.3	27.4	58.0	37.7	39.8	355.8	30.6	186.2	62.3	17.3	12.3	2.7	7.0
Germacrene D Isomer	1462													81.7
SH	1465													10.4
α-Acoradiene	1468	11.9	6.1	3.1	4.7	8.6	38.0	10.1	24.3	14.5	4.5	2.5	0.8	
Dodecanol	1475						2.7		3.8					7.9
γ-Gurjunene	1475		4.7	1.5	0.8		7.0		3.0		0.5			
γ-Muurolene	1478	10.4	11.4	312.5	7.3		78.8	0.9	43.8	2.1	3.4	2.8	50.4	19.3
Germacrene D	1481	1454.8	668.3	2285.8	877.9	1148.7	1993.6	437.6	1533.1	1145.6	326.7	350.0	82.8	451.3
Curcumene	1483	101.1	55.5	4.3	51.1	1.8	9.3	1.8		0.7	64.8	38.0	3.0	
SH	1487	1.3	7.2	3.2	1.6	2.5		0.7	7.2	3.6				
β-Selinene	1488	220.1	114.4	375.2	127.3	232.4	302.7	100.5	214.6	359.2	53.2	66.4	28.0	47.7
α-Selinene	1494			13.3				10.7			9.4		1.0	25.4
α-Muurolene	1497		6.9	11.7	33.6	6.7		5.5	4.5	13.8		4.9		14.5
n-Pentadecane	1500			2.5		1.9	5.8	1.5		2.2				
(E,E)-*α*-Farnesene	1503	13.5	4.6	4.5	2.8	2.4	10.7	0.9	6.5	6.4	0.4			3.0
γ-Cadinene	1508	33.3	22.1	40.8	20.7	54.9	164.2	19.0	115.1	49.2	9.5	3.5	1.5	105.4
cis-*γ*-Bisabolene	1510	7.9		13.2	7.6	12.5	50.2	10.2	24.4	15.2	1.0	1.4	2.2	4.3

Compound	KI	January		April		June				September		December		*In Vitro* shoots
		Hp TA VAp	Hp TM VAp	Hp TA VAp	Hp TM VAp	Hp TA VAp	Hp TA Fl	Hp TM VAp	Hp TM Fl	Hp TA VAp	Hp TM VAp	Hp TA VAp	Hp TM VAp	
SH	1516													9.8
δ-Cadinene	1521	60.8	31.2	53.8	32.1	39.3	104.0	27.7	64.1	82.9	12.4	14.1	8.9	23.9
SH	1527			2.8		0.7		0.6						
E-*γ*-Bisabolene	1530	5.8		2.6	1.2	1.9	7.7	1.9	5.5	3.2	0.5			4.4
α-Cadinene	1534	6.4		2.9	1.9	2.5	12.5	3.0	7.3	5.0	0.5			3.3
SH	1540				1.9	2.5	2.2	1.0						
α-Calacorene	1543			1.8		3.7	7.9	3.0	1.6	2.7	1.9	1.4	1.7	4.3
SO (?)	1549				1.7			34.3	6.2	2.2				2.7
γ-Elemene (?)	1551	3.8		0.5		0.4		3.9		0.8				
E-Nerolidol	1564	8.1	9.7	58.5	26.2	41.8	76.3	52.0	56.0	65.3	13.4	11.3	11.3	2.5
Spathulenol	1574	11.0		6.1	18.2	9.5	10.7	11.1	9.5	11.6		11.4		
cis-3-Hexenyl benzoate	1577	12.4	19.4	38.1	1.8	50.8	9.0	211.8	11.5	20.9	8.4	6.8	10.0	
Caryophyllene Oxide	1580	15.2	8.6	27.5	9.7	46.6	87.2	444.5	107.6	7.0	5.7		2.1	
Globulol	1585	7.9		6.9	3.5	2.1	3.4	2.2	2.3	35.0	1.1	1.2	0.6	
Viridiflorol	1592	10.0	8.1	27.2	9.8	4.3	10.0	2.7	7.1	9.6	2.4	2.3	1.0	
Guaiol	1593	5.0		2.7	2.5	1.6	1.3	3.5	23.1	10.5	0.3			2.5
β-Copaen-4-*α*-ol	1597	36.0	29.7	20.6	38.2	33.2	30.2	42.8	6.4		14.3	18.7	20.2	2.8
Humulene Epoxide II	1603			2.8		1.6	7.1	18.9		0.8				
β-Oplopenone	1610			1.6		1.7	1.4	5.8	1.2					1.3
SO	1612	6.2	6.9	20.4	9.4	4.0		6.1						6.9
epi-*γ*-Eudesmol	1619		7.8	7.6	1.9	1.9	18.4	7.5	11.8	70.1	3.3			
γ-Eudesmol	1624	8.9	5.6	4.9	3.2	1.5	6.6	3.7	4.9	5.6		2.0	1.0	
epi-*α*-Cubenol	1633		4.4	13.6	32.7	9.4	9.3	11.2	8.9	5.6	1.5	18.0	12.1	3.2
epi-*α*-Cadinol	1640	43.8	18.6	66.4	10.2	7.3	269.0	17.3	175.9	93.2	13.2		3.0	22.8
epi-*α*-Muurolol	1642	17.3	14.7	23.1	3.6	3.5	7.2	16.8	8.3	32.5	5.9	5.6	18.9	4.9
δ-Cadinol	1644													6.1
SO	1646					0.5				11.6				
α-Cadinol	1654	51.7	33.9	98.2	46.8	33.5	56.2	28.9	44.9	101.2	20.0	26.8		6.3
β-Bisabolol	1673	3.9		3.7	2.3	17.2	75.4	9.1	4.5	8.0	6.9	1.4	1.1	45.1
10-*epi*-*α*-Bisabolool	1677	4.2		52.9	2.7	13.8	32.8	21.9	44.2	29.7	18.5	3.3	2.3	31.1
n-Tetradecanol	1681		5.7	21.5	7.5	3.8		19.8	19.2				1.9	2.1
Benzyl benzoate	1738					1.1	1.7	1.4	1.5					
n-Heptadecane	1700													2.1
n-Octadecane	1800													1.6
n-Hexadecanol	1881					3.1	5.9	3.2	2.6	7.9	0.4			8.9
n-Nonadecane	1900						11.5		13.0					
n-Eicosane	2000						4.8		3.5					
n-Heneicosane	2100			1.0	1.0	0.4	32.5	0.3	13.2					
Nonadecanal	2111			17.7	27.9	133.0	23.2	79.6	19.6	37.0	101.5			14.8
n-Docosane	2200			1.3	0.8		3.0	0.4	1.2	0.1			0.4	
n-Tricosane	2300	11.5	12.1	13.8	13.1	2.8	12.5	2.4	5.4	5.0	2.4	6.7	5.8	2.0
n-Tetracosane	2400		3.7	2.4	2.6	0.6	1.2	0.6	0.7	1.3	0.8	1.9	1.1	2.7
n-Pentacosane	2500	13.4	8.5	22.2	13.5	4.5	8.6	4.0	5.7	10.2	5.3	7.0	3.5	6.2
n-Hexacosane	2600								0.5					

Compound	KI	January		April		June				September		December		*In Vitro* shoots
		Hp TA VAp	Hp TM VAp	Hp TA VAp	Hp TM VAp	Hp TA VAp	Hp TA Fl	Hp TM VAp	Hp TM Fl	Hp TA VAp	Hp TM VAp	Hp TA VAp	Hp TM VAp	
n-Heptacosane	2700		5.1	3.3	3.7	2.5	13.4	2.2	10.8	5.5	3.2	1.4	1.6	11.0
n-Octacosane	2800								1.8					1.8
n-Nonacosane	2900	2.3				6.3	19.2	2.7	22.7	6.4	4.1			11.0
Monoterpene Hydrocarbons		493.3	259.8	852.3	466.6	697.3	4041.7	925.8	3160.7	1194.8	153.4	334.8	133.8	342.4
Oxygenated Monoterpenes			26.9	46.2	5.7	15.4	30.3	32.8	38.0	37.6	4.5	3.2	2.7	
Sesquiterpene Hydrocarbons		2519.0	1200.0	3824.5	1535.3	2309.4	6328.2	1350.7	4392.8	2493.4	696.1	606.4	222.1	1113.3
Oxygenated Sesquiterpenes		229.2	147.9	444.5	221.8	235.2	702.6	952.1	522.8	499.6	114.8	102.1	83.6	135.4
Alkanes		732.9	237.5	1131.7	546.4	951.7	5466.8	1313.5	3620.9	1254.3	192.5	181.0	28.9	841.6
Others		90.4	104.5	230.7	138.8	307.6	228.3	409.9	232.8	364.1	181.8	67.6	84.9	329.0

VAp- Vegetative aerial parts; **Fl-** Flowers; **KI-** Kovats retention index on a DB-5 column

In the vegetative aerial parts of plants from the Arcos de Valdevez population, the higher EO yield was coincident with the highest accumulation of sesquiterpene hydrocarbons (3.8 mg/g of biomass dry weight) in April. Lower amounts of this group were found in the EO of HpTM-VAp (1.5 mg/g of biomass dry weight) in the same period. Likewise the EO of flowers of *H. perforatum* (common variety), the accumulations of sesquiterpenes hydrocarbons, monoterpene hydrocarbons and *n*-alkanes in EO of flowers of *H. perforatum* 'Topaz' were higher than in the EO of the respective vegetative aerial parts.

Chapter 5

ESSENTIAL OILS OF *HYPERICUM UNDULATUM* SCHOUSBOE EX WILLD.

5.1- Chapter overview

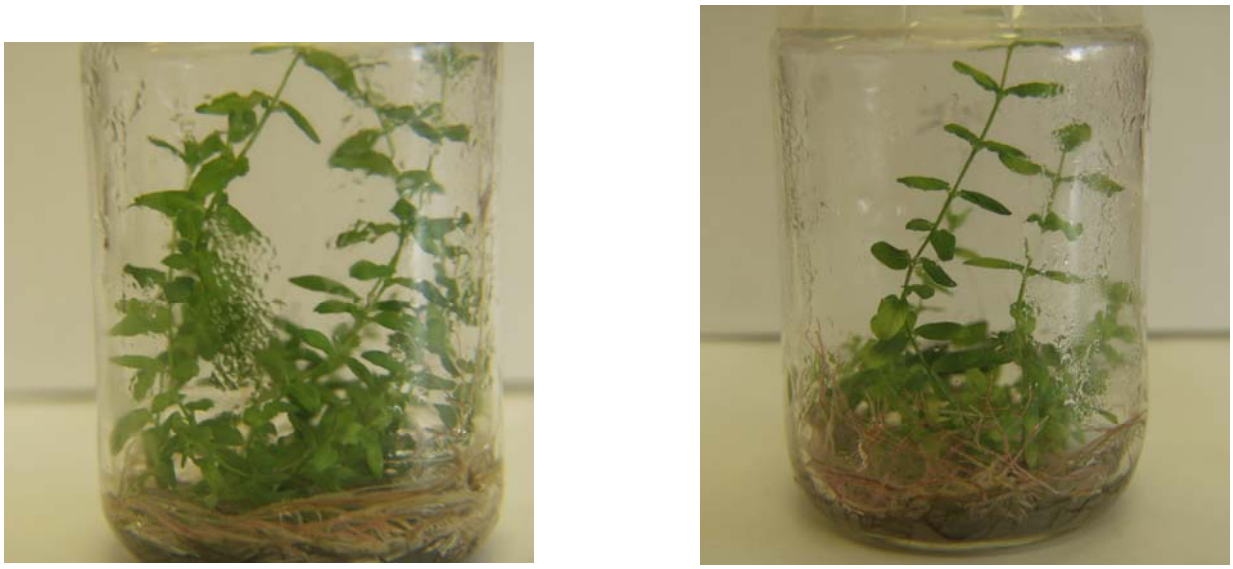
Two of the few phytochemical reports on *Hypericum undulatum* describes the presence of hypericin, quercetin, quercetin sulphate, rutin, mangiferin and chlorogenic acid in its leaves and aerial parts (Seabra *et al.*, 1991; Seabra *et al.*, 1992). However little is known about their essential oils (EO) given the scarce number of papers on this subject (Mathis *et al.*, 1964; Nogueira, 2002). To our knowledge there is no report on *in vitro* cultures of this species.

In this work, *in vitro shoots* cultures of *H. undulatum* were established in two basal media and the respective essential oils were chemically characterized along with the essential oils of in Nature growing plants. The analyses were performed on: (i) essential oils accumulated in the aerial parts of *in vivo* plants, over one year, with interval of 2 months, (ii) essential oils isolated from leaves, stems, ripened seed capsules and flowers of the plants, (iii) essential oils produced by *in vitro* shoots in both culture media and, (iv) essential oils isolated from aerial parts of micropropagated and acclimatized plants.

The *n*-alkanes constituted the group most represented in the EO of almost all the *H. undulatum* plant material, with exception for leaves where the sesquiterpene hydrocarbons were produced and/or accumulated in higher amounts. Undoubtedly, the essential oils of this species, growing wild in the Northern of Portugal is characterized by the high percentage content of *n*-nonane which was the major compound in the EO of all organs of plants and shoots of *H. undulatum*. Over the year fluctuations in the EO composition were registered, namely in what respects the relative contents of the major compounds, 3 of them common to the six samples (*n*-nonane, β-pinene and germacrene D). Except for *n*-nonane, none of the other four most represented compounds in the EO from ripened seed capsules were among the most represented ones of the EO from leaves, stems and flowers. Variations in the composition of EO were observed in shoots grown on the two different media during 60 days of culture. Shoots grown on MS basal medium had a more complex EO than that of shoots grown on Mg basal medium, even though EO contents were identical in both cases. Hydrocarbon terpenic compounds predominated over the oxygenated ones in the EO of in Nature growing plants as well as in shoots and acclimatized plants.

a **b**

Figure 1- a) *Hypericum undulatum* plants. **b)** Micropropagated and acclimatized plants of *Hypericum undulatum* 6 months after transfer to plastic vessels.



a **b**

Figure 2- *In vitro* plantlets of *Hypericum undulatum* grown on **a)** MS basal medium without plant growth regulators after 70 days of culture, and **b)** Mg basal medium without plant growth regulators after 70 days of culture.

Mathis, C. & G. Ourisson (1964). Étude chimio-taxonomique du genre *Hypericum* : II. Identification de constituants de diverses huiles essentielles d'*Hypericum*. *Phytochemistry 3*(1), 115-131.

Nogueira, T. (2002). O género *Hypericum* L. em Portugal Continental. Contribuição para o estudo quimiotaxonómico. Instituto Superior de Agronomia. Lisboa, Universidade Técnica de Lisboa. **PhD. Thesis**.

Seabra, R. M., M. H. Vasconcelos, M. A. C. Costa & A. C. Alves (1992). Phenolic compounds from *Hypericum perforatum* and *Hypericum undulatum*. *Fitoterapia 68*, 473-474.

Seabra, R. M., M. H. Vasconselos & A. C. Alves (1991). Flavonoid sulphates from *Hypericum undulatum*. *Rev. Port. Farm. 12*, 16-18.

5.2- Manuscripts

This chapter comprises the following manuscripts:

Guedes, AP & Fernandes-Ferreira, M. Seasonal Variation of the Essential Oil Content and Composition of *Hypericum undulatum* Schousboe ex Willd.

Guedes, AP & Fernandes-Ferreira, M. Essential oils from leaves, stems and ripened seed capsules of *Hypericum undulatum* Schousboe ex Willd.

Guedes, AP & Fernandes-Ferreira, M. Micropropagation and *in vitro* essential oil production profiles of *Hypericum undulatum* Schousboe ex Willd.

Seasonal Variation of the Essential oil Content and Composition of *Hypericum undulatum* Schousboe ex Willd

Ana P. Guedes and M. Fernandes-Ferreira

CITAB- Centro de Investigação e de Tecnologias Agro-Alimentares e Biológicas, Department of Biology, University of Minho, Campus de Gualtar, 4720-057 Braga, Portugal

Abstract

The composition of *Hypericum undulatum* essential oils (EO) and the respective seasonal variation profile were determined. The EO contents composed of more than 76 components ranged from 3.20 to 5.63 mg/g of biomass dry weight in spring and summer, respectively. From the 76 compounds identified, the *n*-alkanes constituted the major group, mainly due to *n*-nonane, which accounted for more than 37% of the total EO in all samples. The presence of a C_{17} to C_{29} *n*-alkanes series was recorded in the summer of the first year of study. Sesquiterpene hydrocarbons (SH) were the second major group of compounds between June and September, of the first year of study and from April to June of the second one, ranging from 24% to 36% of the total EO. In the winter, the monoterpene hydrocarbons (MH) constituted the second major group instead of SH mainly due to the increase in β-pinene (MH) and decrease of germacrene D (SH) accumulation.

Keywords: *H. undulatum*, essential oils, *n*-alkanes, monoterpene hydrocarbons, sesquiterpene hydrocarbons, *n*-nonane, β-pinene

1- Introduction

Hypericum undulatum, usually grows in wet places and in the riverside edges (Nogueira 2002 and references therein). Due to its reputation as a medicinal plant, this species is among the commercialized *Hypericum* species. Indeed, it is used to treat migraine and heal gall bladder ailments. Its decoctions are also believed to have renal antispasmodic and hepatic protector effects (Ferreira *et al.*, 2006). Recent studies revealed that *H. undulatum* ethanolic extracts can help preventing and alleviating patients suffering from Alzheimer's disease (Ferreira *et al.*, 2006). Previous reports indicated that the phenolic extracts of leaves and aerial parts of this species are mainly composed of hypericin, quercetin, quercetin sulphate, rutin, mangiferin and chlorogenic acid (Seabra *et al.*, 1991; Seabra *et al.*, 1992). However little is known about the *H. undulatum* essential oils (EO). In several species, variations in the EO profile occur by the effect of different factors, such as relative development and maturation of plant organs (Santos-Gomes *et al.*, 2001), climate conditions (Curado *et al.*, 2006), season (Santos-Gomes *et al.*, 2001; Hudaib *et al.*, 2002), and soil mineral fertilization (Zheljazkov *et al.*, 2006). The harvest time (Guedes *et al.*, 2003; Guedes *et al.*, 2004), the culture site (Nogueira, 2002), the organ type (Schwob *et al.*, 2004), as well as conditions of biomass growth (e.g. *in vitro* or *in vivo*) (Guedes *et al.*, 2003) are known to influence the EO content and composition of *Hypericum* plants. The characterization of the EO of *H. perforatum* plants have already been reported (Çakir *et al.*, 1997; Schwob *et al.*, 2002a; Schwob *et al.*, 2004; Pavlović *et al.*, 2006), as well as the EO composition of other species of this genus, namely *H. androsaemum* (Guedes et al., 2003; Guedes et al., 2004), *H. brasiliense* (Abreu et al., 2004), *H. coris* (Schwob et al., 2002b), *H. foliosium* (Santos *et al.*, 1999), *H. hirsutum* (Gudžic et al., 2007), *H. linaroides* (Cakir *et al.*, 2005), *H. maculatum* (Gudžić *et al.*, 2002), *H. olympicum* (Pavlović *et al.*, 2006) and *H. tetrapterum* (Pavlović *et al.*, 2006). However, information on *H. undulatum* EO's composition is negligible. *n*-Nonane was previously reported as the major compound of the EO of this species whereas some authors considered that sesquiterpene hydrocarbons constitute the second most represented group of compounds (Mathis *et al.*, 1964; Nogueira, 2002).

Nowadays, because EO constitutes a pool of potential pharmaceutical substances with medical and commercial value, a detailed identification of the EO components as well as the knowledge of the factors (physiological and environmental) that influence their relative concentrations is of utmost importance. In this work, the EO from aerial parts of *H. undulatum*

were analysed over one year to (i) characterize the broadest range of compounds produced, and (ii) study the variation of their composition profile over the seasonal cycles.

2- Material and Methods

2.1- Plant material

H. undulatum plants were cultivated in Nature (Braga, northern Portugal) and aerial parts of 6-8 plants were randomly collected six times over a period of one year, starting in June of the first year and ending in June of the following year.

2.2- Sampling and hydrodistillation of essential oils

Fresh biomass (~10 g) from the pruned branches of the several samples was submitted to hydrodistillation in a Clevenger type apparatus over 1 h in the presence of a *n*-hexane (1.0 mL) solution containing 5α-cholestane (1mg/mL), for the retention of the hydrodistillate components. The dry weight of the plant material was determined after drying (60ºC, 72h) in a drying stove.

Further analyses of the hydrodistillates were performed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) as described in Chapter 4 (pages 118-119).

3- Results and Discussion

The EO yields obtained in the hydrodistillation of the six samples of aerial parts of *Hypericum undulatum* plants harvested over a period of one year ranged from a maximum of 5.6 mg/g dry weight recorded in June of the first year to a minimum of 3.2 mg/g dry weight, recorded in April of the second year (Figure 1).

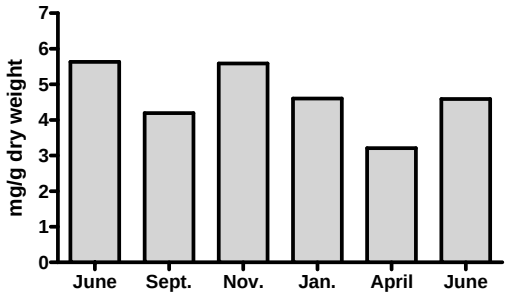


Figure 1 - Essential oil contents (mg/g dry weight) from plants of *Hypericum undulatum* Schousboe ex Willd. cultivated in Braga (Portugal) and harvested at different times over one year.

The lowest EO contents registered in April coincided with the high spring vegetative growth rate of the plant. In this phase of the phenological cycle, carbon and energy are predominantly directed to nutrition and plant growth being, subsequently, less available for the production of secondary metabolites. Low EO contents during the spring were also reported for *H. androsaemum* (Guedes *et al.*, 2004). The number of identified compounds in the hydrodistillates of *H. undulatum* samples, ranged from 49 to 76 depending of the harvest time (Table 1). To our knowledge, up to now, this is the first report describing such a complete composition of the *H. undulatum* EO. Twenty-two compounds were previously identified by Nogueira (2002), 17 from which were also detected in this study.

Table 1 - Composition (%) of the essential oils from plants of *Hypericum undulatum* Schousboe ex Willd. cultivated in Braga (Portugal) and harvested at different times over one year.

Compound	KI(1)	KI(2)	June	Sept.	Nov.	Jan.	April	June
n-Octane	800	800	0.5	0.5	0.6	0.3	1.4	0.5
(E)-2-Hexenal	854	854	2.0	0.2	0.2	tr	0.2	0.1
n-Hexanol	862	863	0.2	0.1	tr	tr	0.2	0.1
n-Nonane	900	900	37.1	41.6	41.5	48.4	42.9	45.9
α-Thujene	929	929	0.1	0.1	0.1	tr	tr	0.1
α-Pinene	937	936	2.4	2.4	5.3	5.4	3.0	2.3
Camphene	952	950	tr	0.1	0.3	0.3	tr	tr
2,6-Dimethyloctane	970	972	0.1	0.1	-	-	-	0.1
Sabinene	976	975	0.2	-	-	-	-	-
β-Pinene	979	978	10.2	7.3	19.8	22.6	11.5	8.1
6-Methyl 5 hepten-2-one	988	987	tr	-	-	-	-	-
Myrcene	991	991	0.2	0.2	0.3	0.3	0.2	0.2
n-Decane	1000	1000	tr	-	tr	tr	-	tr
β-Phellandrene	1026	1024	tr	-	-	-	-	tr

Compound	KI(1)	KI(2)	June	Sept.	Nov.	Jan.	April	June
Limonene	1030	1030	0.3	0.1	0.6	0.3	0.2	0.2
Z-*β*-Ocimene	1038	1040	0.3	0.1	0.3	tr	tr	0.2
E-*β*-Ocimene	1051	1050	5.1	1.6	2.0	1.3	2.3	4.0
γ-Terpinene	1061	1060	tr	tr	tr	-	-	0.1
Terpinolene	1089	1088	0.1	tr	tr	tr	-	0.1
n-Undecane	1100	1100	0.6	1.0	0.6	0.6	0.7	0.8
n-Nonanal	1104	1103	tr	-	-	-	-	tr
2,2,6-Trimethyl hepta-3,5-dione	1165	1162	tr	-	-	-	-	tr
Terpinen-4-ol	1178	1177	tr	0.1	tr	-	0.2	0.1
α-Terpineol	1191	1189	0.1	tr	0.1	tr	-	0.1
n-Decanal	1205	1205	1.0	0.8	1.4	0.3	2.2	1.3
n-Decanol	1273	1276	0.1	-	tr	-	-	0.2
δ-Elemene	1338	1337	0.2	0.2	tr	tr	0.1	0.2
α-Cubebene	1351	1348	0.7	1.6	1.1	1.0	0.7	0.8
α-Copaene	1374	1374	0.2	0.1	-	-	-	0.1
β-Patchoulene	1375	1376	0.5	1.1	0.9	1.0	0.8	0.5
β-Bourbonene	1384	1383	0.6	0.2	0.9	1.0	0.7	0.5
β-Elemene	1390	1390	4.6	7.1	7.0	5.9	6.4	4.4
iso-Italicene	1396	1395	0.2	0.5	0.5	0.3	0.2	0.3
α-Cedrene	1409	1409	0.7	1.2	0.6	0.3	0.6	0.5
(E)-Caryophyllene	1417	1416	7.4	6.0	1.7	1.6	3.6	6.2
β-Gurjunene	1432	1427	0.6	1.0	0.8	0.6	0.7	0.5
Aromadendrene	1443	1442	0.7	1.2	0.6	0.3	0.6	0.5
α-Humulene	1447	1447	0.2	0.4	0.3	0.3	0.2	0.2
allo-Aromadendrene	1452	1450	0.5	0.5	0.3	tr	0.2	0.5
Germacrene D Isomer	1462	1463	tr	0.1	-	-	-	0.1
γ-Muurolene	1476	1473	0.1	0.2	0.2	tr	tr	0.2
Germacrene D	1481	1478	13.2	11.0	3.9	2.5	6.0	11.6
β-Selinene	1482	1482	0.4	0.2	0.2	0.3	tr	0.1
α-Selinene	1494	1486	tr	0.1	-	-	-	0.2
β-Guaiene	1496	1492	1.6	1.4	0.9	0.6	1.0	1.4
α-Muurolene	1498	1497	0.1	0.1	tr	tr	-	0.1
(E,E)-*α*-Farnesene	1506	1498	0.4	0.6	0.6	0.3	0.7	0.4
γ-Cadinene	1512	1508	0.8	0.8	0.5	0.2	0.7	0.4
δ-Cadinene	1522	1521	0.6	0.5	0.3	0.3	0.5	0.5
Dimethyl-4-Isopropyl-Bicyclo(4,4,0)-1,4-Decadiene	1526	1526	0.4	1.2	2.2	1.0	1.7	0.5
α-Calacorene	1543	1543	0.1	tr	-	-	-	tr
E-Nerolidol	1563	1563	0.1	tr	-	-	-	0.1
Spathulenol	1575	1576	0.1	0.4	tr	tr	tr	0.1
Caryophyllene oxide	1581	1578	0.3	0.4	tr	tr	tr	0.3
Globulol	1587	1587	0.1	-	-	-	-	0.1

Compound	KI(1)	KI(2)	June	Sept.	Nov.	Jan.	April	June
Viridiflorol	1591	1592	0.3	0.2	0.3	tr	0.2	0.3
β-Copaen-4-*α*-ol	1596	1597	0.3	0.8	0.6	0.3	0.9	0.4
β-Oplopenone	1612	1607	0.3	0.6	0.3	0.3	0.7	0.4
epi-*α*-Cadinol	1639	1639	0.3	0.1	0.2	tr	tr	0.3
epi-*α*-Muurolol	1643	1642	0.2	0.1	-	-	-	0.2
δ-Cadinol	1653	1650	0.6	0.6	0.5	0.3	0.7	0.9
Z-*α*-Santalol	1670	1668	0.1	0.1	-	-	-	tr
epi-*α*-Bisabolol	1677	1674	0.2	0.2	0.1	tr	-	0.1
n-Heptadecane	1700	1700	tr	tr	-	-	-	tr
n-Octadecane	1800	1800	tr	-	-	-	-	-
n-Nonadecane	1900	1900	tr	-	-	-	-	-
n-Eicosane	2000	2000	tr	-	-	-	-	-
n-Heneicosane	2100	2100	tr	-	tr	-	-	-
n-Docosane	2200	2200	tr	-	-	-	-	-
n-Tricosane	2300	2300	tr	tr	-	tr	0.2	-
n-Tetracosane	2400	2400	tr	tr	tr	tr	0.2	-
n-Pentacosane	2500	2500	0.1	0.2	0.3	0.7	2.1	0.1
n-Hexacosane	2600	2600	tr	tr	-	tr	0.1	-
n-Heptacosane	2700	2700	0.1	0.4	tr	0.7	2.2	-
n-Octacosane	2800	2800	tr	-	-	tr	tr	-
n-Nonacosane	2900	2900	tr	0.1	-	tr	0.9	-

KI- Kovats retention index on a DB-5 column; **KI(1)** – KI for GC-MS; **KI(2)** – KI for GC
tr- trace amounts

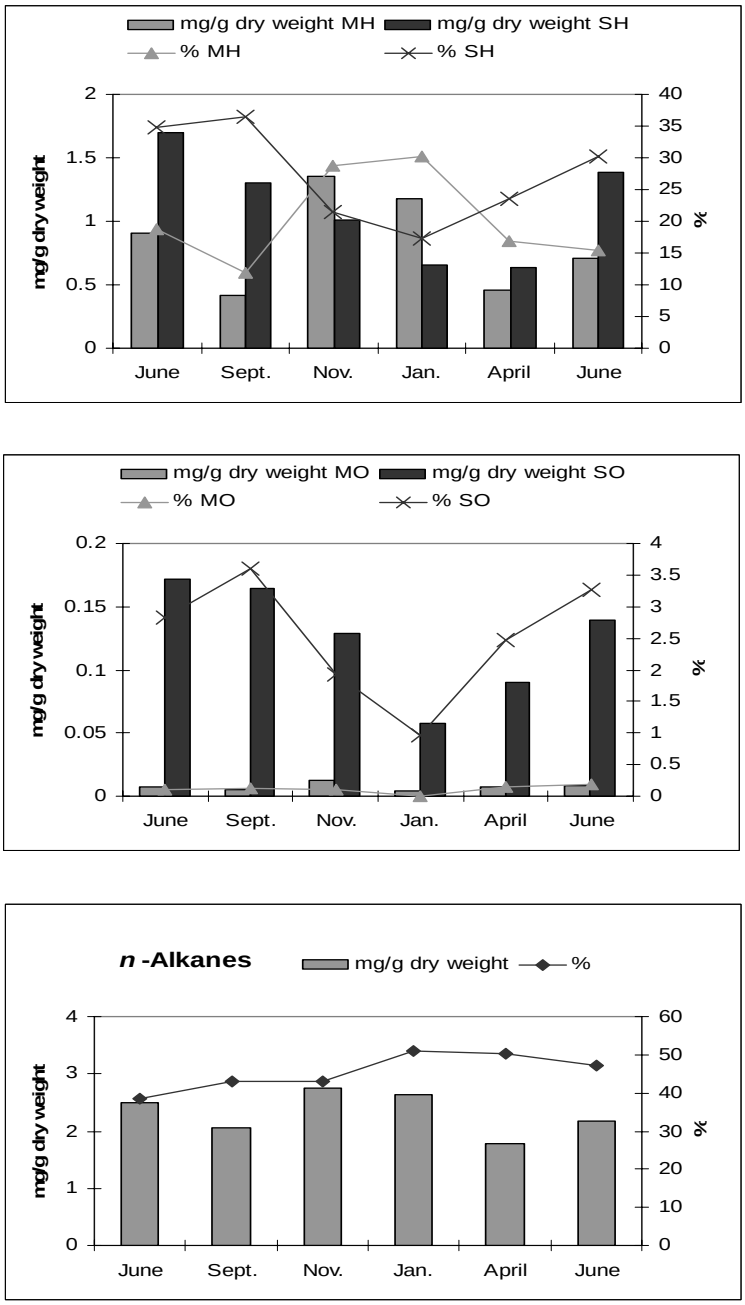


Figure 2 - Specific content (mg/g dry weight) and respective percentages of the five major classes of compounds present in the essential oils isolated from plants of *Hypericum undulatum* Schousboe ex Willd. cultivated in Braga (Portugal) and harvested at different times over one year. **MH**- Monoterpene Hydrocarbons; **MO**- Oxygenated Monoterpenes; **SH**- Sesquiterpene Hydrocarbons; **SO**- Oxygenated Sesquiterpenes

Figure 2 shows the specific and relative contents of the five main groups of *H. undulatum* EO constituents, namely monoterpene hydrocarbons (MH), oxygenated monoterpenes (MO), sesquiterpene hydrocarbons (SH), oxygenated sesquiterpenes (SO) and *n*-alkanes. *n*-Alkanes constitute the major group, corresponding to more than 38% of the total *H. undulatum* EO (Figure 2). *n*-Alkanes had already been considered the major group of

compounds in EO of aerial parts of *H. undulatum* growing wild in Portugal (Nogueira, 2002). Oxygenated sesquiterpenes (SO) and oxygenated monoterpenes constituted the minor groups of the EO of this species (Figure 2). The sesquiterpene hydrocarbons group gathered the most diverse number of compounds, 19 of which were common to all samples. During spring and summer, it was also the second most represented group, overcame by that of monoterpene hydrocarbons, in later autumn and winter. As shown in Figure 2, the variation of the relative contents of these two groups of compounds over the year was opposite. These results are coherent with those reported for *H. perforatum* (Chapter 4). As previously discussed such inverse correlation may reflect differential effects of the environmental conditions on the mevalonate independent pathway, mainly responsible by the biosynthesis of monoterpene compounds in the plastids, and on the acetate/mevalonate pathway leading to sesquiterpene biosynthesis in the cytosol (Davis *et al.*, 2000). Both environmental conditions and plant development might have favoured the sesquiterpene accumulation in spring leading to the increase in its content. During the colder and shorter photoperiod months, the lower growth rate of *H. undulatum* could have allowed a higher maturation of chloroplasts leading to an increase in the accumulation of monoterpenes. Hudaib and co-workers (2002) also found higher amounts of monoterpene hydrocarbons in *Thymus vulgaris* L. plants harvested in December after the vegetative cycle. On can speculate however that such variation may be due to the higher volatile characteristics of the monoterpene hydrocarbons and consequent increased release under the effects of the warmer days of spring and summer. Sesquiterpene hydrocarbons being less volatile are not so easily lost what in parallel with their increased synthesis and accumulation would explain their higher relative contents in June (Figure 2).

As shown in Table 2, the EO from *H. undulatum* plants were richer in hydrocarbon-like compounds rather than in the oxygenated ones.

Table 2 - Relative contents (%) of the five major classes of compounds present in the essential oils isolated from plants of *Hypericum undulatum* Schousboe ex Willd. cultivated in Braga (Portugal) and harvested at different times during over one year.

Compound group	June	Sept.	Nov.	Jan.	April	June
Monoterpene Hydrocarbons	18.8	12.0	28.7	30.2	17.1	15.4
Oxygenated Monoterpenes	0.1	0.1	0.1	<0.1	0.2	0.2
Sesquiterpene Hydrocarbons	34.3	36.4	21.1	16.7	23.7	30.1
Oxygenated Sesquiterpenes	2.8	3.5	1.9	1.0	2.5	3.3
n-Alkanes	38.4	43.8	43.0	50.7	50.7	47.2

Terpinen-4-ol and α-terpineol were the only oxygenated monoterpenes detected, with a proportion of less than 0.2% of the total EO contents. Low amounts of oxygenated monoterpenes in EO of *H. undulatum* had already been reported (Mathis *et al.*, 1964; Nogueira, 2002) as well as in the EO of *H. androsaemum* (Guedes *et al.*, 2003; Guedes *et al.*, 2004). Only 7 of the 12 identified oxygenated sesquiterpene compounds were common to the 6 samples. Among them, β-copaen-4-α-ol, β-oplopenone and δ-cadinol were the major ones, although their respective contributions to the total EO never had reached 1% along the year. Such low contents of oxygenated sesquiterpenes contrasts with the composition of the EO from *Hypericum perforatum* which showed high relative contents of oxygenated sesquiterpenes (Schwob *et al.*, 2004; Radusiene *et al.*, 2005). Indeed, EO from flowers and leaves of *H. perforatum* growing in Lithuania were dominated by oxygenated sesquiterpenes, corresponding to 36-70% of the total EO (Radusiene *et al.*, 2005), while Schowb *et al*. (2004) reported values of around 20% of oxygenated sesquiterpenes in the EO of *H. perforatum* at vegetative, floral budding, flowering and fruiting stages.

In all the studied EO samples, *n*-nonane was found the main compound of *H. undulatum* with a proportion of more than 37% of the total EO (Table 1). This result is in agreement with previous reports on the EO of this species (Mathis *et al.*, 1964; Nogueira, 2002). In EO of *Hypericum foliosum* as well as in EO of *H. caprifoliatum, n*-nonane was also present in high percentages (Santos *et al.*, 1999; Ferraz *et al.*, 2005). However, this feature does not seem to be a specificity of the *Hypericum* genus since *n*-nonane was in low amounts or even undetected in some other species. These are the cases of *H. hirsutum* (Gudžic *et al.*, 2007), *H. androsaemum* (Guedes *et al.*, 2003; Guedes *et al.*, 2004), *H. perforatum* (Schwob *et al.*, 2004; Radusiene *et al.*, 2005), and *H. brasiliense* (Abreu *et al.*, 2004). In the *H. undulatum* EO the lowest level of *n*-nonane was recorded in June of the first year of study and the highest one was recorded in January, in coherence with most of the variation in the level of the total *n*-alkanes (Figure 2). In April, there was increase in *n*-pentacosane and *n*-heptacosane contents. A complete series of *n*-alkanes from C_{17} to C_{29} was identified in the EO extracted from samples obtained in June of the first year of study. Among them, only *n*-pentacosane was common to all the 6 samples obtained over the year. A complete series of long chain *n*-alkanes was also previously described in *H. androsaemum* (Guedes *et al.*, 2003; Guedes *et al.*, 2004). Generally long chain *n*-alkanes are more abundant in plants than the short-chain ones since the first are found in plant epicuticular waxes (Kunst *et al.*, 2003). The role of short-chain alkanes in EO has not been reported yet. Interestingly, the high levels of alkanes from C_7 to C_{11} identified in oleoresins from several species of *Pinus* have been suggested to be a defence mechanism against insect and pathogen

attacks (Savage *et al.*, 1996). Biosynthetically, it was suggested that shorter and long chain length *n*-alkanes do not share all the same pathways being the shorter chain length fatty acids better used by the acyl reduction pathway (Millar *et al.*, 1999), while the long chain *n*-alkanes biosynthesis seems to involve fatty acid elongation followed by decarbonylation (Cheesbrough *et al.*, 1984). Additionally, short-chain alkanes accumulation is interesting because of the excellent combustion properties they share with petrochemical liquid hydrocarbons in gasoline and diesel formulations (Savage *et al.*, 1996).

The relative contents of the compounds that mostly contributed to the variation of the monoterpene hydrocarbons (*α*-pinene, *β*-pinene and *E*-*β*-ocimene) and sesquiterpene hydrocarbons (*β*-elemene, *(E)*-caryophyllene and germacrene D) are shown in Figure 3.

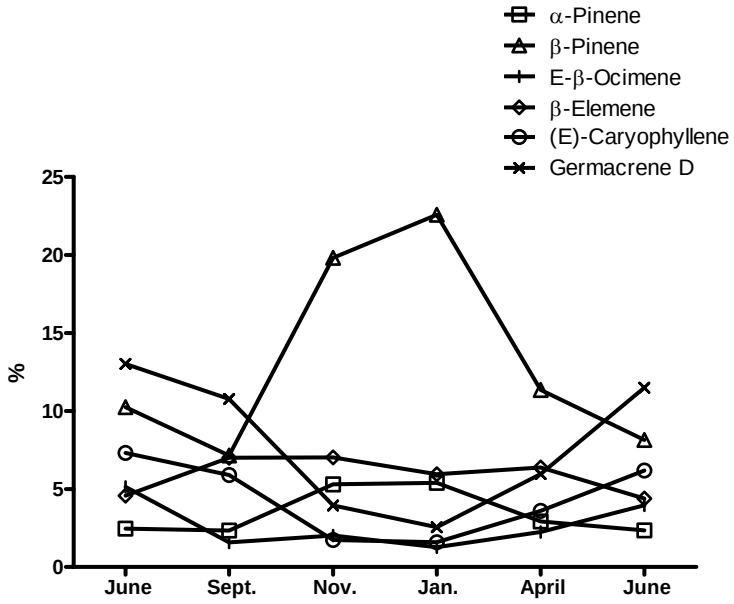


Figure 3 - Relative content (%) of the major monoterpene hydrocarbons and sesquiterpene hydrocarbons of the essential oils from plants of *Hypericum undulatum* Schousboe ex Willd. cultivated at Braga (Portugal) and harvested at different times over one year

From the 5 most represented compounds, 3 of them (*n*-nonane, *β*-pinene and germacrene D) were common to all samples. *β*-Elemene constituted also one of the 5 most represented compounds in the EO of *H. undulatum*, except for plants harvested in June of the first year of study, in which it was substituted by *E*-*β*-ocimene. During the flowering period (May-June) the content of *E*-*β*-ocimene increased two-fold comparing to the other phases of the phenological cycle. Accordingly, Dudareva and co-workers (2003) found high amounts of this monoterpene hydrocarbon in flowers of snapdragon. Between September of the first year of

study and June of the second one the variation of the relative content of germacrene D and *(E)*-caryophyllene was opposite to that of the β-pinene and α-pinene. This variation pattern was consistent with the above mentioned variation of sesquiterpene- and monoterpene-hydrocarbons groups. The increase in the relative content of the total monoterpene hydrocarbons in November and January was essentially due to the increases in β-pinene accumulation and, in lower extent, α-pinene (Figure 3). In these months, β-pinene content reached about 20% of the total EO content and α-pinene was one of the 5 most represented compounds. The accumulation of these 2 monoterpene hydrocarbons, was already demonstrated to be related to leaf development of *Mentha piperita* (Gershenzon *et al.*, 2000).

To conclude, among the numerous EO components described here in *H. undulatum* aerial parts, the *n*-alkanes group, and particularly the short chain alkane *n*-nonane, are predominant. Additionally, our results report a seasonal variation in the composition of the *H. undulatum* EO, correlated with variations in the contents of some of its compounds.

Acknowledgements

This work was sponsored by the Grant SFRH/BD/13283/2003

References

Abreu, I., M. Reis, A. Marsaioli & P. Mazzafera (2004). Essential oil composition of *Hypericum brasiliense* choise. *Flavour and Fragrance Journal*(19), 80-82.

Çakir, A., M. E. Duru, M. Harmandar, R. Ciriminna, S. Passannanti & F. Piozzi (1997). Comparison of the volatile oils of *Hypericum scabrum* L. and *Hypericum perforatum* L. from Turkey. *Flavour and Fragrance Journal 12*(4), 285-287.

Cakir, A., S. Kordali, H. Kilic & E. Kaya (2005). Antifungal properties of essential oil and crude extracts of *Hypericum linarioides* Bosse. *Biochemical Systematics and Ecology 33*(3), 245-256.

Cheesbrough, T. M. & P. E. Kolattukudy (1984). Alkane biosynthesis by decarbonylation of aldehydes catalyzed by a particulate preparation from *Pisum sativum*. *Proceedings of the National Academy of Sciences of the United States of America 81*(21), 6613-6617.

Curado, M. A., C. B. A. Oliveira, J. G. Jesus, S. C. Santos, J. C. Seraphin & P. H. Ferri (2006). Environmental factors influence on chemical polymorphism of the essential oils of *Lychnophora ericoides*. *Phytochemistry 67*(21), 2363-2369.

Davis, E. M. & R. Croteau (2000). Cyclization Enzymes in the Biosynthesis of Monoterpenes, Sesquiterpenes and Diterpenes. *Topics in Current Chemistry 209*, 54-95.

Ferraz, A. B. F., R. P. Limberger, S. A. L. Bordignon, G. L. v. Poser & A. T. Henriques (2005). Essential oil composition of six *Hypericum* species from southern Brazil. *Flavour and Fragrance Journal 20*(3), 335-339.

Ferreira, A., C. Proença, M. L. M. Serralheiro & M. E. M. Araújo (2006). The *in vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Portugal. *Journal of Ethnopharmacology 108*(1), 31-37.

Gershenzon, J., M. E. McConkey & R. B. Croteau (2000). Regulation of Monoterpene Accumulation in Leaves of Peppermint. *Plant Physiology 122*, 205-214.

Gudžić, B., D. Djokovic, V. Vajs, R. Palić & G. Stojanovic (2002). Composition and antimicrobial activity of the essential oil of *Hypericum maculatum* Crantz. *Flavour and Fragrance Journal 17*(5), 392-394.

Gudžic, B., A. Šmelcerović, S. Dordević, N. Mimica-Dukić & M. Ristić (2007). Essential oil

composition of *Hypericum hirsutum* L. *Flavour and Fragrance Journal 22*(1), 42-43.

Guedes, A. P., L. R. Amorim, A. Vicente & M. Fernandes-Ferreira (2004). Variation of the essential oil content and composition in leaves from cultivated plants of *Hypericum androsaemum* L. *Phytochemical Analysis 15*, 146-151.

Guedes, A. P., L. R. Amorim, A. M. S. Vicente, G. Ramos & M. Fernandes-Ferreira (2003). Essential Oils from Plants and *in Vitro* Shoots of *Hypericum androsaemum* L. *J. Agric. Food Chem. 51*(5), 1399-1404.

Hudaib, M., E. Speroni, A. M. Di Pietra & V. Cavrini (2002). GC/MS evaluation of thyme (*Thymus vulgaris* L.) oil composition and variations during the vegetative cycle. *Journal of Pharmaceutical and Biomedical Analysis 29*(4), 691-700.

Kunst, L. & A. L. Samuels (2003). Biosynthesis and secretion of plant cuticular wax. *Progress in Lipid Research 42*, 51-80.

Mathis, C. & G. Ourisson (1964). Étude chimio-taxonomique du genre *Hypericum* : II. Identification de constituants de diverses huiles essentielles d'*Hypericum*. *Phytochemistry 3*(1), 115-131.

Millar, A. A., S. Clemens, S. Zachgo, E. M. Giblin, T. D.C. & L. Kunst (1999). *CUT1*, an *Arabidopsis* gene required for cuticular wax biosynthesis and pollen fertility, encodes a very-long-chain fatty acid condensing enzyme. *The Plant Cell 11*, 825-838.

Nogueira, T. (2002). O género *Hypericum* L. em Portugal Continental. Contribuição para o estudo quimiotaxonómico. Instituto Superior de Agronomia. Lisboa, Universidade Técnica de Lisboa. **PhD. Thesis**.

Pavlović, M., O. Tzakou, P. V. Petrakis & M. Couladis (2006). The essential oil of *Hypericum perforatum* L., *Hypericum tetrapterum* Fries and *Hypericum olympicum* L. growing in Greece. *Flavour and Fragrance Journal 21*(1), 84-87.

Radusiene, J., A. Judzentiene & G. Bernotiene (2005). Essential oil composition and variability of *Hypericum perforatum* L. growing in Lithuania. *Biochemical Systematics and Ecology 33*(2), 113-124.

Santos-Gomes, P. C. & M. Fernandes-Ferreira (2001). Organ and season-dependent variation in the essential oil composition of *Salvia officinalis* L. cultivated in two different sites. *Journal of Agricultural and Food Chemistry 49*, 2908-2916.

Santos, P. A. G., A. C. Figueiredo, J. G. Barroso, L. G. Pedro & J. J. C. Scheffer (1999). Composition of the essential oil of *Hypericum foliosum* Aiton from five Azorean islands. *Flavour and Fragrance Journal 14*(5), 283-286.

Savage, T. J., B. S. Hamilton & R. Croteau (1996). Biochemistry of Short-Chain Alkanes. Tissue-Specific Biosynthesis of *n*-Heptane in *Pinus jeffreyi*. *Plant Physiology 110*, 179-186.

Schwob, I., J.-M. Bessiere, V. Masotti & J. Viano (2004). Changes in essential oil composition in Saint John's wort (*Hypericum perforatum* L.) aerial parts during its phenological cycle. *Biochemical Systematics and Ecology 32*(8), 735-745.

Schwob, I., J.-M. Bessière & J. Viano (2002a). Composition of the essential oils of *Hypericum perforatum* L. from southeastern France. *Comptes Rendus Biologies 325*(7), 781-785.

Schwob, I., J. M. Bessiere, M. Dherbomez & J. Viano (2002b). Composition and antimicrobial activity of the essential oil of *Hypericum coris*. *Fitoterapia 73*(6), 511-513.

Seabra, R. M., M. H. Vasconcelos, M. A. C. Costa & A. C. Alves (1992). Phenolic compounds from *Hypericum perforatum* and *Hypericum undulatum*. *Fitoterapia 68*, 473-474.

Seabra, R. M., M. H. Vasconselos & A. C. Alves (1991). Flavonoid sulphates from *Hypericum undulatum*. *Rev. Port. Farm. 12*, 16-18.

Zheljazkov, V. D., L. E. Craker & B. Xing (2006). Effects of Cd, Pb, and Cu on growth and essential oil contents in dill, peppermint, and basil. *Environmental and Experimental Botany 58*(1-3), 9-16.

Essential oils from leaves, stems and ripened seed capsules of *Hypericum undulatum* Schousboe ex Willd

Ana P. Guedes and M. Fernandes-Ferreira

CITAB- Centro de Investigação e de Tecnologias Agro-Alimentares e Biológicas, Department of Biology, University of Minho, Campus de Gualtar, 4720-057 Braga, Portugal

Abstract

The composition of the essential oils (EO) from fresh leaves, stems, ripened seed capsules and flowers of *Hypericum undulatum* (Clusiaceae) obtained by hydrodistillation was determined by GC and GC-MS. The highest yield, expressed in terms of percentage of EO by biomass dry weight, was obtained from leaves (0.9%), followed by ripened seed capsules (0.8%), flowers (0.6%), and stems (0.3%). The most complex composition profile was that of flowers (97 compounds) followed by ripened seed capsules, leaves and stems with 85, 83 and 47 compounds, respectively. The *n*-alkanes group, from which *n*-nonane was the dominant compound, corresponded to 84%, 42%, 37% and 24% of the EO of stems, flowers, ripened seed capsules, and leaves, respectively. *n*-Alkanes constituted the major group of EO constituents in the *H. undulatum* organs with the exception for leaves where the sesquiterpene hydrocarbons group predominated (59%). A complete series of *n*-alkanes, from C_{22} to C_{29}, was identified in leaves whereas in stems, the *n*-heptacosane and *n*-nonacosane were the only long-chain *n*-alkanes detected. Although the EO from leaves contained a broad range of sesquiterpene hydrocarbons, the dominant compound was *n*-nonane (21.6%). The absolute content of this *n*-alkane corresponded to around 0.2% of the dried biomass of leaves and around 0.3% of the dried biomass of stems, flowers and ripened seed capsules of *H. undulatum*. The second major group of compounds in the EO of leaves was that of *n*-alkanes while in EO of stems, flowers and ripened seed capsules was that of sesquiterpene hydrocarbons which represented around 10%, 41% and 28%, respectively.

Keywords: H. undulatum, essential oils, leaves, flowers, ripened seed capsules, *n*-alkanes, *n*-nonane, sesquiterpenes

1- Introduction

Hypericum undulatum (Clusiaceae) along with *Hypericum androsaemum* L. and *Hypericum perforatum* L., is one of the three species of the *Hypericum* genus most used in Portuguese folk medicine. This species usually grows in wet places and in the riverside edges being also found in United Kingdom, Spain, France and Italy (Nogueira, 2002). Decoctions of its flowers are used in traditional medicine for the treatment of migraine, and in bladder and gall bladder ailments. These decoctions are also believed to have a renal antispasmodic and hepatic protector effect (Ferreira *et al.*, 2006). According to some authors, *H. undulatum* can also help in preventing or alleviating patients suffering from Alzheimer's disease, once its essential oils (EO), ethanolic extracts and decoctions showed both high antioxidant activity and acetylcholinesterase inhibitory capacity (Ferreira *et al.*, 2006). Such type of extracts are mainly composed of phenolic compounds as demonstrated by some authors who identified hypericin, quercetin, quercetin sulphate, rutin, mangiferin and chlorogenic acid in alcoholic extracts of leaves and aerial parts of *H. undulatum* (Seabra *et al.*, 1991; Seabra *et al.*, 1992).

In the past few years, there has been a growing interest in the secondary metabolite production of species of *Hypericum* genus, mainly due to the current widespread use of *H. perforatum* in the treatment of mild to moderate depression. The increasing interest for *H. perforatum* phenolic extracts might be related to their antidepressant properties. Studies on the essential oils (EO) of several *Hypericum* species have been reported, namely of *H. perforatum* (Smelcerovic *et al.*, 2004; Ferraz *et al.*, 2005; Radusiene *et al.*, 2005) and *H. androsaemum* (Guedes *et al.*, 2003; Guedes *et al.*, 2004) some of which describing some of their biological effects namely antimicrobial activities (Toker *et al.*, 2006; Saroglou *et al.*, 2007). Studies on *H. undulatum* are however scant, namely in what respects the EO. To our knowledge the most complete study on *H. undulatum* EO reported up to now, was that of Nogueira (2002) who identified 22 compounds in hydrodistillates from aerial parts of plants from this species.

Given the properties and uses reported to *H. undulatum* we consider of utmost importance a better knowledge on the composition of the extracts and fractions of this species, namely their EO. The present work reports the contents and composition profiles of the EO from leaves, stems, ripened seed capsules and flowers of *H. undulatum* growing in Nature.

2- Material and Methods

2.1- Plant material

H. undulatum plants wild growing in Braga (Northern Portugal) were randomly pruned in September and subsamples of leaves, stems and ripened seed capsules were collected for the EO extraction. The flowers used in the extraction of EO were pruned before, in June, in the same field.

2.2- Sampling and hydrodistillation

Subsamples of 10 g of fresh biomass of leaves, stems and flowers, as well as 5g of fresh biomass of ripened seed capsules of *H. undulatum* were submitted to hydrodistillation in a Clevenger type apparatus over 1 h in the presence of a *n*-hexane (1.0 mL) solution containing 5α-cholestane (1mg/mL), for the retention of the EO components. The dry weight of the plant material was determined after drying (60ºC, 72h) in a drying stove.

Further analyses of the EO were performed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) as described in Chapter 4 (pages 118-119).

3- Results and Discussion

The EO yields obtained by hydrodistillation of the different organs of *H. undulatum* plants ranged from a minimum of 3.4 mg/g of biomass dry weight to a maximum of 8.7 mg/g of biomass dry weight determined for stems and leaves, respectively (Figure 1). Ripened seed capsules and flowers gave yields of 8.1 and 6.5 mg/g of biomass dry weight, respectively (Figure 1).

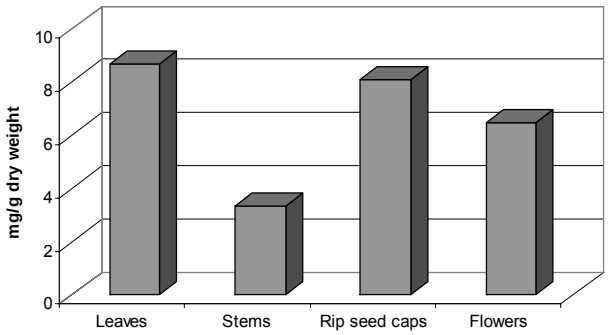


Figure 1 - Essential oil contents (mg/g of biomass dry weight) in leaves, stems, ripened seed capsules (harvested in September) and flowers (harvested in June) of *Hypericum undulatum* Schousboe ex Willd growing in Braga, Northern of Portugal.

Despite being the organ with the highest EO yield, leaves contained a relatively narrow range of compounds compared to the flowers. More than one hundred compounds were found, distributed by the four organs, 89% of which were identified (Table 1). Only forty of all compounds were present in the whole plant including the stems which was the organ with the lowest number of EO constituents (47).

Table 1 - Composition of the essential oils from leaves, stems, ripened seed capsules (harvested in September) and flowers (harvested in June) of *Hypericum undulatum* Schousboe ex Willd. growing in Braga, Northern of Portugal.

COMPOUND	KI	Leaves		Stems		Rip seed caps		Flowers	
		µg/g dry weight	%	µg/g dry weight	%	µg/g dry weight	%	µg/g dry weight	%
n-Octane	800	9.4	0.1	32.6	1.0	71.5	0.8	44.8	0.6
(E)-2-Hexenal	854	47.7	0.4	1.2	tr	-	-	14.6	0.2
n-Hexanol	863	6.4	0.1	6.7	0.2	569.4	6.5	256.6	3.5
n-Nonane	900	2247.9	21.6	2823.7	81.7	2685.9	30.4	2784.7	38.2
α-Thujene	929	2.0	tr	1.3	tr	-	-	0.5	tr
α-Pinene	936	79.0	1.1	17.9	0.7	9.3	0.2	11.5	0.2
Camphene	950	-	-	-	-	5.2	0.1	4.6	0.1
2,6-Dimethyloctane	972	5.7	0.1	7.6	0.2	696.9	8.0	166.5	2.3
Sabinene	975	4.1	tr	-	-	-	-	1.4	0.1
β-Pinene	978	312.8	4.0	74.8	2.9	11.1	0.2	42.2	0.8
6-Methyl-5-hepten-2-one	987	8.9	0.1	-	-	51.2	0.6	7.5	0.1
Myrcene	991	10.7	0.1	3.1	0.1	21.3	0.3	7.5	0.1
n-Decane	1000	4.5	tr	2.8	tr	15.9	0.2	4.0	0.1
n-Octanal	1007	-	-	-	-	6.0	tr	3.1	0.1
α-Terpinene	1018	-	-	-	-	-	-	2.2	0.1
Limonene	1030	11.6	0.1	1.6	tr	6.1	0.2	8.5	0.1
Z-β-Ocimene	1039	15.2	0.2	4.3	0.2	1.2	tr	3.2	0.1
E-β-Ocimene	1050	144.5	1.9	22.6	0.9	3.1	tr	37.8	0.7
γ-Terpinene	1060	1.9	tr	-	-	2.5	tr	4.6	0.1
2-Methyl-decane	1071	-	-	-	-	18.6	0.2	5.5	0.1
trans-Linalool oxide (furanoid)	1073	7.1	0.1	-	-	33.4	0.3	6.5	0.1
2,6-Dimethyl-3,5-Heptanodione	1078	-	-	-	-	42.2	0.5	3.7	0.1
Terpinolene	1087	3.7	tr	-	-	9.5	0.2	12.0	0.2
n-Undecane	1100	113.6	1.1	35.3	1.0	335.4	3.8	190.4	2.6
n-Nonanal	1103	-	-	-	-	9.3	0.2	6.5	0.1
2,2,6-Trimethyl hepta-3,5-dione	1162	6.7	0.1	2.5	tr	27.0	0.3	8.0	0.1

COMPOUND	KI	Leaves		Stems		Rip seed caps		Flowers	
		µg/g dry weight	%	µg/g dry weight	%	µg/g dry weight	%	µg/g dry weight	%
Terpinen-4-ol	1176	4.0	tr	-	-	-	-	2.6	0.1
α-Terpineol	1189	10.6	0.1	-	-	-	-	8.2	0.1
n-Decanal	1205	54.0	0.5	-	-	2.8	tr	7.5	0.1
n-Decanol	1277	5.4	tr	-	-	-	-	2.3	tr
n-Tridecane	1300	-	-	-	-	5.6	tr	-	-
δ-Elemene	1336	51.8	0.7	1.7	tr	-	-	8.1	0.1
α-Cubebene	1348	81.3	1.0	12.3	0.5	431.4	6.6	250.7	4.6
SH	1370	10.4	0.1	-	-	6.9	0.2	4.2	0.1
α-Ylangene	1368	44.0	0.6	-	-	3.4	0.1	2.9	0.1
α-Copaene	1374	60.1	0.7	-	-	420.5	6.3	168.2	3.1
β-Patchoulene	1376	-	-	8.4	0.2	-	-	4.3	0.1
iso-Longifolene	1378	51.8	0.7	-	-	-	-	41.8	0.7
β-Bourbonene	1382	286.1	3.8	12.9	0.5	21.5	0.3	177.3	4.5
β-Elemene	1390	432.6	5.3	70.5	2.7	49.3	0.8	6.1	0.1
iso-Italicene	1394	23.2	0.3	5.3	0.2	112.2	1.7	52.2	1.3
SH	1395	-	-	-	-	13.0	0.2	9.7	0.1
SH	1401	38.2	0.5	-	-	2.3	tr	3.3	0.1
α-Cedrene	1408	411.2	5.7	7.1	0.2	9.5	0.2	125.0	0.5
(E)-Caryophyllene	1416	779.0	9.6	50.5	1.9	145.2	2.2	282.1	6.6
β-Gurjunene	1427	63.6	0.8	7.8	0.2	252.7	3.9	76.3	1.8
SH	1435	39.2	0.6	-	-	7.9	0.2	21.0	0.3
Aromadendrene	1442	92.9	1.2	7.2	0.2	5.9	0.2	26.6	0.6
α–Humulene	1447	47.0	0.6	3.1	0.1	5.8	0.2	10.9	0.2
allo-Aromadendrene	1450	61.0	0.8	4.5	0.2	7.2	0.2	14.5	0.2
SH	1458	31.1	0.4	-	-	2.4	tr	18.6	0.5
Germacrene D isomer	1463	15.2	0.2	2.9	0.1	5.7	0.1	14.9	0.2
γ-Gurjunene	1467	-	-	-	-	4.2	tr	14.7	0.2
γ-Muurolene	1475	460.8	6.4	2.6	0.2	30.7	0.5	1.3	0.1
γ-Curcumene	1483	-	-	-	-	5.4	0.1	22.9	0.4
Germacrene D	1478	1045.5	12.9	53.7	2.1	64.8	1.0	421.2	7.7
SH	1480	-	-	-	-	-	-	31.2	0.4
β-Selinene	1482	23.5	0.3	3.8	0.2	5.5	0.2	5.8	0.1
α-Selinene	1485	59.8	0.8	-	-	44.1	0.7	5.7	0.1
Valencene	1487	-	-	-	-	-	-	36.9	0.7
β-Guaiene	1492	121.3	1.5	8.7	0.2	-	-	49.0	0.9

COMPOUND	KI	Leaves		Stems		Rip seed caps		Flowers	
		µg/g dry weight	%	µg/g dry weight	%	µg/g dry weight	%	µg/g dry weight	%
α-Muurolene	1497	26.5	0.4	-	-	11.8	0.2	25.3	0.5
(E,E)-*α*-Farnesene	1498	72.9	0.9	5.4	0.2	6.5	0.2	24.5	0.5
SH	1499	-	-	-	-	-	-	8.9	0.1
SH	1503	-	-	-	-	-	-	106.4	1.5
γ-Cadinene	1508	87.8	1.1	1.8	tr	89.7	1.4	29.8	0.5
δ-Cadinene	1521	57.5	0.7	3.8	0.2	30.9	0.5	67.0	1.2
Dimethyl-4-Isopropyl-Bicyclo-(4,4,0)-1,4-Decadiene	1526	28.8	0.4	2.5	tr	2.9	tr	39.4	0.5
trans-Cadina-1(2),4-diene	1530	-	-	-	-	-	-	8.0	0.1
α-Cadinene	1535	5.3	0.1	-	-	2.1	tr	5.1	0.1
SH	1538	-	-	-	-	27.5	0.4	3.0	0.1
α-Calacorene	1543	8.9	0.1	0.7	tr	8.5	0.2	4.2	0.1
γ-Elemene (?)	1546	2.9	tr	-	-	12.6	0.2	3.5	0.1
trans-Nerolidol	1562	10.7	0.1	-	-	175.0	2.0	17.5	0.2
SO	1568	39.5	0.4	-	-	15.1	0.2	-	-
cis-3-Hexenyl Benzoate	1572	34.9	0.3	-	-	15.2	0.2	13.7	0.1
SO	1569	-	-	-	-	8.8	0.2	-	-
Spathulenol	1575	65.0	0.6	2.7	tr	30.1	0.3	9.2	0.1
Caryophyllene oxide	1577	30.7	0.3	5.1	0.2	463.6	5.2	27.5	0.3
Globulol	1587	16.3	0.1	-	-	12.5	0.2	4.5	0.1
Viridiflorol	1591	67.1	0.6	2.5	0.1	11.8	0.2	9.6	0.1
SO	1585	-	-	-	-	-	-	7.8	0.1
β-Copaen-4-*α*-ol	1597	50.8	0.5	5.3	0.2	16.7	0.2	35.6	0.5
α-Guaiol	1598	12.0	0.1	2.6	0.1	14.1	0.2	20.0	0.2
Tetradecanal	1605	-	-	-	-	24.8	0.3	-	-
β-Oplopenone	1606	76.9	0.8	4.1	0.1	71.9	0.8	38.7	0.5
1-*epi*-Cubenol	1628	3.4	tr	-	-	54.5	0.7	15.8	0.2
epi-*α*-Cadinol	1638	27.1	0.3	0.7	tr	51.6	0.5	66.9	0.9
epi-*α*-Muurolol	1642	22.3	0.2	-	-	81.5	0.9	55.0	0.7
δ-Cadinol	1650	85.2	0.8	5.4	0.2	18.7	0.2	93.1	1.3
Z-*α*-Santalol	1667	14.8	0.1	-	-	53.6	0.6	10.2	0.1
epi-*α*-Bisabolol	1674	25.7	0.2	-	-	18.5	0.2	16.0	0.2
n-Heptadecane	1700	6.4	0.1	-	-	11.8	0.2	4.8	0.1
n-Octadecane	1800	2.8	tr	-	-	-	-	-	-
Hexahydrofarnesyl acetone	1849	-	-	-	-	11.0	0.1	11.0	0.1
n-Nonadecane	1900	-	-	-	-	5.6	tr	6.2	0.1

COMPOUND	KI	Leaves		Stems		Rip seed caps		Flowers	
		µg/g dry weight	%	µg/g dry weight	%	µg/g dry weight	%	µg/g dry weight	%
n-Heneicosane	2100	-	-	-	-	12.5	0.2	3.8	0.1
n-Docosane	2200	3.1	tr	-	-	-	-	-	-
n-Tricosane	2300	6.0	0.1	-	-	7.7	0.1	10.8	0.1
n-Tetracosane	2400	7.5	0.1	-	-	-	-	1.2	tr
n-Pentacosane	2500	45.0	0.4	-	-	11.6	0.2	7.8	0.1
n-Hexacosane	2600	6.6	0.1	-	-	2.7	tr	-	-
n-Heptacosane	2700	61.3	0.6	1.7	tr	37.7	0.4	-	-
n-Octacosane	2800	3.3	tr	-	-	8.9	0.2	-	-
n-Nonacosane	2900	17.6	0.2	1.4	tr	61.8	0.8	1.7	tr
Monoterpene Hydrocarbons		586.9	7.5	125.5	4.8	69.3	1.0	136.5	2.5
Oxygenated Monoterpenes		21.7	0.2			33.4	0.3	17.3	0.2
Sesquiterpene Hydrocarbons		4592.4	59.0	274.4	10.2	1834.6	28.2	2193.4	41.1
Oxygenated Sesquiterpenes		547.4	5.2	28.3	0.9	1098.3	12.2	427.5	5.6
***n*-Alkanes**		2534.9	24.4	2897.5	83.6	3274.5	37.2	3060.2	42.0
Others		375.0	3.8	20.5	0.5	1795.7	21.0	627.9	8.7

KI- Kovats retention index on a DB-5 column; **tr-** trace amounts
Rip seed caps- Ripened seed capsules

Some constituents of *H. undulatum* EO were associated to a given type of organ. Those are the cases of *n*-octadecane and *n*-docosane, only identified in leaves, *n*-tridecane and tetradecanal, present only in ripened seed capsules, and *α*-terpinene, valencene and *trans*-cadina-1(2),4-diene, exclusive constituents from the EO of flowers. The EO composition of *H. undulatum* had already been studied by Nogueira (2002) who identified a maximum of 22 compounds. *n*-Alkanes was the major group found in stems, ripened seed capsules and flowers, while in leaves, the sesquiterpene hydrocarbons predominated (Table 1). The other constituents distributed by monoterpene hydrocarbons (MH), oxygenated monoterpenes (MO) and oxygenated sesquiterpenes (SO) were present in the different types of organs, excepting for stems that lacked oxygenated monoterpenes (MO).

n-Alkanes represented more than 80%, 37% and 40% of the total EO of stems, ripened seed capsules and flowers, respectively (Figure 2). The predominance of this group was mainly due to the high amount of *n*-nonane, the major compound in the four types of organs (Figure 3). In leaves, the *n*-alkanes constituted the second group most represented accounting for 24% of the total EO. Nogueira (2002) had already reported the predominance of alkanes in the EO of

H. undulatum, with *n*-nonane, 3-methyl-nonane and 2-methyl-octane, contributing to the abundance of this group. In the study here reported, 2-methyl-octane and 3-methyl-nonane were not detected. However, 2,6-dimethyloctane was identified as the second major compound of the EO from ripened seed capsules. The group of alkanes was also present, in significant percentage, in the EO of *Hypericum perforatum* and *Hypericum tetrapterum* grown in Greece even though not as the major group (Pavlović *et al.*, 2006). However, the authors reported that in the EO of *Hypericum olympicum* the alkanes group accounted only for 9% of the total EO.

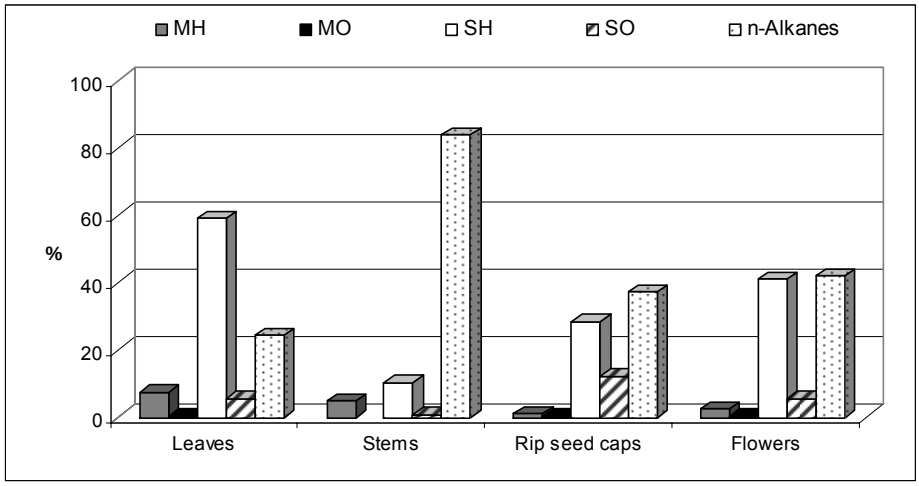


Figure 2 – Relative content (%) of the main groups of compounds in the essential oils from leaves, stems, ripened seed capsules (harvested in September) and flowers (harvested in June) of *Hypericum undulatum* Schousboe ex Willd growing in Braga, Northern of Portugal. **MH**- Monoterpene Hydrocarbons; **MO**- Oxygenated Monoterpenes; **SH**- Sesquiterpene Hydrocarbons; **SO**- Oxygenated Sesquiterpenes.

The presence of a complete series of *n*-alkanes, between C_{22} and C_{29}, was found in hydrodistillates of *H. undulatum* leaves (Table 1). In hydrodistillates from ripened seed capsules, a series of *n*-alkanes from C_{17} to C_{29} was identified, with exception of *n*-docosane and *n*-tetracosane. *n*-Heptacosane and *n*-nonacosane were the only long-chain *n*-alkanes common to the four type of organs. These *n*-alkanes are usually found in plant epicuticular waxes along with other long-chain aliphatic compounds (Kunst *et al.*, 2003). The presence of series of *n*-alkanes, from C_{18} to C_{28} and from C_{20} to C_{29}, in the EO of *Hypericum androsaemum* had already been reported (Guedes *et al.*, 2003; Guedes *et al.*, 2004). A longer series (C_{19}-C_{33}) was detected by Stojanovic *et al.* (2003) in EO of *Hypericum perforatum*. The same authors identified almost all the compounds from the same series (C_{19}-C_{33}) in the EO of *H. maculatum* and *H. olympicum*. *n*-Alkanes with shorter chains, *n*-octane, *n*-decane and *n*-undecane, were

identified in all organs. The predominance of *n*-nonane in the EO of this species (Figure 3) was also reported by Mathis and Ourisson (1964) and Nogueira (2002). This compound was among the major ones in the EO of *Hypericum foliosum* (Santos *et al.*, 1999), *H. caprifoliatum* (Ferraz *et al.*, 2005) and of other species of the genus, although in a great range of contents, as reported for *H. carinatum*, *H. myrianthum*, *H. polyanthemum, H. ternum* and *H. connatum* (Ferraz *et al.*, 2005) as well as *H. perforatum*, *H. tetrapterum* and *H. olympicum* (Pavlović *et al.*, 2006). Depending on the season, this compound can range greatly in the EO of *Hypericum* plants, as reported for *H. androsaemum* (0.6-9.1%) (Guedes *et al.*, 2004) and, in some cases, as in *H. perforatum*, it is present in trace amounts (Radusiene *et al.*, 2005) or even absent, as in the EO of *H. scabrum* and *H. perforatum* grown in Turkey (Çakir *et al.*, 1997) and Uzbekistan (Baser *et al.*, 2002), *H. tormentosum* and *H. humifusum* (Nogueira *et al.*, 1998), *H. perforatum* grown in France (Schwob *et al.*, 2002b; Schwob *et al.*, 2004), *H. rumeliacum* (Couladis *et al.*, 2003), and *H. perfoliatum* (Touafek *et al.*, 2005).

Sesquiterpene hydrocarbons, constituted the major group of compounds in EO of *H. undulatum* leaves being the second most represented in the EO of the other three *H. undulatum* organs accounting for 60% in leaves, 41 % in flowers, 28% in ripened seed capsules and 10% in stems. Nogueira (2002) had already reported this group as the second major one in the EO from *H. undulatum* with relative amounts ranging from 10% to 22%. Sesquiterpene hydrocarbons was also reported as the predominant group in EO of plants and *in vitro* shoots of *H. androsaemum* (Guedes *et al.*, 2003; Guedes *et al.*, 2004) as well as in the EO of *H. perforatum*, *H. tetrapterum* and *H. olympicum* (Pavlović *et al.*, 2006). Other authors showed also that the composition of the EO from aerial parts of *H. perforatum* varies during the phenological cycle, with predominance of the sesquiterpene hydrocarbons at the floral budding, flowering and fruiting phases (Schwob *et al.*, 2004).

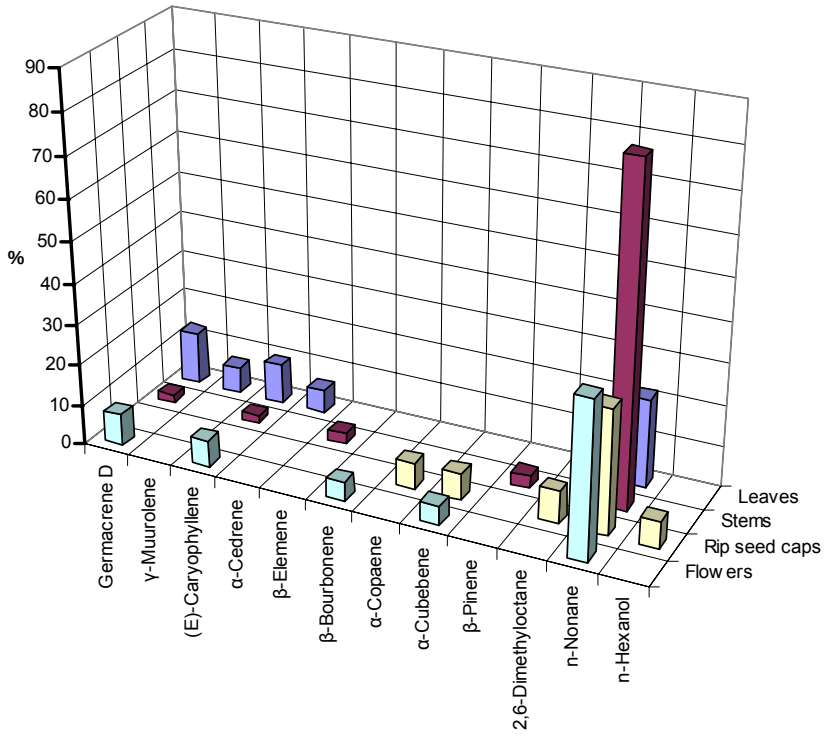


Figure 3 – Percentage content of the five major compounds of the essential oils from leaves, stems, ripened seed capsules (harvested in September) and flowers (harvested in June) of *Hypericum undulatum* Schousboe ex Willd growing in Braga, Northern of Portugal.

The relative amount of the total monoterpene hydrocarbons in the EO of *H. undulatum* leaves (7.5%) was higher than that found in the EO of each one of the other type of organs. This group was the major one in *H. rumeliacum* (Couladis *et al.*, 2003) as well as in *H. perforatum* and *H. scabrum* grown in Turkey (Çakir *et al.*, 1997), being also well represented in the EO of *H. foliosum* (Santos *et al.*, 1999). From all the monoterpene hydrocarbons detected and identified, only 6 were common to the four types of organs. *β*-Pinene, was the main monoterpene hydrocarbon, accounting for 2.9% of the total EO of stems and 4% of the total EO content from the leaves. This compound, however, did not reach 1% of the total EO of ripened seed capsules and flowers (Figure 3). Nogueira (2002) reported *β*-pinene as one of the less represented compounds ranging from 0.4% to 1.4% of the *H. undulatum* EO. *α*-Pinene, a monoterpene hydrocarbon reported as a major compound in different species of *Hypericum* (Çakir *et al.*, 1997; Nogueira *et al.*, 1998; Baser *et al.*, 2002; Couladis *et al.*, 2003; Pavlović *et al.*, 2006), was poorly represented in *H. undulatum* EO, accounting for less than 1.2% of its content. The group of oxygenated monoterpenes was poorly represented, being constituted by *trans*-linalool oxide, terpinen-4-ol and *α*-terpineol in the EO of leaves and flowers, and only by *trans*-linalool oxide in the EO of ripened seed capsules. No oxygenated monoterpene was

detected in the stems (Table 1). These results are coherent with those reported by Nogueira (2002) who found values of oxygenated monoterpenes lower than 1%. The author reported values of oxygenated sesquiterpenes of 1-2% in the EO of the aerial parts of *H. undulatum.* In the present study oxygenated sesquiterpenes accounted for less than 1% of the EO of stems, reaching, however, 5.2%, 5.6% and more than 12% in EO of leaves, flowers and ripened seed capsules, respectively.

Considering the five most represented compounds in the four types of organs studied, only *n*-nonane was common to all of them (Figure 3). *(E)*-Caryophyllene and germacrene D were common to leaves, stems and flowers, while α-cubebene was among the five most abundant compounds of EO extracted from ripened seed capsules and flowers. The list of the five most represented compounds was completed with γ-muurolene and α-cedrene for leaves; β-pinene and β-elemene for stems; 2,6-dimethyloctane, *n*-hexanol and α-copaene for ripened seed capsules; and β-bourbonene for flowers. Germacrene D was the second major compound in the EO of leaves (12.9%) and flowers (7.7%), and the fourth in the EO of stems (2.1%), representing however, 1% of the total EO of the ripened seed capsules of *H. undulatum*. Nogueira (2002) reported the presence of this compound, in contents not exceeding 1%, in EO of different *H. undulatum* accessions with one exception in which it reached 6%. *(E)*-Caryophyllene is usually one of the most represented sesquiterpene hydrocarbons in the EO of several *Hypericum* species, such as *H. carinatum* and *H. ternum* at flowering stage (Ferraz *et al.*, 2005) and *H. perforatum* (Baser *et al.*, 2002). In the EO of leaves and flowers of *H. undulatum* here reported, *(E)*-caryophyllene was the third major compound, accounting for 9.6% and 6.6%, respectively, while in the ripened seed capsules and in the stems it was lower than 3%. Lower relative amounts of *(E)*-caryophyllene in EO of *H. undulatum* had been reported before (0.5% to 2%) (Nogueira, 2002). In EO of *H. androsaemum*, a seasonal variation in the *(E)*-caryophyllene content from 9% to 17% was reported (Guedes *et al.*, 2004). The reported contents of *(E)*-caryophyllene in EO of different *Hypericum* species has not been consistent, which may be related to its role as metabolic intermediary, sharing metabolic pathways with other EO compounds, such as caryophylladienol and caryophyllene oxide (Schwob *et al.*, 2004). The caryophylladienol was not detected in *H. undulatum* EO, but the caryophyllene oxide was identified in the hydrodistillates of the four organs studied. In the ripened seed capsules this compound was the major oxygenated sesquiterpene (5.2%). From the 16 oxygenated sesquiterpenes detected in this study, 14 were common to the EO of leaves, ripened seed capsules and flowers of *H. undulatum* while a half of these ones were common to

stems EO. *n*-Hexanol, the fourth most represented compound in the ripened seed capsules (6.5%), represented only 0.1%, 0.2% and 3.5% of the total EO of leaves, stems and flowers, respectively. This compound was not detected in the EO of *H. undulatum* analyzed by Nogueira (2002).

As concluding remarks the present study revealed that the EO profiles of *H. undulatum* are quite different from the EO profiles of other *Hypericum* species already studied. Although the major compound (*n*-nonane) was common to the different organs, this report clearly shows that each *H. undulatum* organ has its specific EO profile. Indeed, EO from stems, ripened seed and flowers are richer in *n*-alkanes, while the EO of leaves have the sesquiterpene hydrocarbons as the most abundant group of compounds. The ripened seed capsules contain high levels of oxygen-containing sesquiterpenes, mainly due to the high amount of caryophyllene oxide.

References

Baser, K. H. C., T. Ozek, H. R. Nuriddinov & A. B. Demirci (2002). Essential oils of two *Hypericum* species from Uzbekistan. *Chemistry of Natural Compounds. 38*(1), 54-57.

Çakir, A., M. E. Duru, M. Harmandar, R. Ciriminna, S. Passannanti & F. Piozzi (1997). Comparison of the volatile oils of *Hypericum scabrum* L. and *Hypericum perforatum* L. from Turkey. *Flavour and Fragrance Journal 12*(4), 285-287.

Couladis, M., I. B. Chinou, O. Tzakou & P. V. Petrakis (2003). Composition and antimicrobial activity of the essential oil of *Hypericum rumeliacum* subsp. *apollinis* (Boiss. & Heldr.). *Phytotherapy Research 17*(2), 152-154.

Ferraz, A., D. H. Faria, M. N. Benneti, A. Brondani da Rocha, G. Schwartsmann, A. Henriques & G. L. von Poser (2005). Screening for antiproliferative activity of six southern Brazilian species of *Hypericum*. *Phytomedicine 12*(1-2), 112-115.

Ferraz, A. B. F., R. P. Limberger, S. A. L. Bordignon, G. L. v. Poser & A. T. Henriques (2005). Essential oil composition of six *Hypericum* species from southern Brazil. *Flavour and Fragrance Journal 20*(3), 335-339.

Ferreira, A., C. Proença, M. L. M. Serralheiro & M. E. M. Araújo (2006). The *in vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Portugal. *Journal of Ethnopharmacology 108*(1), 31-37.

Guedes, A. P., L. R. Amorim, A. Vicente & M. Fernandes-Ferreira (2004). Variation of the essential oil content and composition in leaves from cultivated plants of *Hypericum androsaemum* L. *Phytochemical Analysis 15*, 146-151.

Guedes, A. P., L. R. Amorim, A. M. S. Vicente, G. Ramos & M. Fernandes-Ferreira (2003). Essential Oils from Plants and *in Vitro* Shoots of *Hypericum androsaemum* L. *J. Agric. Food Chem. 51*(5), 1399-1404.

Kunst, L. & A. L. Samuels (2003). Biosynthesis and secretion of plant cuticular wax. *Progress in Lipid Research 42*, 51-80.

Nogueira, T. (2002). O género *Hypericum* L. em Portugal Continental. Contribuição para o estudo quimiotaxonómico. Instituto Superior de Agronomia. Lisboa, Universidade Técnica de Lisboa. **PhD. Thesis**.

Nogueira, T., F. Duarte, F. Venâncio, R. Tavares, M. Lousã, C. Bicchi & P. Rubiolo (1998). Aspectos Quimiotaxonómicos do Género *Hypericum* L. em Portugal. *Silva Lusitana 6*(1), 55-61.

Pavlović, M., O. Tzakou, P. V. Petrakis & M. Couladis (2006). The essential oil of *Hypericum perforatum* L., *Hypericum tetrapterum* Fries and *Hypericum olympicum* L. growing in Greece. *Flavour and Fragrance Journal 21*(1), 84-87.

Radusiene, J., A. Judzentiene & G. Bernotiene (2005). Essential oil composition and variability of *Hypericum perforatum* L. growing in Lithuania. *Biochemical Systematics and Ecology 33*(2), 113-124.

Santos, P. A. G., A. C. Figueiredo, J. G. Barroso, L. G. Pedro & J. J. C. Scheffer (1999). Composition of the essential oil of *Hypericum foliosum* Aiton from five Azorean islands. *Flavour and Fragrance Journal 14*(5), 283-286.

Saroglou, V., P. D. Marin, A. Rancic, M. Veljic & H. Skaltsa (2007). Composition and antimicrobial activity of the essential oil of six *Hypericum* species from Serbia. *Biochemical Systematics and Ecology 35*(3), 146-152.

Schwob, I., J.-M. Bessiere, V. Masotti & J. Viano (2004). Changes in essential oil composition in Saint John's wort (*Hypericum perforatum* L.) aerial parts during its phenological cycle. *Biochemical Systematics and Ecology 32*(8), 735-745.

Schwob, I., J. M. Bessiere, M. Dherbomez & J. Viano (2002b). Composition and antimicrobial activity of the essential oil of *Hypericum coris*. *Fitoterapia 73*(6), 511-513.

Seabra, R. M., M. H. Vasconcelos, M. A. C. Costa & A. C. Alves (1992). Phenolic compounds from *Hypericum perforatum* and *Hypericum undulatum*. *Fitoterapia 68*, 473-474.

Seabra, R. M., M. H. Vasconselos & A. C. Alves (1991). Flavonoid sulphates from *Hypericum undulatum*. *Rev. Port. Farm. 12*, 16-18.

Smelcerovic, A., N. Mimica-Dukic & S. Djordjevic (2004). Essential oil composition of *Hypericum perforatum* L. ssp angustifolium from South Serbia. *J. Essent. Oil Bear. Plants 7*, 275–278.

Toker, Z., G. Kizll, H. Ç. Özen, M. Kizll & S. Ertekin (2006). Compositions and antimicrobial activities of the essential oils of two *Hypericum* species from Turkey. *Fitoterapia 77*(1), 57-60.

Touafek, O., A. Nacer, A. Kabouche & Z. Kabouche (2005). Analysis of the essential oil of algerian *Hypericum perfoliatum* (L). *Flavour and Fragrance Journal 20*(6), 669-670.

Micropropagation and *in vitro* essential oil production profiles of *Hypericum undulatum* Schousboe ex Willd

Ana P. Guedes and M. Fernandes-Ferreira

CITAB- Centro de Investigação e de Tecnologias Agro-Alimentares e Biológicas, Department of Biology, University of Minho, Campus de Gualtar, 4720-057 Braga, Portugal

Abstract

In vitro cultures of *Hypericum undulatum* Schousboe ex Willd were established on two different basal media (MS and Mg) devoid of growth regulators from one nodal primary explant after its surface-sterilization. MS basal medium induced a higher number of shoots and roots per explant, as well as longer shoots and roots than did the Mg medium. The essential oil (EO) yields ranged from 0.5 to 1.0% and from 0.4 to 1.0% (w/w) by hydrodistillation of *H. undulatum* shoots grown on MS and Mg media, respectively. The hydrodistillation of micropropagated and acclimatized plants, 6 and 8 months after the plantlets transfer to plastic pots gave yields of 1.1%. The EO were predominantly constituted by *n*-alkanes whose relative contents constituted 63% (w/w) of the EO of micropropagated and acclimatized plants, 57-83% and 71-86% of the EO of shoots growing on MS and on Mg media, respectively. *n*-Nonane was the major constituent, representing 58-59% of the EO of micropropagated and acclimatized plants, 50-75% and 63-79% of the EO of shoots growing on MS and on Mg media, respectively.

Keywords: Hypericum undulatum; *in vitro* shoots; acclimatized plants; essential oils; *n*-alkanes; *n*-nonane

1- Introduction

Hypericum undulatum, usually grows in wet places and in the riverside edges (Nogueira, 2002), being used to treat migraine and heal gall bladder ailments. Its decoctions are also believed to have renal antispasmodic and hepatic protector effects (Ferreira *et al.*, 2006). In Portugal, *H. undulatum* together with *H. perforatum* and *H. androsaemum* is one of the most used and commercialized medicinal *Hypericum* species. In view of the increasing demand for

medicinal plants, there is a need to develop approaches for efficient propagation. Micropropagation by *in vitro* meristems has been reported as an effective tool for obtaining genetically uniform plants, which can be the source of important secondary compounds.

The growth rate of *in vitro* plant tissue and organ cultures is a function of the net assimilation rate resulting from uptake of the medium nutrients (Kothari *et al.*, 2004). As the morphogenesis of plant tissues under *in vitro* conditions is also largely influenced by the composition of the culture medium, the right set up of a basal medium is of utmost importance for the establishment of an efficient tissue culture system for plant regeneration. Minerals are the major component of plant tissue culture media, which comprises the essential macro- and micronutrients and a supplement of carbon source and vitamins. The composition of macro- and microelements in most standard media has been developed through empirical manipulations of one or more combinations of existing formulations (Ramage *et al.*, 2002; Kothari *et al.*, 2004). Generally, the use of plant growth regulators is also required for optimal growth and regeneration rates of *in vitro*-cultured plant tissues. The morphogenic response of the explant depends on the interaction between the nutrient salts and the organic constituents, especially plant growth regulators (Chauhan *et al.*, 2004). However, the effects of the ratio and concentrations of these growth regulators depend on the plant species and the type of culture. It has been reported that the nutrient levels in the medium greatly affect plant regeneration (Ramage *et al.*, 2002). Regardless the growth regulator used, plant regeneration can be improved by modifying the salt composition (Kothari *et al.*, 2004). Modifications of the basal medium have been found to influence *callus* induction and plant regeneration on species such as wheat (Purnhauser *et al.*, 1993), indica rice (Sahrawat *et al.*, 1999), recalcitrant indian barley (Chauhan *et al.*, 2004), hazelnut (Nas *et al.*, 2004), *Paspalum scrobiculatum* and *Eleusine coracana* (Kothari-Chajer *et al.*, 2008), and *Stevia rebaudiana* (Jain *et al.*, 2009). Therefore, the ratios of plant growth regulators cannot be viewed as the sole mechanism controlling *in vitro* developmental processes (Ramage *et al.*, 2002). The optimal nutrient composition in the medium affects the sensitivity of the explants in response to plant growth regulators (Chauhan *et al.*, 2004). It has also been reported that appropriate levels of nutrients may partially substitute plant growth regulators in the culture medium (Preece, 1995). For plant regeneration from embryogenic *callus* of *Eleusine coracana* the addition of higher concentrations of NH_4NO_3 can substitute the growth regulator NAA requirement in the medium (Poddar *et al.*, 1997).

Plant regeneration of *Hypericum* species has been achieved using whole seedlings or their excised parts (Cellarova *et al.*, 1995), such as hypocotyl sections (Murch *et al.*, 2000) and

leaves (Pretto *et al.*, 2000) as primary explants. However, to our knowledge, no report on the establishment of *in vitro* cultures of *H. undulatum* was published up to now. On the other hand little information is available on essential oils (EO) produced by *H. undulatum* plants in which, alkanes, namely *n*-nonane, and sesquiterpene hydrocarbons were reported as the major groups of compounds (Mathis *et al.*, 1964a; Nogueira, 2002).

In this work we established *in vitro* cultures of *H. undulatum* on MS culture medium and on a basal medium settled by us (Mg) by modification of that of $N_{30}K$ (Margara, 1984). The micropropagated plants were acclimatized and the respective EO composition profiles together with those of *in vitro* shoots were determined. The results from these studies are here reported.

2- Material and Methods

2.1- Plant material

A total of 200 explants constituted by leaves, nodal and internodal segments of *H. undulatum* plants, grown in a field located in Braga (Northern Portugal), were excised and immersed in ethanol (70%) for 2 minutes, before surface sterilization by immersion in a sodium hypochlorite solution (5% or 10%) for 10 or 15 minutes. To remove traces of chlorine, leaves and stem segments were washed three times with sterile distilled water and then placed on Murashige and Skoog (Murashige and Skoog, 1962) basal medium (MS) supplemented with 2% sucrose, without any growth regulators. The pH of the medium was adjusted to 5.7 and it was solidified with 0.8% agar prior to autoclaving at 15 psi for 20 min at 121ºC. Sterile primary explants were incubated at 25±2ºC under a photoperiod 16 h light/8 h dark. Illumination was supplied by cool white fluorescent tubes with a light intensity of 52μmol $m^{-2}s^{-1}$.

2.2- Establishment and development of in vitro *cultures*

To evaluate the effect of different basal media on shoot multiplication, around 50 explants were distributed on MS and Mg [basal medium containing the macroelements of the $N_{30}K$ mineral formulation (Margara, 1984) with MS microelements and vitamins (Murashige *et al.*, 1962)] basal media, both supplemented with 3% sucrose and without any growth regulators (Table 1).

Table 1- Macroelements composition of the MS and Mg culture media

Macroelements	MS (mg/L)	Mg (mg/L)
KNO_3	1900	1313
NH_4NO_3	1650	480
$Ca(NO_3)_2.4H_2O$	-	590
$CaCl_2.2H_2O$	440	-
$MgSO_4.7H_2O$	370	246
KCl	-	74.5
KH_2PO_4	170	136

The efficacy of each basal medium on the plantlets proliferation and growth was determined after 30 and 60 days of culture by recording the number of induced shoots *per* explant, their length and their biomass dry weight. For plant multiplication, in both culture conditions, nodal segments were transferred every 8 weeks to fresh culture medium and incubated at 25±2ºC under a photoperiod 16 h light/8 h dark. Illumination was supplied by cool white fluorescent tubes with a light intensity of 52µmol $m^{-2}s^{-1}$.

2.3- Growth profiles and acclimatization of the in vitro *regenerated plantlets*

The growth profiles were determined over the 6th subculture period. Samples were constituted by 3 culture flasks, harvested every 10 days, in which shoots from the flasks of each basal media were separated from roots. The dry weight of the shoots and roots was determined after freeze drying at 0.05 mbar for 72 h. The study was carried out in a completely randomised design with three replicates per time of culture.

Plantlets with 60 days of culture, grown on MS or Mg basal media were transferred to plastic pots containing an organic soil mixture substrate. Plantlets were kept under laboratory environmental conditions at room temperature and close to a window. Initially, plantlets were covered with a plastic bag, in which holes were made after 2 weeks. Several holes were periodically made until the plastic was removed after 8 weeks.

2.4- Essential oil composition and production profiles

The composition and production profiles of the essential oils were determined over the 6th subculture period. Shoots from about 5 flasks of each basal media were separated from roots, at every 10 days, and the respective fresh biomass was gathered and submitted to hydrodistillation for essential oil recovery. Essential oils from the aerial parts of 6 and 8 months

old acclimatized plants were isolated by the same way. Hydrodistillation was performed in a Clevenger type apparatus, over 1 h in the presence of a *n*-hexane (1.0 mL) solution containing 5α-cholestane (1mg/mL), for the retention of the hydrodistillate components. The dry weight of the plant material was determined after drying (60ºC, 72h) in a drying stove.

Further analyses of the hydrodistillates were performed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) in the conditions described before (pages 118-119).

3- Results and Discussion

3.1- Establishment of in vitro *cultures*

From the different methods of surface sterilization applied to the vegetative primary explants of *H. undulatum* only the treatment with 5% hypochlorite solution during 15 minutes allowed shoot regeneration (Table 2).

Table 2- Effect of the method of surface-sterilization of primary explants (leaves, internodal and nodal segments) from in Nature growing *Hypericum undulatum* plants on the regeneration of *in vitro* plantlets.

Culture medium	Surface-sterilized method	Contamination (%)	Death (%)	Regeneration (%)
MS basal without growth regulators	Ethanol 70% (2 min) Sodium Hypochlorite 5% (10 min) 3 washes with sterile distilled water	76	24	0
	Ethanol 70% (2 min) Sodium Hypochlorite 5% (15 min) 3 washes with sterile distilled water	25	74.5	0.5
	Ethanol 70% (2 min) Sodium Hypochlorite 10% (10 min) 3 washes with sterile distilled water	20	80	0
	Ethanol 70% (2 min) Sodium Hypochlorite 10% (15 min) 3 washes with sterile distilled water	10	90	0

In three of the four assays no one shoot regenerated due to a generalized high rate of contamination or explants' death. Indeed, the plant regeneration occurred from only one nodal segment giving a plantlet without contamination. This only plantlet was cloned and multiplied successively through nodal segment subcultures giving origin to all *H. undulatum* plantlets used in all studies involving *in vitro* cultures of this species. Increasing the sodium hypochlorite concentration or time of exposure led to a reduction in the contamination rate but induced the

death of the explants by oxidation/browning (Table 2). Most of the *in vitro* cultures of *Hypericum* species have been established from seeds. Those are the cases of *H. brasiliense* (Cardoso et al., 1996) and *H. perforatum* (Murch *et al.*, 2000; Bais *et al.*, 2002; Murch *et al.*, 2002; Smith *et al.*, 2002; Walker *et al.*, 2002; Pasqua *et al.*, 2003; Zobayed *et al.*, 2005). An efficient protocol of surface sterilization of *H. perforatum* was obtained when leaves were surface-sterilized in a 20% (v/v) commercial sodium hypochlorite solution containing 0.04% of Tween 20 for 20 min (Pretto *et al.*, 2000). The use of bleach (10%) for 20 min together with $HgCl_2$ (0.2%) for 5 min and Tween 20 (0.01%) was reported to be the most suited to the sterilization of stem sections of *H. foliosium* (Moura, 1998). No reports have been published however on the establishment of *in vitro* cultures of *H. undulatum*.

Nodal segments of the aseptically regenerated shoot of *H. undulatum* were cultivated on MS and Mg basal media without growth regulators. Shoot initiation was observed within 1-2 weeks of culture in both culture media. This study was carried out to assess the effect of two basal media (MS and Mg) on sprouting of shoots from nodal segments of aseptic plants. More than 90% of subcultured nodal segments developed in complete plantlets on both basal media (Table 3).

Table 3- Effect of basal media (Mg and MS) on the regeneration of *in vitro* plantlets of *Hypericum undulatum* by subculturing nodal segments. All the results are the mean and standard deviation of 3 replicas, determined at the end of 30 and 60 days of culture.

Days of culture	**30 days**		**60 days**	
Culture medium	**Mg**	**MS**	**Mg**	**MS**
Nº shoots/ explant	2.42±0.76	3.08±0.80	2.08±0.44	3.50±0.75
Shoots length (mm)	21.8±0.38	36.2±0.79	87.4±3.44	97.0±3.97
Shoots dry weight/ flask (mg)	12±0.00	15.22±0.03	70.2±3.71	62.19±0.00
Nº roots/ explant	1.25±0.43	2.50±0.95	2.75±0.66	4.08±0.29
Roots length (mm)	7.68±2.98	4.42±1.98	15.17±4.82	24.26±8.76
Roots dry weight/ flask (mg)	0.69±0.34	0.19±0.13	11.51±0.68	13.58±0.74

The number of shoots and roots produced from nodal explants, after 30 and 60 days of culture on MS basal medium was higher than that obtained with Mg medium (Table 3). On MS basal medium the shoots showed a faster linear growth, reaching 97mm after 60 days of culture. The roots from the plantlets grown on MS medium at 30th day were shorter than those grown on Mg medium, but longer at the 60th day (Table 3). At the 60th day of culture, plantlets grown on Mg basal medium were in lower number per explant and had shorter and thicker shoots than

those grown on MS medium. MS and Mg basal media differed in the macronutrients composition (Table 1). Mg basal medium was constituted by macronutrients of the $N_{30}K$ mineral formulation, having a lower ionic concentration than that of MS medium. Besides the differences shown in Table 3, on can say that there were no high discrepancies in terms of plantlet growth between the two basal media. Some authors have shown good results in terms of plant regeneration on media with ionic concentrations lower than that of MS medium. Moura (1998) tested the effect of five different basal media in the culture of *H. foliosium*, showing that the best results were obtained in the culture medium, whose MS macronutrient solution was diluted to 2/5. The high ionic concentration of the MS culture medium in some cases, seems to inhibit the growth of several woody species, and cause vitrification, as observed in cultures of *Fragaria* (Trigiano *et al.*, 1992). Vitrification could be a physiological response to the high content of ammonium nitrate present in the MS culture medium. In our study no signs of vitrification were recorded in *in vitro* plantlets of *H. undulatum*. Trigiano and co-workers (1992) suggested the use of macronutrients of the $N_{30}K$ mineral formulation when plant species are sensitive to high concentrations of mineral salts.

Notwithstanding the requirement of plant growth regulators in the regeneration of some species, no one was needful for development of *H. undulatum* plantlets from nodal segments. *In vitro* culture of *H. foliosium* on culture medium free of growth regulators induced a slight increase in the number of nodes per explant in the initiation stage of culture. The shoot length and number of nodes per shoot in the elongation stage were similar in both hormonal supplemented or non-supplemented culture media (Moura, 1998). Maximum multiplication rates from shoot apices of cotton was also obtained by culturing the explants on MS salts with modified B5 vitamins free of growth regulators (Zapata *et al.*, 1999). A propagation system was devised for several *Medicago truncatula* genotypes, in which the addition of growth regulators was restricted to the induction phase, therefore reducing the risks of epigenetic and somaclonal variation (Neves *et al.*, 2001). The results herein reported show that nodal segments of *H. undulatum* have the sufficient endogenous growth regulators to support growth and development without hormonal amendment in the culture media.

All the plantlets grown on MS medium and acclimatized in the conditions here described survived, showing that the methodology assayed was completely successful. There was no branching of shoots observed, as well as no detectable/visible variation among the "acclimatized" plants with respect to morphological characteristics. The plantlets grown on Mg basal medium submitted to acclimatization by the same methodology died 1-2 months after transfer to the vessels.

3.2- Essential oils from in vitro *shoots*

The EO contents ranged from 4.9 to 10.4 mg/g of biomass dry weight in plantlets grown on MS medium (Figure 1) and from 4.1 to 9.5 mg/g of biomass dry weight in plantlets grown on Mg medium (Figure 2). These variation ranges were higher than those recorded for the EO isolated from the aerial parts of *in vivo* plants of this species. Figures 1 and 2 show the EO specific content profiles of *in vitro* shoots during a culture cycle of 60 days, as well as the dry biomass growth, during the same time period on MS and Mg media, respectively.

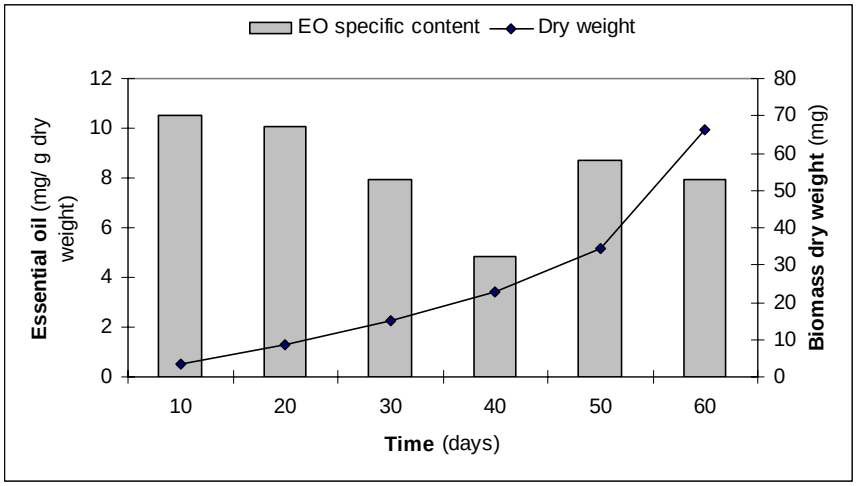


Figure 1 - Essential oil contents (mg/g of biomass dry weight) and biomass dry weight per flask (mg) of *in vitro* shoot cultures of *Hypericum undulatum* grown on MS basal medium without plant growth regulators, over a culture cycle of 60 days.

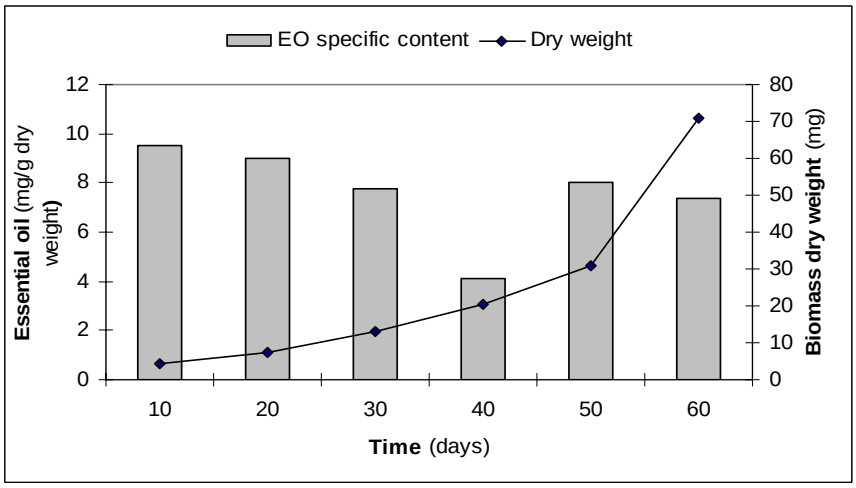


Figure 2 - Essential oil contents (mg/g of biomass dry weight) and biomass dry weight per flask (mg) of *in vitro* shoot cultures of *Hypericum undulatum* grown on Mg basal medium without plant growth regulators, over a culture cycle of 60 days.

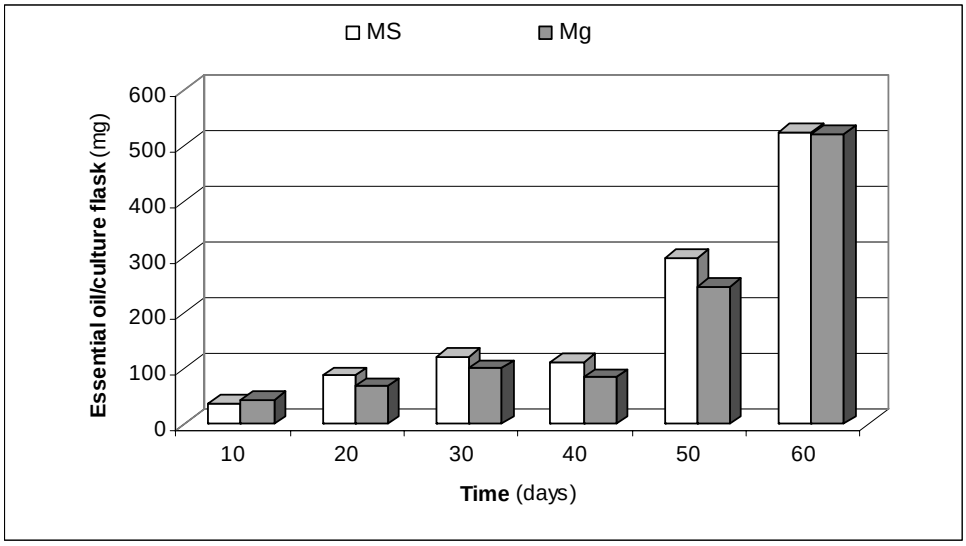


Figure 3- Essential oil contents (mg) by culture flask of *in vitro* shoots of *Hypericum undulatum* grown on MS and Mg basal media without plant growth regulators, over a culture cycle of 60 days.

The highest specific EO contents were 10.4 and 9.5 mg/g of biomass dry weight, recorded at the 10th day of culture, in plantlets grown on MS and Mg media, respectively. Thereafter, the EO contents decreased in inverse correlation with biomass dry weight, reaching the minimum of 4.9 and 4.1 mg/g of dry weight at 40th day in plantlets grown on MS and Mg media, respectively (Figures 1 and 2). Between the 40th and 50th day of culture, however, the EO contents increased almost 2-fold, decreasing again between the 50th and 60th day of culture, while the biomass dry weight increased at a higher growth ratio (Figures 1 and 2). On can say that during the first 40 days of culture, the developing plantlets were preferentially using the carbon and energy sources for growth and plant development, in detriment of the production of secondary metabolites. The increase in the EO specific content between the 40th and the 50th days, however, is not consistent with this interpretation. On the other hand, essential oils are usually produced in specialized structures, whose differentiation may be delayed in the time, relatively to shoot growth, contributing thereby to the overall decrease in the EO specific content. In *H. perforatum* plants these structures can be translucent glands and/or type B cannals structures (Ciccarelli *et al.*, 2001; Maffi *et al.*, 2003).

Over the 60 days of culture, some variations occurred in composition of the EO produced by *in vitro* shoots of *H. undulatum* on the two basal media (Table 4).

Table 4- Composition (µg/g of biomass dry weight) of the essential oils from *in vitro* shoots of *Hypericum undulatum* grown over 60 days on MS or Mg basal media without any growth regulator.

Compounds	KI	10 days		20 days		30 days		40 days		50 days		60 days	
		MS	Mg	MS	Mg	MS	Mg	MS	Mg	MS	Mg	MS	Mg
n-Octane	800						92.4		18.5		62.9		53.8
(E)-2-Hexenal	854					35.5	84.5	21.9	31.2	38.2	54.1	49.7	47.1
n-Hexanol	863					33.9		20.5		47.7		30.2	
n-Nonane	900	7402.5	7433.5	5925.2	6433.2	4215.1	4699.2	3000.6	2619.5	3800.8	5717.4	4051.2	4930.3
α-Pinene	936	207.5	360.7	307.6	204.9	188.7	189.3	112.1	80.9	126.6	119.4	147.2	154.7
Camphene	950					16.5		9.9	2.3	13.5	80.1	11.8	49.4
β-Pinene	978	542.8	312.2	784.2	908.8	440.6	583.1	263.9	248.3	314.7	335.3	420.6	415.5
Myrcene	991					33.4		7.0		12.0		17.5	
n-Decane	1000					15.4		7.4				13.0	
Limonene	1030					12.4		7.4		10.1		9.5	
E-*β*-Ocimene	1051			64.7		18.0	5.2	7.3	2.0	16.9	3.3	16.7	10.4
n-Undecane	1100	702.1	693.1	429.5	409.5	418.1	508.2	242.0	210.2	394.1	383.6	250.6	256.2
α-Terpineol	1190					21.1		5.6		33.1		24.6	
n-Decanal	1205	89.6		56.9		24.9		8.4		8.6		8.2	
n-Tridecane	1300	115.1		68.2		28.3		10.2		11.3		10.1	
δ-Elemene	1337			146.5		96.0	88.5	35.2	27.9	136.7	82.4	104.8	67.5
α-Cubebene	1348	177.3	38.2	148.2	302.9	145.3	213.4	103.6	149.4	127.7	185.3	123.3	236.6
α-Copaene	1374	100.5	47.6	84.1	115.8	80.7	143.4	57.7	78.5	69.1	118.5	67.1	101.8
β-Bourbonene	1383					23.4	4.9	10.2	2.9	13.0	3.2	14.0	25.4
β-Elemene	1390	213.2	438.6	246.3	207.1	183.4	198.2	123.9	152.4	157.4	184.6	160.5	240.7
SH	1392		49.3	57.3	34.3	42.8	91.5	31.3	32.5	35.9	53.2	36.5	77.8
iso-Italicene	1395					8.9		5.7		23.6		21.9	
Italicene	1405					15.3		5.1		33.7		2.8	
α-Cedrene	1409			9.2	8.0	40.4	93.3	26.6	31.2	36.2	54.2	24.3	52.5
(E)-Caryophyllene	1417					51.6	45.1	15.7	50.5	19.8	81.3	18.5	76.2
β-Gurjunene	1427			54.3		46.0	141.4	35.3	52.1	44.8	82.5	42.3	66.2
Aromadendrene	1442					32.3	7.3	26.6	3.0	34.3	24.0	32.7	50.6
α-Humulene	1447									13.2		12.6	
allo-Aromadendrene	1450	31.0		70.1		51.2		26.3		42.1		37.9	
Germacrene D	1480	74.2		101.6	213.6	57.3	39.5	48.2	33.3	11.2	22.2	10.1	5.8
β-Selinene	1482				4.9		3.1		9.2	72.1	120.5	73.1	165.4
α-Selinene	1486									15.4		16.4	
β-Guaiene	1492					34.5	48.3	14.8	21.2	13.3	16.1	21.0	49.3
α-Muurolene	1497			18.3		10.9		13.5		9.7		11.8	
(E,E)-*α*-Farnesene	1498					165.4	407.5	1.9	8.8	6.1	25.0	50.0	48.3
γ-Cadinene	1508	228.6		210.1		105.0		74.6		105.0		129.9	
δ-Cadinene	1521	215.7	163.8	259.1	112.5	113.1	88.3	116.5	91.4	80.7	79.8	88.3	49.7
SH	1530					19.2		14.6		19.1		137.6	
α-Cadinene	1534			268.5		311.0		173.4		2519.7		145.3	
E-Nerolidol	1563			26.7		11.6							
Viridiflorol	1591			119.5		176.8		7.0		8.7		6.5	
β-Copaen-4-*α*-ol	1596					38.3		6.2		24.8		7.6	
β-Oplopenone	1612									6.2		8.2	
epi-*α*-Bisabolol	1674			79.2		63.6		7.0		13.7		8.3	
Z-*α*- *trans*-Bergamotol	1694			61.7		21.6		7.7		11.6		8.0	
n-Heptadecane	1700			29.9		57.1		5.0				2.7	
n-Octadecane	1800			24.0		33.8		3.6		5.5		4.5	
n-Nonadecane	1900			37.0		23.8		2.7		4.8		6.0	

Compounds	KI	10 days		20 days		30 days		40 days		50 days		60 days	
		MS	Mg	MS	Mg	MS	Mg	MS	Mg	MS	Mg	MS	Mg
n-Eicosane	2000	117.1		40.5		28.6		5.6		3.8		11.8	
n-Heneicosane	2100			21.4		22.2		2.6		3.7		4.5	
n-Docosane	2200			25.9		31.1		3.0		4.4		10.9	
n-Tricosane	2300	55.6		50.3		23.0		1.8		5.0		1.1	
n-Tetracosane	2400			31.2		32.8		6.0		5.4		10.1	
n-Pentacosane	2500			25.3		25.9		5.5		6.1		12.2	
n-Hexacosane	2600			18.9		20.1		1.9		3.5		4.5	
n-Heptacosane	2700	130.8		72.1	12.9	65.6	11.4	45.2	89.1	54.5	83.0	49.6	67.1
n-Octacosane	2800			23.8		24.6		6.8		7.5		6.8	
n-Nonacosane	2900	97.4		79.6	17.8	71.6	5.2	55.4	55.1	46.5	79.6	28.7	57.9
Monoterpene Hydrocarbons		750.4	672.9	1156.5	1113.7	709.7	777.6	407.7	333.6	493.7	538.1	623.2	630.0
Oxygenated Monoterpenes								5.6		33.1		24.6	
Sesquiterpene Hydrocarbons		1040.6	737.5	1673.7	999.1	1688.8	1613.7	961.0	744.1	3639.8	1132.6	1379.7	1313.8
Oxygenated Sesquiterpenes				287.0		311.9		27.9		53.4		38.5	
***n*-Alkanes**		8620.5	8126.6	6902.7	6873.4	5108.8	5316.3	3405.4	2992.2	4368.4	6326.5	4478.4	5365.3
Others				56.9		94.2	84.5	50.8	31.2	94.5	54.1	88.1	47.1

The EO isolated from *in vitro* cultures were less complex than those from *in vivo* plants. As can be seen, in the EO from shoots grown on MS basal medium, 17 to 56 compounds were detected over the culture cycle, while in the EO from shoots grown on the Mg basal medium, the number of compounds detected ranged from 9 to 25. All the compounds detected in the EO from shoots grown on the Mg basal medium were found in the EO of shoots grown on MS basal medium. Almost all of the compounds identified in the EO from *in vitro* shoots are constituents of the EO of in Nature wild growing *H. undulatum* plants. The exception was *(Z)-α-trans*-bergamotol which was not identified in any of the organs from *in vivo* plants. The less complexity of *in vitro* essential oils may be due to the poor differentiation and/or juvenility of the shoots, characteristic of this type of cultures. In shoots grown on both culture media, the number of detected compounds increased with the time of culture reaching their maximum at the end of 60 days of culture on MS basal medium, and at the end of 40 days of culture on Mg basal medium (Table 4).

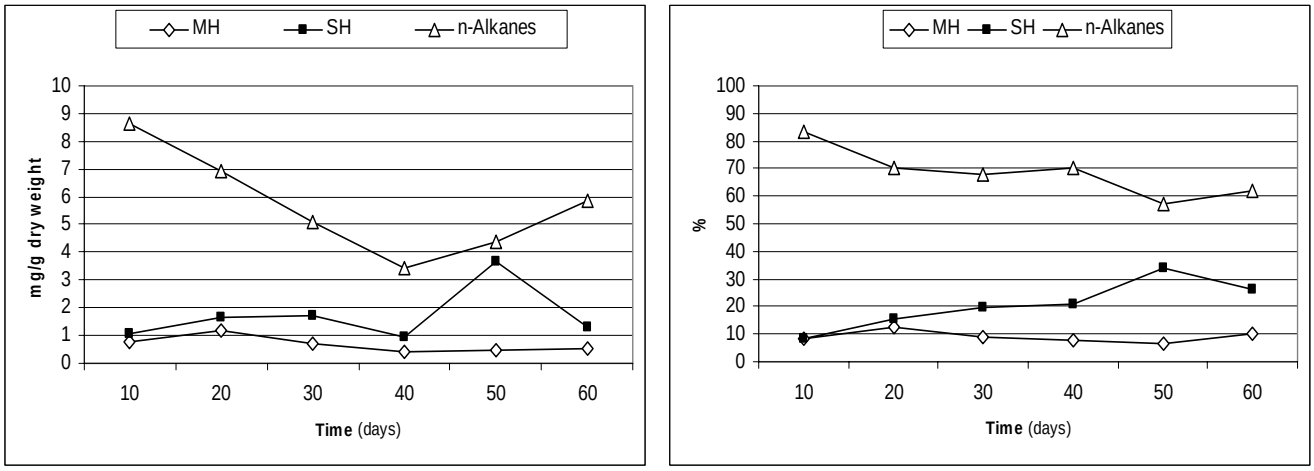


Figure 4a - Specific contents (mg/g of biomass dry weight) and percentage amounts (%) of the main compound groups from the essential oils of *in vitro* shoots of *Hypericum undulatum* grown on MS medium without plant growth regulators, over a culture cycle of 60 days. **MH**- Monoterpene Hydrocarbons; **SH**- Sesquiterpene Hydrocarbons.

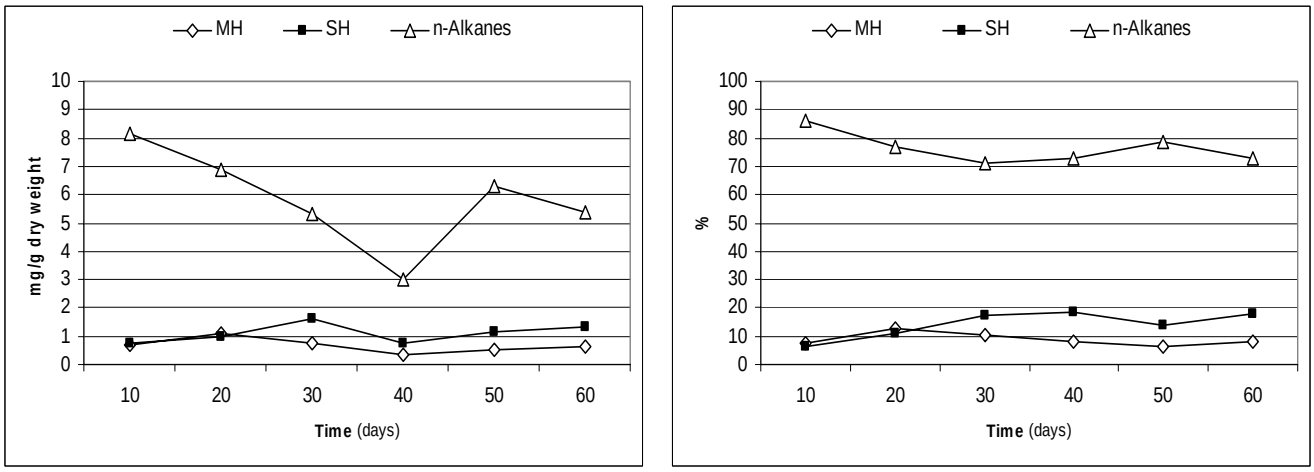


Figure 4b - Specific contents (mg/g of biomass dry weight) and percentage amounts (%) of the main compound groups from the essential oils of *in vitro* shoots of *Hypericum undulatum* grown on Mg medium without plant growth regulators, over a culture cycle of 60 days. **MH**- Monoterpene Hydrocarbons; **SH**- Sesquiterpene Hydrocarbons.

The *n*-alkanes constituted the main group of constituents representing 57.4-83.4% and 71.3-86.0% of the total EO from *H. undulatum* shoots grown on MS and Mg basal media, respectively (Figures 4a and 4b). Likewise the EO extracted from aerial parts of in Nature growing *H. undulatum* plants, *n*-nonane was the most represented compound, ranging from 49.9% to 75.1% and from 63.2% to 78.5% of the EO of shoots grown on MS and on Mg basal media, respectively. *n*-Undecane, was the second most represented compound at the 10th day of culture in both basal media as well as at the 50th day of culture on the Mg medium. The

accumulation of substantial amounts of short-chain *n*-alkanes were also found in members of the pine family, as Jeffrey pine (*Pinus jeffreyi*), in which *n*-heptane was the dominant compound in the oleoresin secretion (Savage *et al.*, 1996a; Savage *et al.*, 1996b). This secretion is formed as a constitutive and inducible defence of conifers against insect pests (e.g. bark beetles) and pathogens (Trapp *et al.*, 2001; Keeling *et al.*, 2006; Keeling *et al.*, 2008). Little is known about the biosynthesis of short-chain *n*-alkanes, although it probably does not follow the same pathway of the terpenoids (Bohlmann *et al.*, 2008), as apparently they are dependent of different metabolic precursors (Savage *et al.*, 1996a; Savage *et al.*, 1996b). Not only the biosynthetic pathways of short-chain *n*-alkanes are still unclear, but also the functions of these molecules in EO are not well understood. A complete series of long-chain *n*-alkanes from *n*-octadecane to *n*-nonacosane was found in the EO from shoots grown on MS basal medium since the 20th day until the 60th day of culture (Table 4). However, in shoots grown on Mg basal medium only *n*-heptacosane and *n*-nonacosane were detected. During the culture cycle, these last two *n*-alkanes were the most represented in the EO from shoots grown on MS medium. Long-chain *n*-alkanes were found also in the EO obtained from aerial parts and different organs of in Nature growing *H. undulatum*.

The sesquiterpene hydrocarbons constituted the second major group at the 20th and 30th days in the EO of shoots grown on the MS and Mg media, respectively (Figures 4a and 4b). Over the first days the 2nd major group was that of monoterpene hydrocarbons whose major constituents were α- and β-pinene over all the growth cycle in shoots grown on both media. Beginning on the 20th day there was an increase in the percentage amount of sesquiterpene hydrocarbons in inverse correlation with monoterpene hydrocarbons whose percentage amount decreased up to 60th day (Figures 4a and 4b). Sesquiterpenes are derived from farnesyl diphosphate (FPP) by a group of enzymes termed sesquiterpene synthases, and encoded by members of the *Tps* gene family, while monoterpenes derive from geranyl diphosphate (GPP). The gradual increase in the accumulation of sesquiterpenes comparing to the monoterpenes, after the 20th day in *H. undulatum* shoots, regardless the basal culture medium, may reflect a higher efficiency in the synthesis of FPP from isopentenyl diphosphate (IPP) and its conversion into sesquiterpenes in detriment of the conversion of GPP into monoterpenes. Similarly, over the year, this same pattern of variation was registered in EO from aerial parts of in Nature growing *H. undulatum*. Therefore, it is possible that specific physiological and environmental conditions can modulate the production of EO compounds favouring one or another class of compounds.

α-Terpineol was the only oxygenated monoterpene detected in the EO from shoots growing on MS medium after the 30th day of culture. This class of compounds is usually synthesised from limonene, which shares with all the other monoterpene hydrocarbons the same precursor, GPP (Croteau *et al.*, 1994). In shoots such as in Nature growing plants of *H. undulatum* the biosynthetic pathway of oxygenated monoterpenes is less favoured than that of sesquiterpenes.

3.3- Essential oils from micropropagated and acclimatized plants

The EO yield obtained from the micropropagated plants, eight months after their acclimatization (10.5 mg/g of biomass dry weight) was similar to that maximum obtained from *in vitro* shoots grown on MS medium at 10th day and higher than those obtained from in Nature wild growing plants. Such as it was recorded for *in vitro* shoots and in Nature wild growing plants, the EO of acclimatized plants were also characterized by a high content of *n*-alkanes (62.8%) with *n*-nonane as the major one (58 to 59%) of the 54 compounds detected (Table 5). Sesquiterpene hydrocarbons, gathering 24 compounds, and monoterpene hydrocarbons, gathering 10 compounds were the second and the third constituent groups representing around 19-20% and 16% of the EO, respectively (Table 5).

Table 5 - Composition (µg/g of biomass dry weight and %) of the essential oils from micropropagated and acclimatized plants of *Hypericum undulatum*, 6 and 8 months after transfer to plastic vessels.

Compounds	**KI**	**6 months**		**8 months**	
		µg/g dry weight	%	µg/g dry weight	%
(E)-2-Hexenal	854	24.6	0.2	19.1	0.2
n-Nonane	900	6031.6	57.9	6164.1	58.9
α-Thujene	929			7.9	tr
α-Pinene	936	331.0	3.2	338.7	3.2
Camphene	950	24.6	0.2	25.4	0.2
2,6-Dimethyl-octane	972			19.8	0.2
β-Pinene	978	1123.4	10.8	1156.8	11.0
Myrcene	991	24.2	0.2	23.7	0.2
n-Decane	1000			7.6	tr
Limonene	1030	27.4	0.2	29.3	0.3
Z-*β*-Ocimene	1038			8.3	0.0
E-*β*-Ocimene	1051	102.6	1.0	95.0	0.9
n-Undecane	1100	207.0	1.9	217.7	2.1
Terpinen-4-ol	1178			5.9	tr
α-Terpineol	1190	4.2	tr	4.8	tr
n-Decanal	1206	3.7	tr	29.4	0.1
*n.*Tridecane	1300	29.5	0.2	7.6	tr
δ-Elemene	1337	134.1	1.3	134.1	1.3

Compounds	KI	6 months		8 months	
		µg/g dry weight	%	µg/g dry weight	%
α-Cubebene	1348	166.8	1.6	169.6	1.6
α-Copaene	1374	118.7	1.2	120.8	1.2
β-Bourbonene	1382	33.9	0.3	25.2	0.2
SH	1388			39.5	0.4
β-Elemene	1390	522.8	5.1	448.0	4.3
SH	1392	56.0	0.5	57.9	0.6
iso-Italicene	1395	13.0	0.2	12.5	0.2
Italicene	1405	19.0	0.2	19.3	0.2
α-Cedrene	1410	55.9	0.5	58.4	0.6
(E)-Caryophyllene	1417	87.0	0.8	94.7	0.9
β-Gurjunene	1427	70.9	0.7	72.2	0.7
Aromadendrene	1442	56.6	0.5	59.6	0.6
α-Humulene	1447	17.8	0.2	21.6	0.2
allo-Aromadendrene	1450	43.2	0.4	42.4	0.4
Germacrene D	1480	11.4	0.2	13.2	0.2
β-Selinene	1482	141.7	1.4	94.5	0.9
α-Selinene	1486	21.3	0.2	22.4	0.2
β-Guaiene	1492	33.2	0.3	11.0	0.2
α-Muurolene	1497	37.9	0.4	13.9	0.2
(E,E)-α-Farnesene	1499	117.5	1.2	105.9	1.0
δ-Cadinene	1521	8.6	tr	8.1	tr
(E)-γ-Bisabolene	1530	29.4	0.3	27.8	0.3
α-Cadinene	1534	312.7	3.0	321.4	3.1
Viridiflorol	1591	43.6	0.4	30.4	0.3
β-Copaen-4-*α*-ol	1596	52.9	0.5	52.6	0.5
β-Oplopenone	1612	25.1	0.2	26.9	0.3
δ-Cadinol	1650	6.2	0.1	9.9	0.1
epi-*α*-Bisabolol	1675	8.1	tr	7.3	tr
n-Eicosane	2000	5.7	tr	5.3	tr
n-Docosane	2200	0.7	tr	4.3	tr
n-Tricosane	2300	3.0	tr	3.6	tr
n-Tetracosane	2400	1.3	tr	7.1	0.1
n-Pentacosane	2500	66.5	0.6	19.6	0.2
n-Heptacosane	2700	139.4	1.5	103.8	1.2
n-Octacosane	2800	6.7	0.1	14.6	0.2
n-Nonacosane	2900	39.7	0.5	33.4	0.3
Monoterpene Hydrocarbons		1633.1	15.6	1704.9	16.2
Oxygenated Monoterpenes		4.2	<0.1	10.7	<0.1
Sesquiterpene Hydrocarbons		2109.4	20.4	2001.7	19.3
Oxygenated Sesquiterpenes		135.9	1.1	127.1	1.2
n-Alkanes		6531.1	62.7	6581.0	63.0
Others		28.3	0.2	48.6	0.3

2,6-Dimethyloctane and *n*-decane as well as two monoterpene hydrocarbons (*α*-thujene and *Z*-*β*-ocimene), terpinen-4-ol and a sesquiterpene hydrocarbon were detected in the EO from the 8 months old acclimatized plants but not detected in the samples harvested 2 months before.

β-Elemene, α-cadinene and α-cubebene were the three major sesquiterpene hydrocarbons while β-pinene and α-pinene were the two major monoterpene hydrocarbons.

Oxygenated terpenes were present in much lower amounts than the terpene hydrocarbons, likewise in Nature growing plants and *in vitro* shoots. In the EO of acclimatized plants, terpinen-4-ol and α-terpineol, in trace amounts, were the only oxygenated monoterpenes detected, and oxygenated sesquiterpenes contents represented less than 1.5% with β-copaen-4-α-ol being the most represented one (0.5%).

A similar number of compounds was detected in the EO obtained from shoots, after 60 days of culture on MS basal medium and EO from acclimatized plants. However, the contents of EO found in acclimatized plants were higher than those recorded for 60 days old *in vitro* shoots. A different result was described for *Salvia fruticosa*, whose content of EO recorded *in vitro* was higher than that observed in the greenhouse-grown plants (Arikat *et al.*, 2004). The authors attributed such difference to the hormonal supplementation used in the culture medium suggesting that the increased yield of EO *in vitro* would be a consequence of the increased percentage of glandular hairs at the secretory stage as a result of the addition of benzyladenine (BA). Positive effects of BA on the capacity of *Lavandula dentata in vitro* growing plantlets in the production and/or accumulation of EO were also shown (Sudriá *et al.*, 1999). Similarities in the EO composition between the parent *in vivo* plants and *in vitro* cultures has been reported for several medicinal plants, such as *Lavandula angustifolia* and *Rosmarinus officinalis* (Webb *et al.*, 1984), *Mentha spicata* (Hirata *et al.*, 1990) and *Minthostachys mollis* (Chebel *et al.*, 1998).

As concluding remarks, in the work here reported differences in the composition of the EO from *in vitro* shoots were recorded as a consequence of the different mineral composition on the basal media as well as differences in the composition depending on the conditions of production, *in vitro* vs *in vivo*, with the *in vitro* shoots and acclimatized plants producing EO less complex than those produced by Nature growing wild plants.

References

Arikat, N. A., F. M. Jawad, N. S. Karam & R. A. Shibli (2004). Micropropagation and accumulation of essential oils in wild sage (*Salvia fruticosa* Mill.). *Scientia Horticulturae 100*(1-4), 193-202.

Bais, H. P., T. S. Walker, J. J. McGrew & J. M. Vivanco (2002). Factors affecting growth of cell suspension cultures of *Hypericum perforatum* L. (St. John's wort) and production of hypericin. *In Vitro Cellular and Developmental Biology - Plant 38*(1), 58-65.

Bohlmann, J. & C. I. Keeling (2008). Terpenoid biomaterials. *The Plant Journal 54*(4), 656-669.

Cardoso, M. & D. De Oliveira (1996). Tissue culture of *Hypericum brasiliense* choisy: Shoot multiplication and callus induction. *Plant cell, tissue and organ culture 44*(2), 91-94

Cellarova, E., K. Kimáková, Z. Daxnerová & P. Mártonfi (1995). *Hypericum perforatum* (St. John's Wort): *In vitro* culture and the production of hypericin and other secondary metabolites. *Biotechnology in Agriculture and Forestry*. Y. P. S. Bajaj. Berlin Heidelberg, Springer-Verlag. **33:** 261-275.

Chauhan, M. & S. Kothari (2004). Optimization of nutrient levels in the medium increases the efficiency of callus induction and plant regeneration in recalcitrant indian barley (*Hordeum vulgare* L.) *in vitro*. *In Vitro Cellular & Developmental Biology - Plant 40*(5), 520-527.

Chebel, A., A. Koroch, H. Juliani, H. Juliani & V. Trippi (1998). Micropropagation of *Minthostachys mollis* (H.B.K.) grieseb. and essential oil composition of clonally propagated plants. *In Vitro Cellular & Developmental Biology - Plant 34*(3), 249-251.

Ciccarelli, D., A. C. Andreucci & A. M. Pagni (2001). Translucent Glands and Secretory Canals in *Hypericum perforatum* L. (Hypericaceae): Morphological, Anatomical and Histochemical Studies During the Course of Ontogenesis. *Annals of Botany 88*(4, Part 1), 637-644.

Croteau, R., W. R. Alonso, A. E. Koepp & M. A. Johnson (1994). Biosynthesis of Monoterpenes: Partial Purification, Characterization, and Mechanism of Action of 1,8-Cineole Synthase. *Archives of Biochemistry and Biophysics 309*(1), 184-192.

Ferreira, A., C. Proença, M. L. M. Serralheiro & M. E. M. Araújo (2006). The *in vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Portugal. *Journal of Ethnopharmacology 108*(1), 31-37.

Hirata, T., S. Murakami, K. Ogihara & T. Suga (1990). Volatile monoterpenoid constituents of the plantlets of *Mentha spicata* produced by shoot tip culture. *Phytochemistry 29*(2), 493-495.

Jain, P., S. Kachhwaha & S. L. Kothari (2009). Improved micropropagation protocol and enhancement in biomass and chlorophyll content in *Stevia rebaudiana* (Bert.) Bertoni by using high copper levels in the culture medium. *Scientia Horticulturae 119*(3), 315-319.

Keeling, C. & J. Bohlmann (2008). Terpenoids in Plants. *Wiley Encyclopedia of Chemical Biology*, John Wiley & Sons, Inc.

Keeling, C. I. & J. Bohlmann (2006). Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. *New Phytol. 170*, 657–675.

Kothari-Chajer, A., M. Sharma, S. Kachhwaha & S. Kothari (2008). Micronutrient optimization results into highly improved *in vitro* plant regeneration in kodo (*Paspalum scrobiculatum* L.) and finger (*Eleusine coracana* (L.) Gaertn.) millets. *Plant Cell, Tissue and Organ Culture 94*(2), 105-112.

Kothari, S. L., K. Agarwal & S. Kumar (2004). Inorganic Nutrient Manipulation for Highly Improved *in Vitro* Plant Regeneration in Finger Millet-*Eleusine coracana* (L.) Gaertn. *In Vitro Cellular and Developmental Biology - Plant 40*(5), 515-519.

Maffi, L., L. Camoni, R. B. Fornasiero & A. Bianchi (2003). Morphology and development of secretory structures in *Hypericum perforatum* and H. richeri. *Nordic Journal of Botany 23*(4), 453-461.

Margara, J. (1984). *Bases de la Multiplication Vegetative*. Versalles. Paris, INRA.

Mathis, C. & G. Ourisson (1964a). Étude chimio-taxonomique du genre *Hypericum* : II. Identification de constituants de diverses huiles essentielles d'*Hypericum*. *Phytochemistry 3*(1), 115-131.

Moura, M. (1998). Conservation of *Hypericum foliosum* aiton, an endemic azorean species, by micropropagation. *In Vitro Cellular & Developmental Biology - Plant 34*(3), 244-248.

Murashige, T. & F. Skoog (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plantarum 15*, 473-497.

Murch, S. J., K. L. Choffe, J. M. R. Victor, T. Y. Slimmon, S. KrishnaRaj & P. K. Saxena (2000). Thidiazuron-induced plant regeneration from hypocotyl cultures of St. John's wort (*Hypericum perforatum*. cv 'Anthos'). *Plant Cell Reports 19*(6), 576-581.

Murch, S. J., H. P. V. Rupasinghe & P. K. Saxena (2002). An *in vitro* and hydroponic growing system for hypericin, pseudohypericin, and hyperforin production of St. John's Wort (*Hypericum perforatum* CV New Stem). *Planta Medica 68*, 1108-1112.

Nas, M. N. & P. E. Read (2004). A hypothesis for the development of a defined tissue culture medium of higher plants and micropropagation of hazelnuts. *Scientia Horticulturae 101*(1-2), 189-200.

Neves, L. O., L. Tomaz & M. P. S. Fevereiro (2001). Micropropagation of *Medicago truncatula* Gaertn. cv. Jemalong and *Medicago truncatula* ssp. Narbonensis. *Plant Cell, Tissue and Organ Culture 67*, 81-84.

Nogueira, T. (2002). O género *Hypericum* L. em Portugal Continental. Contribuição para o estudo quimiotaxonómico. Instituto Superior de Agronomia. Lisboa, Universidade Técnica de Lisboa. **PhD. Thesis**.

Pasqua, G., P. Avato, B. Monacelli, A. R. Santamaria & M. P. Argentieri (2003). Metabolites in cell suspension cultures, *calli*, and *in vitro* regenerated organs of *Hypericum perforatum* cv. Topas. *Plant Science 165*(5), 977-982.

Poddar, K., R. K. Vishnoi & S. L. Kothari (1997). Plant regeneration from embryogenic callus of finger millet *Eleusine coracana* (L.) Gaertn. on higher concentrations of NH_4NO_3 as a replacement of NAA in the medium. *Plant Science 129*(1), 101-106.

Preece, J. E. (1995). Can nutrient salts partially substitute for plant growth regulators? *Plant Tiss. Cult. Biotechnol. 1*, 26–37.

Pretto, F. R. & E. R. Santarém (2000). Callus formation and plant regeneration from *Hypericum perforatum* leaves. *Plant Cell, Tissue and Organ Culture 62*(2), 107-113.

Purnhauser, L. & G. Gyulai (1993). Effect of copper on shoot and root regeneration in wheat, triticale, rape and tobacco tissue cultures. *Plant Cell, Tissue and Organ Culture 35*(2), 131-139.

Ramage, C. & R. Williams (2002). Mineral Nutrition and Plant Morphogenesis. *In Vitro Cell. Dev. Biol.—Plant 38*, 116–124.

Sahrawat, A. & S. Chand (1999). Stimulatory effect of copper on plant regeneration in indica rice (*Oryza sativa* L.). *Journal of Plant Physiology 154*(4), 517-522.

Savage, T. J., B. S. Hamilton & R. Croteau (1996a). Biochemistry of Short-Chain Alkanes. Tissue-Specific Biosynthesis of *n*-Heptane in *Pinus jeffreyi*. *Plant Physiology 110*, 179-186.

Savage, T. J., M. K. Hristova & R. Croteau (1996b). Evidence for an Elongation/Reduction/C1-Elimination Pathway in the Biosynthesis of *n*-Heptane in Xylem of Jeffrey Pine. *Plant Physiol. 111*(4), 1263-1269.

Smith, M., H. Kobayashi, M. Gawienowski & D. Briskin (2002). An *in vitro* approach to investigate medicinal chemical synthesis by three herbal plants. *Plant Cell, Tissue and Organ Culture 70*, 105-111.

Sudriá, C., M. T. Piñol, J. Palazón, R. M. Cusidó, R. Vila, C. Morales, M. Bonfill & S. Cañigueral (1999). Influence of plant growth regulators on the growth and essential oil content of cultured *Lavandula dentata* plantlets. *Plant Cell, Tissue and Organ Culture 58*(3), 177-184.

Trapp, S. & R. Croteau (2001). Defensive Resin Biosynthesis in Conifers. *Annual Review of Plant Physiology & Plant Molecular Biology 52*(1), 689.

Trigiano, R., R. Geneve, S. Merkle & J. Preece (1992). Tissue and cell cultures of woody legumes. *Horticultural Reviews*. **14:** 265-332.

Walker, T. S., H. Pal Bais & J. M. Vivanco (2002). Jasmonic acid-induced hypericin production in cell suspension cultures of *Hypericum perforatum* L. (St. John's wort). *Phytochemistry 60*(3), 289-293.

Webb, J. K., D. V. Banthorpe & D. G. Watson (1984). Monoterpene synthesis in shoots regenerated from callus cultures. *Phytochemistry 23*(4), 903-904.

Zapata, C., M. Srivatanakul, S.-H. P. S-H, B.-M. Lee, M. Salas & R. Smith (1999). Improvements in shoot apex regeneration of two fiber crops: cotton and kenaf *Plant Cell, Tissue and Organ Culture 56*(3), 185-191.

Zobayed, S. M. A., F. Afreen & T. Kozai (2005). Temperature stress can alter the photosynthetic efficiency and secondary metabolite concentrations in St. John's wort. *Plant Physiology and Biochemistry 43*(10-11), 977-984.

5.3 Supplementary data

An approximation of the absolute quantification of all the essential oils (EO) constituents from plants of *Hypericum undulatum* Schousboe ex Willd. cultivated in Braga (Portugal) and harvested at different times during the year is shown in Table S1.

Table S1 - Composition (μg/g dry weight) of the essential oils from plants of *Hypericum undulatum* Schousboe ex Willd. cultivated in Braga (Portugal) and harvested at different times during the year.

Compound	KI(1)	KI(2)	June	Sept.	Nov.	Jan.	April	June
n-Octane	800	800	30.3	21.9	43.2	22.6	41.3	30.4
(E)-2-Hexenal	854	854	128.2	8.5	11.2	5.2	6.0	11.0
n-Hexanol	862	863	13.7	5.6	4.9	1.8	11.1	7.5
n-Nonane	900	900	2411.5	1951.5	2642.8	2519.7	1518.3	2792.0
α-Thujene	929	929	3.2	2.2	6.4	5.1	1.7	3.0
α-Pinene	937	936	117.6	82.0	249.4	206.1	76.2	105.4
Camphene	952	950	1.4	4.1	13.9	11.9	4.2	1.0
2,6-Dimethyloctane	970	972	4.6	6.2	-	-	-	9.3
Sabinene	976	975	8.6	-	-	-	-	-
β-Pinene	979	978	489.8	253.8	932.9	873.6	299.4	366.5
6-Methyl-5-hepten-2-one	988	987	1.6	-	-	-	-	-
Myrcene	991	991	11.3	6.7	16.4	12.1	6.1	10.5
n-Decane	1000	1000	2.1	-	2.2	2.0	-	1.5
β-Phellandrene	1026	1024	1.2	-	-	-	-	1.5
Limonene	1030	1030	13.2	6.5	21.5	15.7	6.7	11.6
Z-β-Ocimene	1038	1040	13.7	5.2	8.9	5.8	4.1	11.0
E-β-Ocimene	1051	1050	245.6	55.1	98.7	47.3	62.3	178.4
γ-Terpinene	1061	1060	2.0	1.4	3.1	-	-	4.0
Terpinolene	1089	1088	2.5	1.5	3.5	2.0	-	3.4
n-Undecane	1100	1100	41.0	49.7	44.4	36.6	18.6	45.5
n-Nonanal	1104	1103	2.1	-	-	-	-	2.9
2,2,6-Trimethyl hepta-3,5-dione	1165	1162	0.8	-	-	-	-	1.8
Terpinen-4-ol	1178	1177	2.2	3.2	3.6	-	7.4	3.6
α-Terpineol	1191	1189	4.3	2.5	9.2	3.7	-	6.7
n-Decanal	1205	1205	62.4	35.4	88.8	24.1	71.2	82.3
n-Decanol	1273	1276	5.3	-	5.0	-	-	13.1
δ-Elemene	1338	1337	10.6	6.8	5.4	2.7	3.6	9.1
α-Cubebene	1351	1348	35.1	55.4	50.0	34.7	20.6	35.5
α-Copaene	1374	1374	8.6	6.2	-	-	-	5.4
β-Patchoulene	1375	1376	24.9	40.1	46.4	35.4	20.9	23.1
β-Bourbonene	1384	1383	29.0	9.2	40.1	31.2	19.5	24.5
β-Elemene	1390	1390	225.6	249.5	330.7	224.5	173.4	199.0
iso-Italicene	1396	1395	11.8	18.3	20.3	13.0	7.9	13.4
α-Cedrene	1409	1409	33.9	43.0	30.2	15.7	12.4	21.7

Compound	KI(1)	KI(2)	June	Sept.	Nov.	Jan.	April	June
(E)-Caryophyllene	1417	1416	357.3	210.7	86.6	57.4	95.2	279.3
β-Gurjunene	1432	1427	27.0	34.9	34.2	25.3	15.7	23.2
Aromadendrene	1443	1442	34.0	41.6	26.6	16.9	12.7	21.5
α-Humulene	1447	1447	9.9	13.7	10.9	6.7	4.9	7.5
allo-Aromadendrene	1452	1450	25.5	16.8	9.5	5.2	7.7	21.4
Germacrene D Isomer	1462	1463	2.7	4.3	-	-	-	1.8
γ-Muurolene	1476	1473	5.6	7.7	5.3	5.2	0.7	8.0
Germacrene D	1481	1478	642.7	383.9	183.6	101.4	156.0	517.9
β-Selinene	1482	1482	17.6	7.9	6.9	7.3	0.7	3.7
α-Selinene	1494	1486	2.1	2.0	-	-	-	7.4
β-Guaiene	1496	1492	75.7	48.5	37.9	22.8	29.1	61.7
α-Muurolene	1498	1497	6.3	6.3	4.3	2.0	-	5.7
(E,E)-*α*-Farnesene	1506	1498	17.8	22.7	31.0	16.6	15.8	15.6
γ-Cadinene	1512	1508	37.5	29.4	23.8	10.5	14.9	19.1
δ-Cadinene	1522	1521	30.0	16.9	12.8	8.0	10.2	24.7
Dimethyl-4-Isopropyl-Bicyclo(4,4,0)-1,4-Decadiene	1526	1526	28.7	55.0	140.3	35.2	61.7	25.1
α-Calacorene	1543	1543	4.3	2.6	-	-	-	2.0
E-Nerolidol	1563	1563	5.0	2.1	-	-	-	4.3
Spathulenol	1575	1576	6.2	17.6	6.3	2.9	5.1	9.7
Caryophyllene oxide	1581	1578	16.4	18.2	3.4	2.2	2.6	20.8
Globulol	1587	1587	6.4	-	-	-	-	7.9
Viridiflorol	1591	1592	22.2	36.8	33.3	13.9	30.6	24.8
β-Copaen-4-*α*-ol	1596	1597	19.0	9.6	11.4	4.6	9.4	18.4
β-Oplopenone	1612	1607	19.3	27.9	24.4	9.6	20.7	21.2
epi-*α*-Cadinol	1639	1639	20.9	7.2	9.1	2.0	2.7	20.2
epi-*α*-Muurolol	1643	1642	10.9	5.4	-	-	-	10.9
δ-Cadinol	1653	1650	42.9	26.7	33.0	19.1	19.5	50.2
Z-*α*-Santalol	1670	1668	5.7	3.1	-	-	-	2.6
epi-*α*-Bisabolol	1677	1674	13.1	9.8	8.5	2.9	-	6.5
n-Heptadecane	1700	1700	1.0	2.7	-	-	-	0.8
n-Octadecane	1800	1800	0.2	-	-	-	-	-
n-Nonadecane	1900	1900	0.2	-	-	-	-	-
n-Eicosane	2000	2000	0.2	-	-	-	-	-
n-Heneicosane	2100	2100	0.4	-	2.4	-	-	-
n-Docosane	2200	2200	0.8	-	-	-	-	-
n-Tricosane	2300	2300	1.6	1.3	-	2.1	6.7	-
n-Tetracosane	2400	2400	0.8	1.4	3.1	4.3	7.8	-
n-Pentacosane	2500	2500	6.7	9.2	15.4	18.7	74.0	2.7
n-Hexacosane	2600	2600	0.3	1.2	-	1.7	5.2	-
n-Heptacosane	2700	2700	4.9	17.1	5.5	14.8	72.6	-
n-Octacosane	2800	2800	0.1	-	-	1.3	3.2	-
n-Nonacosane	2900	2900	0.6	3.6	-	3.7	26.6	-

Compound	KI(1)	KI(2)	June	Sept.	Nov.	Jan.	April	June
Monoterpene Hydrocarbons			909.7	417.9	1351.6	1179.7	460.6	710.7
Oxygenated Monoterpenes			7.3	5.7	12.8	3.7	7.4	8.9
Sesquiterpene Hydrocarbons			1693.7	1298.8	1015.0	651.7	630.3	1386.3
Oxygenated Sesquiterpenes			171.3	164.4	129.3	57.2	90.6	139.5
***n*-Alkanes**			2501.8	2056.9	2759.0	2627.5	1774.1	2168.6
Others			348.8	248.7	319.5	80.2	615.9	941.5

KI- Kovats retention index on a DB-5 column; **KI(1)** – KI for GC-MS; **KI(2)** – KI for GC

The EO of *H. undulatum* were characterized by high contents of *n*-alkanes. The highest amount of those compounds occurred in November followed by monoterpene hydrocarbons. However, over the year, with the exception of later autumn and winter, sesquiterpene hydrocarbons were the second most represented group of compounds. Over the year, the constituents that most contributed to the variation of the major compound groups in the essential oils of plants of *H. undulatum* were: *n*-nonane (*n*-alkanes); α-pinene, β-pinene and *E*-β-ocimene (Monoterpene Hydrocarbons); and β-elemene, (*E*)-caryophyllene and germacrene D (Sesquiterpene Hydrocarbons).

The percentage composition of the essential oils of *in vitro* shoots of *H. undulatum* is shown in Table S2.

Table S2 - Composition (%) of the essential oils from *in vitro* shoots of *Hypericum undulatum* grown over 60 days on MS or Mg basal media without any growth regulator.

Compound	KI	10 days		20 days		30 days		40 days		50 days		60 days	
		MS	Mg	MS	Mg	MS	Mg	MS	Mg	MS	Mg	MS	Mg
n-Octane	800						1.3		0.5		0.8		0.9
E-2-Hexenal	854					0.9	1.2	0.4	0.8	0.6	0.7	0.8	0.9
Hexanol	863					0.9		0.7		0.6		0.6	
n-Nonane	900	75.1	78.5	65.1	72.2	57.6	63.2	62.2	63.6	49.9	71.2	54.3	66.6
α-Pinene	936	4.2	3.8	2.7	2.3	2.6	2.5	2.3	2.0	1.6	1.5	2.7	1.8
Camphene	950					tr		tr	tr	0.3	1.0	0.4	0.9
β-Pinene	978	4.2	3.8	9.2	10.2	6.0	7.8	5.3	6.2	4.2	4.2	6.3	5.6
Myrcene	991					0.3		tr		0.1		0.4	
n-Decane	1000					tr		tr				0.2	
Limonene	1030					tr		tr		tr		tr	
E-β-Ocimene	1051			0.8		tr	tr	tr	tr	0.3	tr	0.4	tr
n-Undecane	1100	8.3	7.5	4.3	4.6	5.8	6.8	5.1	5.2	5.1	4.7	4.6	3.6
α-Terpineol	1190					tr		tr		0.4		0.4	
n-Decanal	1205	tr		tr		tr		tr		0.1		0.1	
n-Tridecane	1300	tr		tr		tr		0.2		0.1		0.1	
δ-Elemene	1337			1.4		1.1	1.2	0.8	0.7	1.7	1.0	1.9	0.9
α-Cubebene	1348	tr	tr	2.2	3.4	1.7	2.8	2.1	3.7	1.6	2.3	2.3	3.3

Compound	KI	10 days		20 days		30 days		40 days		50 days		60 days	
		MS	Mg	MS	Mg	MS	Mg	MS	Mg	MS	Mg	MS	Mg
α-Copaene	1374	tr	tr	0.6	1.3	0.9	1.9	1.3	2.0	0.9	1.5	1.2	1.5
β-Bourbonene	1383					tr	tr	tr	tr	0.3	tr	0.4	tr
β-Elemene	1390	4.2	4.4	2.2	2.3	2.6	4.1	2.5	3.7	2.1	2.3	3.0	3.3
SH	1392		tr	tr	tr	0.9	1.2	0.7	0.8	0.5	0.7	0.7	0.9
iso-Italicene	1395					tr		0.2		0.3		0.4	
Italicene	1405					tr		0.2		0.3		0.1	
α-Cedrene	1409			tr	tr	0.9	1.2	0.7	0.8	0.5	0.7	0.7	0.9
(E)-Caryophyllene	1417					0.3	0.6	0.4	1.3	0.3	1.0	0.4	0.9
β-Gurjunene	1427			tr		0.9	1.9	0.7	1.3	0.6	1.0	0.7	0.9
Aromadendrene	1442					0.6	tr	0.7	tr	0.4	0.3	0.7	0.9
α-Humulene	1447									0.2		0.4	
allo-Aromadendrene	1450	tr		tr		0.9		0.7		0.6		0.7	
Germacrene D	1480	tr		1.4	2.3	0.6	0.6	1.0	0.8	0.1	0.3	0.2	tr
β-Selinene	1482				tr		tr		0.2	0.9	1.5	1.5	2.1
α-Selinene	1486									0.3		0.4	
β-Guaiene	1492					0.3	0.6	0.4	0.5	0.1	0.2	0.4	0.9
α-Muurolene	1497			tr		tr		0.5		0.1		0.2	
(E,E)-*α*-Farnesene	1498					1.0	tr	tr	0.2	0.1	0.3	0.8	0.9
γ-Cadinene	1508	1.3		2.9		1.1		1.7		1.3		2.1	
δ-Cadinene	1521	2.7	2.0	2.2	1.3	1.7	1.2	2.3	2.0	1.0	1.0	1.9	0.9
SH	1530					tr		0.2		0.3		1.9	
α-Cadinene	1534			2.7		4.3		3.7		19.3		3.3	
E-Nerolidol	1563			tr		tr							
Viridiflorol	1591			1.4		1.0		tr		tr		tr	
β-Copaen-4-*α*-ol	1596					0.3		0.2		0.3		tr	
β-Oplopenone	1612									tr		0.1	
epi-*α*-Bisabolol	1674			tr		0.3		0.2		0.1		0.1	
n-Heptadecane	1694			0.6		0.3		tr				tr	
Z-*α*- *trans*-Bergamotol	1700			0.6		0.3		0.2		0.1		0.1	
n-Octadecane	1800			tr		0.3		0.2		tr		0.1	
n-Nonadecane	1900			tr		0.3		tr		tr		tr	
n-Eicosane	2000	tr		tr		0.3		tr		0.1		0.1	
n-Heneicosane	2100			tr		0.3		tr		tr		tr	
n-Docosane	2200			tr		0.3		tr		0.1		0.1	
n-Tricosane	2300	tr		tr		tr		tr		tr		tr	
n-Tetracosane	2400			tr		0.3		tr		0.1		0.1	
n-Pentacosane	2500			tr		0.3		tr		tr		0.1	
n-Hexacosane	2600			tr		tr		tr		tr		tr	
n-Heptacosane	2700	tr		tr	tr	1.2	tr	1.1	2.0	1.0	1.0	1.1	0.9
n-Octacosane	2800			tr		tr		tr		0.1		0.1	
n-Nonacosane	2900	tr		tr	tr	1.5	tr	1.3	1.3	0.8	1.0	0.6	0.8
Monoterpene Hydrocarbons		8.34	7.6	12.7	12.5	8.8	10.4	7.7	8.3	6.5	6.6	10.0	8.3
Oxygenated Monoterpenes								<0.1		0.4		0.4	
Sesquiterpene Hydrocarbons		8.23	6.4	15.4	10.7	19.6	17.2	20.7	18.3	33.9	14.0	26.2	18.1
Oxygenated Sesquiterpenes				2.0		1.8		0.4		0.4		0.2	
***n*-Alkanes**		83.43	86.0	70.0	76.8	68.1	71.3	70.2	72.7	57.4	78.7	61.7	72.7
Others				<0.1		1.7	1.2	1.1		1.3	0.7	1.6	0.9

The essential oils of *in vitro* shoots grown on MS and Mg basal media were characterized by high amounts of *n*-alkanes, followed by the terpene hydrocarbons and oxygenated terpenes. Over the 60 days of culture, the constituents that most contributed to the variation of the major compound groups in the essential oils of *in vitro* shoots grown on the two culture media were: *n*-nonane and *n*-undecane (*n*-alkanes), α-pinene and β-pinene (Monoterpene Hydrocarbons), and β-elemene (Sesquiterpene Hydrocarbons).

Chapter 6

IN VITRO APPROACHES FOR *HYPERICUM* GENETIC TRANSFORMATION

6.1- Chapter overview

Gene transfer by *Agrobacterium* is the method of choice for the genetic transformation of most plant species and a possible strategy to enhance production of secondary metabolites in plant cultures. *A. rhizogenes* infection induces the production of hairy roots in plant cells, which according to some authors hold high growth rates and genetic stability (Canto-Canché *et al.*, 1999). Nevertheless, several parameters are known to affect T-DNA transfer and integration into the plant genome. Thus, a transformation protocol depends on the establishment of a reliable plant regeneration system as well as on the efficiency of *Agrobacterium*-plant interaction. The transformation of *H. perforatum* mediated by *A. rhizogenes* was already reported (Di Guardo *et al.*, 2003; Vinterhalter *et al.*, 2006). According to the authors, spontaneous shoot regeneration occurred from the hairy roots.

In order to establish a transformation protocol of *H. androsaemum, H. perforatum* and *H. undulatum* mediated by *A. rhizogenes* A4, several approaches were tried by manipulating the following parameters: z explant pre-culture, (ii) bacterial density, (iii) type of explant, (iv) explant wounding, (v) addition of acetosyringone to the bacterial suspension and co-culture medium, and (vi) co-culture period.

Notwithstanding the above-mentioned reports, on successful production of *H. perforatum* hairy roots, in this work, the production of hairy roots was not achieved. The efforts made included the use of leaves, internodal segments and root segments of the studied species as start material for genetic transformation. A reduction on the bacteria viability during co-cultivation with plant cells, probably caused by the release of antimicrobial substances from the plant cells, has been previously pointed out as a possible reason for *H. perforatum* recalcitrance to *A. rhizogenes* A4 (Franklin *et al.*, 2008). In this work, the antimicrobial activity of *H. androsaemum, H. perforatum* and *H. undulatum* against *A. rhizogenes* A4 was assayed using powdered dried biomass added to the bacteria growth medium culture. The results from these preliminary assays, however, were inconclusive.

Canto-Canché, B. & V. Loyola-Vargas (1999). Chemicals from roots, hairy roots, and their application. *Chemicals via higher plant bioengineering*. Shahidi. NewYork, Kluwer Academic/Plenum Publishers. **23:** 5−244.

Di Guardo, A., E. Cellàrovà, J. Koperdakova, L. Pistelli, B. Ruffoni, A. Allavena & A. Giovannini (2003). Hairy root induction and plant regeneration in *Hypericum perforatum* L. *J. Genet. Breed. 57*, 269–278.

Franklin, G., L. Conceição, E. Kombrink & A. Dias (2008). *Hypericum perforatum* plant cells reduce *Agrobacterium* viability during co-cultivation. *Planta 227*, 1401–1408.

Vinterhalter, B., S. Ninkovic, A. Cingel & D. Vinterhalter (2006). Shoot and root culture of *Hypericum perforatum* L. transformed with *Agrobacterium rhizogenes* A4M70GUS. *Biologia Plantarum 50*(4), 767-770.

In vitro Approaches for *Hypericum* Genetic Transformation

1- Introduction

Agrobacterium rhizogenes, a gram-negative, soil-borne plant pathogen has the ability to insert foreign DNA sequences into the plant genome. The major advantage of *Agrobacterium*-mediated gene transfer over biolistics is its ability to integrate fewer copies of foreign inserts into the plant genome, thereby reducing the risk of transgene rearrangements and gene silencing (Veluthambi *et al.*, 2003). Genetic transformation by *A. rhizogenes* is characterized by the production of neoplastic roots which are known to have a high growth rate and genetic stability (Canto-Canché *et al.*, 1999). The *rolA*, *rolB* and *rolC* oncogenes, isolated from T-DNA of the *A. rhizogenes* A4-Ri plasmids, were shown to induce root formation in transformed plant cells (Cardarelli *et al.*, 1987; Spena *et al.*, 1987). In fact, hairy root disease following agrobacterial infection is caused by the integration in the plant genome of the *rolA*, *rolB*, *rolC* and *rolD* genes that are carried on plasmids of *A. rhizogenes* (Giri *et al.*, 2000; Bulgakov, 2008). The *rol* genes may affect either the metabolism of plant hormones or the sensitivity of plant cells to hormones (Meyer *et al.*, 2000). As a group, *rol* genes have been suggested to cause an increase in auxin sensitivity (Spena *et al.*, 1987), as in *rolABC* transformed tobacco (Spanò *et al.*, 1988), tomato (van Altvorst *et al.*, 1992) and rose leaves (Spanò *et al.*, 1988). This discovery stimulated investigations that aimed to understand the effects of these genes upon plant development. Nowadays, a new function of the *rol* genes in plant-*Agrobacterium* interaction became apparent with the discovery that these genes are potential activators of secondary metabolism in transformed cells from the Solanaceae, Araliaceae, Rubiaceae, Vitaceae and Rosaceae plant families (Bulgakov, 2008). It was found that the accumulation of secondary metabolites in *rolABC*-transformed roots of tobacco and *Catharanthus roseus* was similar to that found in hairy roots induced by *Agrobacterium* wild type (Palazón *et al.*, 1997; Hong *et al.*, 2006). Roots of tobacco lines transformed with *rolABC* together or with *rolC* alone showed increased growing capacity and produced more nicotine, an alkaloid synthesized in the roots, than did the controls (Palazón *et al.*, 1998). According to Bulgakov (2008), the ability of hairy roots to produce high amounts of secondary metabolites is an interesting natural phenomenon, which raises the question of which gene loci of T-DNA are responsible for this effect. Considering that T-DNA *rol* gene loci have a large impact on diverse biochemical processes in transformed plant cells, it is reasonable to consider that the essential genetic determinants are the *rol* genes of *A. rhizogenes.* Although a possible impact of additional T-DNA genes on secondary

metabolism in hairy roots cannot be excluded, the influence of such hypothetical genes seems to be less prominent (Bulgakov, 2008).

Hairy roots of several plant species have already been reported for their capacity to produce a large variety of phytochemicals, some of those with putative interest to the pharmaceutical industry. For example, scopolamine, the most valuable tropane alkaloid used in medicine, is produced in the roots of several species, such as *Hyoscyamus muticus*. Transformation of this species with *A. rhizogenes* containing the gene encoding the enzyme that synthesizes the desired metabolite resulted in the production of hairy roots with enhanced levels of scopolamine (Jouhikainen *et al.*, 1999). Other examples are the *A. rhizogenes*-mediated transformation of *Valeriana wallichii* and *Panax ginseng*, in which induced hairy roots produced a two- to threefold increase in valepotriates and ginsenoside levels, respectively (Banerjee *et al.*, 1998; Bulgakov *et al.*, 1998).

Transformation of plants with *A. rhizogenes* can also result on the production of transgenic regenerated plants from hairy roots under *in vitro* culture conditions as it has been already reported for some species (Oksman-Caldentey *et al.*, 1991; Handa, 1992; Christey, 2001; Vinterhalter *et al.*, 2006). Such transgenic plants regenerated from hairy roots display a characteristic phenotype which includes reduced apical dominance in both stems and roots, shortened internodes, wrinkled and wider leaves, adventitious root production, altered flower morphology, late flowering and reduced pollen and seed production (Tepfer, 1984; Christey, 2001). These combined symptoms are termed hairy-root phenotype and can be observed in a number of species, although to varying degrees, depending on the species or the clone within the same species, being also possible the formation of morphological variants from the same root clone (Tepfer, 1984). The typical hairy-root phenotype has also been reported to be inheritable, although in some cases lateral shoots of hairy-root plants revert to the normal phenotype without losing the T-DNA (Tepfer, 1984). The advantage of Ri (root-inducing) plasmid based gene transfer is that spontaneous shoot regeneration is obtained avoiding the callus phase and genetic somaclonal variations. Ri plasmid-based gene transfer affords also a higher rate of transformation and regeneration of transgenic plants without the use of a selection agent, thereby allowing high rate of co-transfer of genes on binary vector without selection (Giri *et al.*, 2000). Further, *Agrobacterium tumefaciens* mediated transformation results in a high frequency of escapes whereas *A. rhizogenes* mediated transformation consistently yields only transformed cells that can be obtained after several cycles of root tip cultures. The hairy roots can be maintained by subculturing for a long time and subsequent shoot regeneration can be obtained (Giri *et al.*, 2000). Rapid growth of hairy roots on hormone-free medium followed

by high plantlet regeneration allows the clonal propagation of elite plants. The indirect effect of Ri plasmid T-DNA on the increase in essential oils, and thus on the fragrance of certain plants, may be an additional benefit of *rol* genes when introduced into ornamental plants such as lemon geranium (Pellegrineschi *et al.*, 1994). Spontaneous shoot regeneration from hairy roots of lemon geranium with increased production of geraniol and other aromatic substances was reported (Pellegrineschi *et al.*, 1997). The use of *rol* genes, together with other genes codifying for enzymes from biosynthetic pathways of odorous components, opens new perspectives on improving scent in ornamental flowers (Casanova *et al.*, 2005). In some cases variability of secondary metabolite production in transgenic plants can occur, as suggested by some authors, as a consequence of the insertion of different numbers of gene copies and/or different insertion sites of the foreign T-DNA in the plant genome or yet, as a consequence of the silencing of genes involved in the secondary metabolism (Giri *et al.*, 2000).

Nevertheless, developing a transformation system requires not only a reliable regeneration system, but also an efficient cell transformation procedure, a mechanism for selection of the transformed cells, and antibiotic conditions for the inhibition of *Agrobacterium* growth in the *in vitro* culture post-infection (Ellis *et al*., 1989). Yet it is essential that the conditions used for each of these steps do not interfere with plant tissue regeneration. For *H. perforatum* two transformation protocols mediated by *A. rhizogenes* has been published (Di Guardo *et al.*, 2003; Vinterhalter *et al.*, 2006). In both transformation procedures the spontaneous regeneration of shoots from hairy roots was observed. Previous studies showed the presence of hypericin in two transformed plant lines of *H. perforatum* obtained by hairy roots induced from *in vitro* root and leaf tissues with the *A. rhizogenes* ATCC 15834 strain (Di Guardo *et al.*, 2003; Kornfeld *et al.*, 2007). However, no study has been published on the genetic transformation of *H. androsaemum* and *H. undulatum*. Since our initial attempts to develop an *Agrobacterium*-mediated transformation system were hampered by explants browning and subsequent death, we focused on exploring the hypothetical factors that may influence *A. rhizogenes*-mediated transformation of *H. androsaemum*, *H. perforatum* and *H. undulatum*. Thus, in the present study, several approaches were assayed in order to evaluate several parameters, such as:

- sensitivity of *H. androsaemum*, *H. perforatum* and *H. undulatum* explants to the antibiotics cefotaxime and carbenicillin;
- influence of preculturing period;
- influence of the methods of co-cultivation.

2- Material and Methods

2.1- Plant materials and tissue culture conditions

In vitro shoot cultures of *H. androsaemum*, *H. perforatum* and *H. undulatum* were maintained on MS basal medium free of growth regulators in a growth room at 25±2ºC with a photoperiod of 16h light/8h dark. Leaves, internodal segments and root segments of 30-40 days old shoots of the three *Hypericum* species were excised, under aseptic conditions, cultivated on MS basal medium and used in the transformation procedures mediated by the wild type *Agrobacterium rhizogenes* A4.

2.2- Assays with the antibiotics, cefotaxime and carbenicillin on Hypericum in vitro *cultures*

Cefotaxime and carbenicillin, the antibiotics used in this work, are commonly used for counter-selection of *Agrobacterium* (Yepes *et al.*, 1994; Shackelford *et al.*, 1996). To investigate its effects in the plant regeneration, leaves, internodal segments and root portions of *in vitro* shoots, from the three *Hypericum* species, were cultured on MS basal medium with both antibiotics at varying concentrations ranging from 0, 150 and 200µg/ml. The excised explants were cultured on the MS basal medium free of growth regulators and supplemented with both cefotaxime (0, 150 and 200µg/ml) and carbenicillin (0, 150 and 200µg/ml). Both antibiotics were filter sterilized through a 0.22 µm membrane (Millipore). Aliquots of stock solutions of all these antibiotics were dispensed individually into 1.5 capacity eppendorf tubes and stored at -20ºC till further use. Antibiotic solutions were aseptically added to the autoclaved MS basal medium before the media were poured into the Petri dishes. The explants inoculated in Petri dishes were kept at 25ºC under 16 h photoperiod of white fluorescent lamps (52 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Regeneration was registered after 30 days of culture.

2.3- Agrobacterium rhizogenes *culture*

The wild type *A. rhizogenes* strain A4 used for *Hypericum* sp. transformation was grown in MYB medium [5 g/L yeast extract, 8 g/L mannitol, 0.5 g/L Casamino acids, 2 g/L $(NH_4)_2SO_4$ and 5 g/L NaCl, pH 6.6], solidified with agar (20g/L) for 48h. The suspension culture for tissue inoculation was prepared by growing a single *A. rhizogenes* colony in 5.0 ml of MYB liquid medium overnight at 28ºC with continuous rotary shaking (200 rpm). Subsequently, 1.0 ml of the bacterial suspension was transferred into 9.0 ml of fresh MYB liquid medium and maintained under similar conditions. After the bacterial culture reached the

optical density (OD) of 0.6 at 600 nm, it was diluted (1:3) in liquid MS basal medium without growth regulators and used to infect plant explants.

2.4- Assays for A. rhizogenes-*mediated transformation of* H. androsaemum, H. perforatum *and* H. undulatum

In preliminary experiments (designated assay ECO2 in Table 1), explants (leaves, internodal segments and roots) were infected by wounding the tissues with a scalpel previously immersed in the diluted bacterial solution. Co-cultivation was performed on MS basal medium without growth regulators in dark for 3 days. After co-cultivation, all the explants were transferred to MS basal medium supplemented with the antibiotics carbenicillin (150µg/ml) and cefotaxime (150µg/ml) to eliminate *A. rhizogenes*. Control explants were cultured in the same conditions, after the respective tissues have been wounded with the scalpel previously immersed in liquid MS basal medium without growth regulators. After 30 days, roots developed from control and from co-cultivated explants were excised from the explants and cultivated on MS basal medium without growth regulators. The number of explants with regenerated roots and the number of browning death explants were counted after that period.

2.5- Approaches for the optimization of a transformation protocol of H. androsaemum, H. perforatum *and* H. undulatum *species by* A. rhizogenes

In order to get a suitable transformation protocol for hairy roots induced by *A. rhizogenes* of the three species of *Hypericum*, several parameters were evaluated. Table 1 summarizes the various assays performed using leaves (L), internodal segments (In) and root segments (R) obtained from *in vitro* shoots of *H. androsaemum*, *H. perforatum* and *H. undulatum* as plant material target of the genetic transformation. In assays EPC2 and EPC4, the pre-culture of explants was performed by culturing leaves, internodal segments and root segments on MS basal medium devoid of growth regulators and maintained in a growth room at 25±2ºC with a photoperiod of 16h light/8h dark, for 2 and 4 days, respectively, before *Agrobacterium* infection. Ten *in vitro* plantlets of each one of the three *Hypericum* species were also wounded with the scalpel blade previously immersed in a diluted (1:3) bacterial suspension in liquid MS basal medium without growth regulators.

Table 1 - Different transformation conditions of *H. androsaemum*, *H. perforatum* and *H. undulatum* mediated by the wild type *A. rhizogenes* strain A4

Assay	Bacterial concentration (OD at 600nm)	Pre-culture period (days)	Co-cultivation period (hours)	[AS] bacterial suspension (µM)	[AS] co-culture medium (µM)	Explant	Infection method
ECO1	0.6	-	48	-	-	L / In / R	wounding
ECO2	0.6	-	72	-	-	L / In / R	wounding
ECO3	0.6	-	96	-	-	L / In / R	wounding
ECO4	0.6	-	120	-	-	L / In / R	wounding
EPC0	0.6	0	72	-	-	L / In / R	wounding
EPC2	0.6	2	72	-	-	L / In / R	wounding
EPC4	0.6	4	72	-	-	L / In / R	wounding
EAS0	0.6	-	72	0	-	L / In / R	wounding
EAS20	0.6	-	72	20	-	L / In / R	wounding
EAS100	0.6	-	72	100	-	L / In / R	wounding
EAS200	0.6	-	72	200	-	L / In / R	wounding
EAC20	0.6	-	72	-	20	L / In / R	wounding
EAC100	0.6	-	72	-	100	L / In / R	wounding
EAC200	0.6	-	72	-	200	L / In / R	wounding
EASC20	0.6	-	72	20	20	L / In / R	wounding
EASC100	0.6	-	72	100	100	L / In / R	wounding
EASC200	0.6	-	72	200	200	L / In / R	wounding
EB0.4	0.4	-	72	-	-	L / In / R	wounding
EB0.6	0.6	-	72	-	-	L / In / R	wounding
EB0.8	0.8	-	72	-	-	L / In / R	wounding
EB1.0	1.0	-	72	-	-	L / In / R	wounding
EI10	0.6	-	72	-	-	L / In / R	immersion (10 min)
EI20	0.6	-	72	-	-	L / In / R	immersion (20 min)
EI30	0.6	-	72	-	-	L / In / R	immersion (30 min)
EIASC20	0.6	-	72	20	20	L / In / R	immersion (20 min)
EIASC100	0.6	-	72	100	100	L / In / R	immersion (20 min)
EIASC200	0.6	-	72	200	200	L / In / R	immersion (20 min)
EIB0.6	0.6	-	72	-	-	L / In / R	immersion (20 min)
EIB0.8	0.8	-	72	-	-	L / In / R	immersion (20 min)
EIB1.0	1.0	-	72	-	-	L / In / R	immersion (20 min)

OD- Optical density

[AS]- Acetosyringone concentration

L- leaves; In- internodal segments; R- root segments

2.6- Assays for the evaluation of antibacterial activity of Hypericum sp.

In order to determine an hypothetical antimicrobial activity of *Hypericum* against *A. rhizogenes* A4, *in vitro* cultures of *H. androsaemum, H. perforatum* and *H. undulatum* were freeze dried and powdered and 2g of biomass powder of each one were directly added to the MYB medium (15ml). In the first experiment, the dried powder of each *Hypericum* species was aseptically added to the autoclavated MYB medium before the media were poured into the Petri dishes. In a second experiment, the dried powder of each *Hypericum* species was added to the MYB medium before being autoclavated at 121ºC for 20 min.

The suspension culture of the wild type *A. rhizogenes* strain A4 used for inoculation in the MYB medium with the plant powder was prepared in the same way as for the transformation procedure. When the bacterial culture reached the optical density (OD) of 0.6 at 600 nm, it was diluted (1:3) in liquid MYB medium and inoculated in MYB medium with the plant powder. After 48 hours the bacterial growth was evaluated.

3- Results

3.1- Sensitivity of H. androsaemum, H. perforatum *and* H. undulatum *explants to the antibiotics cefotaxime and carbenicillin*

The effects of cefotaxime and carbenicillin on the *in vitro* cultures of *H. androsaemum*, *H. perforatum* and *H. undulatum*, 4 weeks after the beginning of the culture, are summarised in the Tables 2, 3 and 4.

Table 2 – Organ formation (%) from *H. androsaemum* explants (leaves, internodal segments and root segments) after 4 weeks of culture on MS basal medium devoid of growth regulators and supplemented with both cefotaxime and carbenicillin.

Explant	Cef/Carb (µg/ml)	Nº of explants	Explants with shoots (%)	Explants with roots (%)	Explants with *callus* (%)	Necrotic explants (%)
L	0/0 (CT)	30	0	40	0	60
	150/150	30	0	33	0	67
	200/200	30	0	20	0	80
In	0/0 (CT)	30	20	0	80	0
	150/150	30	17	0	73	10
	200/200	30	0	0	60	40
R	0/0 (CT)	30	0	20	10	70
	150/150	30	0	10	10	80
	200/200	30	0	7	0	93

L- leaves; In- internodal segments; R- root segments

cef- Cefotaxime; carb- Carbenicillin

The results from the treatment of leaves of *H. androsaemum* with 150 μg/ml of cef/carb were not significantly different from the control, as root regeneration occurred in similar ratio in both culture conditions. Shoots and *calli* were induced from internodal segments in control cultures as well as in those ones cultivated with cef/carb (150μg/ml). However, as it can be seen in Table 2 there was no induction of roots in this type of explant, in any of the tested conditions. Root segments were the explants with a lower capacity of regeneration in all the three conditions of culture. However, the increase in the antibiotics concentration influenced negatively the responses of the explants to the medium. Comparing to the control, the treatments with cef/carb 200/200 μg/ml decreased roots formation from 40 to 20% in leaves and from 20 to 7% in root explants and inhibited completely the shoot regeneration from internode explants (Table 2).

Table 3 – Organ formation (%) from *H. perforatum* explants (leaves, internodal segments and root segments) after 4 weeks of culture in MS basal medium devoid of growth regulators supplemented with both cefotaxime and carbenicillin.

Explant	Cef/Carb (μg/ml)	Nº of explants	Explants with shoots (%)	Explants with roots (%)	Explants with *callus* (%)	Necrotic explants (%)
L	0/0 (CT)	30	0	47	0	53
	150/150	30	0	30	10	60
	200/200	30	0	17	0	83
In	0/0 (CT)	30	10	17	70	3
	150/150	30	10	10	73	7
	200/200	30	7	0	70	23
R	0/0 (CT)	30	0	7	20	73
	150/150	30	0	10	17	73
	200/200	30	0	0	7	93

L- leaves; In- internodal segments; R- root segments
cef- Cefotaxime; carb- Carbenicillin

Such as it happened with *H. androsaemum*, no shoot regeneration occurred from *H. perforatum* leaves independently of the presence or absence of cefotaxime and carbenicillin (Table 3). A decreasing effect on roots formation, from the petiole zone was however recorded with the increasing of the antibiotics. Root segments were that ones with the highest rate of explant death. More than 20 internodal segments had the formation of *callus* in all the culture conditions. The regenerated *calli* were mainly localized in the borders of the explants. Similarly to *H. androsaemum,* shoot regeneration occurred only from internodal explants of *H. perforatum* decreasing however in the presence of 200/200 μg/ml of antibiotics.

Table 4 – Organ formation (%) from *H. undulatum* explants (leaves, internodal segments and root segments) after 4 weeks of culture in MS basal medium devoid of growth regulators supplemented with both cefotaxime and carbenicillin.

Explant	Cef/Carb (µg/ml)	Nº of explants	Explants with shoots (%)	Explants with roots (%)	Explants with *callus* (%)	Necrotic explants (%)
L	0/0 (CT)	30	0	40	0	80
	150/150	30	0	35	0	75
	200/200	30	0	15	0	90
In	0/0 (CT)	30	20	0	77	3
	150/150	30	13	0	63	10
	200/200	30	3	0	67	30
R	0/0 (CT)	30	0	10	0	90
	150/150	30	0	7	0	83
	200/200	30	0	0	3	97

L- leaves; In- internodal segments; R- root segments

cef- Cefotaxime; carb- Carbenicillin

No shoots regenerated from leaves and root segments of *H. undulatum* such as it happened with the other two *Hypericum* species (Table 4). Shoot regeneration occurred from nodal segments decreasing however, proportionately with the antibiotics concentration (Table 4). Induction of *calli* was favoured from internodal segments (more than 60% in the presence of both concentrations of antibiotics). However, the induction of roots was not observed from this type of explant. In previous reports on *H. perforatum* transformation, only one antibiotic was used. Di Guardo *et al*. (2003) and Vinterhalter *et al.* (2006) eliminated the bacteria used in the transformation protocol with cefotaxime at the concentrations of 100mg/L and 200mg/L, respectively, while Franklin and co-workers (2007) used ticarcillin clavulanate (250 mg/L) for *Agrobacterium* elimination. The results obtained in this work might be related with the effect of antibiotics on plant morphogenesis. In fact, cefotaxime-sodium salt (cephalosporin) as well as carbenicillin-disodium salt belong to the β-lactam group of antibiotics, which bind to the 50S subunits of the prokaryote ribosome thereby interfering with protein synthesis. Both antibiotics inhibit cell wall synthesis in dividing bacterial cells resulting in their lysis (Young *et al.*, 1984; Mathias *et al.*, 1986). Cefotaxime has a broader antimicrobial spectrum and higher pharmacological activity than carbenicillin (Wise *et al.*, 1978; Doerr *et al.*, 1980). But apart from their anti-microbial activity, previous reports showed that these antibiotics influence the growth of the plant cultures, due to their plant hormone-like effects (Nauerby *et al.*, 1997). Indeed, cefotaxime was shown to influence morphogenesis in barley (Mathias *et al.*, 1986), wheat (Yu *et al.*, 2008), and apple (Yepes *et al.*, 1994), among others, while carbenicillin

inhibited the maturation of somatic embryos in *Picea sitchensis* (Sarma *et al.*, 1995). The carbenicillin effects on morphogenesis might be related with the formation of the degradation product from carbenicillin, phenylacetic acid, with auxin activity (Sarma *et al.*, 1995). It is conceivable that auxins produced by the breakdown of carbenicillin when combined with endogenously synthesized auxins can explain the positive effect of this antibiotic on the organogenesis of several species (Nakano *et al.*, 1993; Hammerschlag *et al.*, 1997; Yu *et al.*, 2001), as well as its negative effect on others (Yepes *et al.*, 1994; Nauerby *et al.*, 1997). On the other hand, it was suggested that cefotaxime was not broken down into auxin-like substances, being its effects explained by another mode of action (Holford *et al.*, 1992). Mathias and Boyd (1986) reported the possibility of metabolites, with plant growth regulatory activity, generated from cefotaxime by plant esterases, to be responsible for the above mentioned effects. In conclusion, although, cefotaxime and carbenicillin have been reported to be the best antibiotics for selective killing of *Agrobacterium* in several transformation experiments, any new plant culture system should be screened for antibiotic sensitivity prior to their use in eradicating *Agrobacterium* after co-cultivation. The optimum concentration of the selective agent should be that one that prevents regeneration without being toxic to the target explant (Yepes *et al.*, 1994).

Generally, after hairy roots induction, concentration of the antibiotic is gradually decreased in the next subcultures until no one is added to the culture medium. In conclusion, comparing with the control results, with the concentration of 150 μg/ml for both cefotaxime and carbenicillin all the explants had a positive response. However, the rate of regeneration from internodal segments of *Hypericum* sp. decreased when they were incubated on MS basal medium with both antibiotics at a concentration of 200 μg/ml. According to the results here described, cefotaxime and carbenicillin both at 150 μg/ml were used in the following approaches for genetic transformation of *H. androsaemum*, *H. perforatum* and *H. undulatum*, mediated by *A. rhizogenes* A4. For the three *Hypericum* species, among leaves, stem fragments, and root fragments, the best explants to be used in plant transformation seemed to be internodal segments, since they were the ones with a higher regeneration frequency even in the presence of the antibiotics cefotaxime and carbenicillin.

3.2- Influence of pre-cultivation period

During the pre-cultivation period, explants are usually cultivated in plant culture medium prior to bacterial infection, which can improve transformation efficiency because it accelerates cell division of explants and maintain cell activity, consequently favouring cell growth after transformation. For *Petunia* leaf explants, pre-cultivation in a medium with 2,4-D

and BAP during 2 to 3 days before co-cultivation with *Agrobacterium* drastically enhanced genetic transformation (Villemont *et al.*, 1997). The authors suggested that during pre-culture, cells were actively dividing and duplicating DNA, and at the moment of bacterial inoculation phytohormone-activated cell nuclei were being recruited into S-phase of cell cycle. Pre-culture periods seemed to attenuate the drastic decrease in regeneration capacity after co-cultivation (Dronne *et al.*, 1999). Proper phytohormone treatments during pre-culture period have also been used to overcome the reduced transformation susceptibility of the *Arabidopsis* mutant *uvh1*, which was previously considered resistant to *Agrobacterium*-mediated transformation (Chateau *et al.*, 2000). Pre-cultured leaf blade, petiole, stem and root segment explants of *H. perforatum* on MS medium supplemented with BA (0.5mg/l) and IAA (1.0 mg /l), for 24h, were unable to induce the formation of transgenic shoots after *Agrobacterium* transformations (Franklin *et al.*, 2007). On the other hand, hairy root production and regeneration of shoots from hairy roots of *H. perforatum* were achieved in the absence of a pre-culture period (Di Guardo *et al.*, 2003; Vinterhalter *et al.*, 2006). In the experiments here reported, the tentative made either with pre-culture periods ranging from 2 to 5 days or without it were not succeed as there was no hairy roots induced independently of the treatment used (Table 1).

3.3- Influence of the methods of co-cultivation

Co-cultivation is one of the most important steps for genetic transformation of plants, as it is during this step, that T-DNA is incorporated into plant genomic DNA. However, several factors can influence it.

3.3.1- Bacterial density

Different bacterial concentrations (0.4-1.0 of OD_{600}) of wild type *A. rhizogenes* A4 were used in the attempt to transform leaves, internodal segments and roots of *H. androsaemum*, *H. perforatum* and *H. undulatum* (Table 1). However, none of those experiments were succeed in the formation of hairy roots from any of the tested explants of any *Hypericum* species. Even for the higher concentrations of *A. rhizogenes* A4, the usually observed overgrowth of the bacteria during co-cultivation period was not registered. Lack of *H. perforatum* hairy roots production after inoculation with *A. rhizogenes* wild type strains A4 and LBA9402 (0.8-1.0 of OD_{660}) was also reported by Franklin and co-workers (2007). The same bacterial concentrations were also unsuccessfully used in the transformation of *H. perforatum* with *A. tumefaciens* LBA4404 and EHA105 (Franklin *et al.*, 2007). However, DiGuardo and co-workers reported the production of hairy roots of *H. perforatum* after inoculation of roots and leaf segments with bacterial suspensions of wild type *A. rhizogenes* ATCC 15834 diluted 1:10 (0.1 OD_{550}). Indeed, bacterial

concentration plays an important role in the production of transformed roots. Studies on other species has shown that suboptimal concentrations resulted in low availability of bacteria for transforming the plant cells while high concentrations decreased their potential by competitive inhibition (Kumar *et al.*, 1991; Tao *et al.*, 2006).

Besides bacterial density, genetic transformation mediated by *Agrobacterium* is also affected by different strains of the bacteria. Differences in the ability for infection of several *A. rhizogenes* strains in a given plant species have already been reported showing that *A. rhizogenes* strains vary in their transforming efficiency (Tepfer, 1990; Maldonado-Mendoza *et al.*, 1992; Vanhala *et al.*, 1995; Zehra *et al.*, 1998; Królicka *et al.*, 2001). Therefore, different bacterial strains have different hairy roots generating capacity. Nguyen and co-workers suggested that differences in virulence and morphology could be explained by the plasmids harboured by bacterial strains (Nguyen *et al.*, 1992). According to some authors the presence of specific signals from specific *Agrobacterium* strains, may be recognized by specific plant genotypes (Owens *et al.*, 1985; Byrne *et al.*, 1987; Hobbs *et al.*, 1989; Fillipone *et al.*, 1992). In addition, extensive morphological variation in individual hairy root cultures may be explained by differential expression of T-DNA genes present in the transformed roots, variable copy numbers of T-DNA inserts and positional integration effects of the T-DNA in the host genome (Cho *et al.*, 1998). In our study, wild type *A. rhizogenes* strain A4 was that one used in the attempts to transform *H. androsaemum*, *H. perforatum* and *H. undulatum*. However, both our results and those reported by Franklin *et al.* (2007) showed the incapacity of this strain for hairy roots induction from *Hypericum* explants. *A. rhizogenes* strain A4M70GUS as well as the wild type *A. rhizogenes* ATCC 15834, were shown to induce hairy roots formation in *H. perforatum* (Di Guardo *et al.*, 2003; Vinterhalter *et al.*, 2006). The first strain contains a GUS construct [*uidA* sequence under the 70S promoter (enhancer-doubled 35S Ca MV promoter), followed by NOS polyadenylation sequence] integrated into the TL region of the cointegrative plasmid pRiA4 (Tepfer *et al.*, 1987).

3.3.2- Effect of the type of explant in hairy root production of *Hypericum*

Previous results had shown that internodal segments of *H. androsaemum*, *H. perforatum* and *H. undulatum* would be the best explant for a plant transformation protocol, due to its higher regeneration frequencies, even in the presence of cefotaxime and carbenicillin. However, leaves and root segments of the three plant species were also infected with *A. rhizogenes* A4 (Table 1). In none of the explants, the *Agrobacterium* infection induced the formation of hairy roots, likewise the results reported by Franklin and co-workers (2007). Given the absence of

hairy roots in the *in vitro* explants, 10 *in vitro* plantlets of the three plant species were infected by wounding the stem with the scalpel blade previously immersed in the bacterial suspension. Nevertheless, once more, no hairy roots were induced. A similar infection method was used by Vinterhalter *et al.* (2006), who reported the formation of hairy roots by that approach. Di Guardo *et al.* (2003) obtained hairy roots from leaf and root fragments with another wild agropine type *A. rhizogenes* strain ATCC15834.

3.3.3- Effect of wounding the plant explants

Among the different strategies used to increase *Agrobacterium* transformation frequency, wounding plant tissues prior to inoculation was found be one of the most important. According to some authors, plant wounding seems to be a key factor in the interaction between *Agrobacterium* and the host plant cells, thereby affecting the efficiency of *Agrobacterium*-mediated gene transfer (Stachel *et al.*, 1985). Wounding stimulates DNA replication and proliferation of plant cells, improving the integration of the T-DNA (Villemont *et al.*, 1997). Wounding of explants also allows *Agrobacterium* to infect the target tissue as well as to produce the phenolic chemical signals (e.g. acetosyringone and α-hidroxiacetosyringone) that induce the T-DNA transfer (Stachel *et al.*, 1985). Hairy roots were induced from *H. perforatum* explants when they were wounded with a lancet blade (Di Guardo *et al.*, 2003) and from shoots wounded with a needle dipped in the bacterial suspension (Vinterhalter *et al.*, 2006). Two methods of infection of leaves, internodal segments and root segments were used in the work here reported: (a) immersion of the explants from *H. androsaemum*, *H. perforatum* and *H. undulatum* in the *A. rhizogenes* A4 suspension (1:3 dilution) for 10 to 30 minutes, and (b) explants wounding with a scalpel previously immersed in the bacterial suspension (Table 1). However, once again, no hairy roots were induced. Immersion of different explants from *H. perforatum* during 5, 10, 20 and 30 min in the bacterial suspension also resulted in the absence of hairy root induction (Franklin *et al.*, 2007). Several alternative approaches can be used to wound the target tissues. Some researchers reported that the wounding of the explant could be enhanced by subjecting the plant tissue to sonication (Trick *et al.*, 1997; Tang, 2003; Weber *et al.*, 2003). This technology or so-called sonication assisted *Agrobacterium*-mediated transformation (SAAT) tremendously improved the transformation efficiency of several crops that were recalcitrant to *Agrobacterium*-mediated transformation. The enhanced transformation efficiency by this method is probably due to the microwounding that occurs on the surface and inside of the target tissue which allows the bacterial cells to reach the proper target tissue and

stimulate the production of signalling molecules involved in the T-DNA transfer (Finer *et al.*, 2008).

3.3.4- Effect of acetosyringone in the bacterial and co-cultivation media

Addition of acetosyringone and related compounds to the bacteria and co-cultivation media are reported to improve *Agrobacterium* mediated transformation. In the work here reported, exogenous acetosyringone at 20 to 200μM (Table 1), in both inoculation and co-cultivation media had no beneficial effect on the transformation, since no induction of hairy roots was got from any of the tested explants of *H. androsaemum*, *H. perforatum* and *H. undulatum* under the influence of that compound. Acetosyringone which is a plant wound-exuded chemical, known to function as signal molecule inducing *vir* gene expression, and thereby activating T-DNA transfer (Stachel *et al.*, 1985; Cangelosi *et al.*, 1990), has been reported to improve transformation efficiency of several medicinal plant species, such as *Salvia miltiorrhiza* (Hu *et al.*, 1993), *Podophyllum hexandrum* (Giri *et al.*, 2001), *Digitallis minor* (Sales et al., 2003) and *Ruta graveolens* (Lièvre *et al.*, 2005). Nevertheless, several studies demonstrated that pre-induction with exogenous acetosyringone may not be essential if the phenolic compounds exudates by the wounded tissue are sufficient to activate *vir* genes induction of *Agrobacterium* during co-cultivation (Hiei *et al.*, 1997; Rao *et al.*, 2007) (Park et al., 1996; Cheng et al., 1997). In the transformation protocols established by DiGuardo *et al.* (2003) and Vintherhalter *et al.* (2006), hairy roots were induced in the absence of acetosyringone. Other reports showed that for species such as *Torenia fournieri* (Tao *et al.*, 2006) and *Pisum sativum* (De Kathen et al., 1990), addition of high concentrations of acetosyringone can even have a detrimental effect.

3.3.5- Effect of co-cultivation period

The time of co-cultivation can also affect gene transfer frequency and consequently transformation efficiency. Co-cultivation periods from 1-5 days were tested for transformation of the three *Hypericum* species (Table 1), but none of them led to the formation of hairy roots. In general, most of the reported studies shows better levels of expression and higher transformations frequencies using co-culture periods of 48 to 72 hours (Gilbert *et al.*, 1996; Tzvi Tzfira, 1997; Tao *et al.*, 2006). Even though, longer co-cultivation periods can negatively affect transformation by reducing bacterial affinity to the plant cell or by leading to an overgrowth of the bacteria inducing competitive inhibition (Tao *et al.*, 2006), hairy roots of *H. perforatum* were obtained from shoots after 7-10 days of co-cultivation (Vinterhalter *et al.*,

2006). In the work here reported, a period of 72 hours of co-culture was used in all of the attempts to transform *H. androsaemum*, *H. perforatum* and *H. undulatum* without positive results.

Several difficulties were found in attempts made in the work here reported to the establishment of a transformation protocol of *Hypericum* species mediated by *A. rhizogenes*. Several reports have been published on the high necrosis and poor survival rate of target plant tissues during the process of *Agrobacterium*-mediated T-DNA transfer. The lack of hairy root induction with *A. rhizogenes*, could be a consequence of induced death in the plant *Hypericum* cells. Indeed, there are several reports indicating a high level of necrosis and poor survival rate of target plant tissues during the process of *Agrobacterium*-mediated T-DNA transfer (Pu *et al.*, 1992; Deng *et al.*, 1995; Mercuri *et al.*, 2000; Chakrabarty *et al.*, 2002; Das *et al.*, 2002). Some authors have also demonstrated the induction of programmed cell death in plant cells after co-cultivation with *Agrobacterium* (Hansen, 2000; Parrott *et al.*, 2002). On the other hand, plants can modulate their gene expression in response to *Agrobacterium* infection which by its side can trigger the plant defence machinery maybe as a consequence of plant's hypersensitive reaction to *Agrobacterium* infection as already suggested (Ditt *et al.*, 2001; Ditt *et al.*, 2005). Hypersensitive reaction is described as one of the plant defence responses, generally characterized by the accumulation of antimicrobial agents, as well as the rapid and localized cell death around the infection site (Hammond-Kosack *et al.*, 1996; Richter *et al.*, 2000). Necrosis of the collapsed cells is, sometimes, a consequence of the sequence of events during hypersensitive reaction (Goodman *et al.*, 1994). In *H. perforatum* suspension cells infected with *Agrobacterium*, a drastic reduction in the bacteria viability during co-cultivation period has been found (Franklin *et al.*, 2008). The same authors also verified antimicrobial potential of the cell-free liquid medium of *Agrobacterium*-treated plant cell cultures suggesting the release of some antimicrobial substance(s) by the *H. perforatum* cells to the culture media. In fact, it seems that *H. perforatum* cells recognize *Agrobacterium* as a potential pathogen, triggering its defence mechanism responses, and leading to the reduction of bacteria viability.

3.4- Culture of Agrobacterium rhizogenes *A4 in presence of* Hypericum *biomass*

Plant powder of the three *Hypericum* species were tested directly for their antibacterial activity against *A. rhizogenes*. The plant powder of *H. androsaemum* and *H. undulatum* incorporated in the culture medium showed no effect on the *A. rhizogenes* A4 growth. In the Petri dish a layer of bacteria colonies appeared. For *H. perforatum*, even though an uncountable number of bacterial colonies was registered, a slight reduction in the bacteria growth occurred.

However, after 48 hours of *A. rhizogenes* A4 inoculation in the MYB medium with powders previously sterilized by autoclave the number of colonies were uncountable. Comparing the results from the two experiments a lower growth of *A. rhizogenes* was found in Petri dishes with not autoclavated plant powder. It is possible that the putative active compounds that might exert any antibacterial activity against *A. rhizogenes* A4 might not be active after autoclaving. A similar procedure, in which plant powder was incorporated in the agar was used to test the antibacterial activity of black myrobalan (*Teminalia chebula* Retz) against *Helicobacter pylori* (Malekzadeh *et al.*, 2001). According to the authors, plant powder gave higher MIC and MBC values (150 and 175 mg/l, respectively), than the water extracts. The authors suggested that black myrobalan extracts contain a heat stable agent(s) with possible therapeutic potential, since water extract was active after autoclaving for 30 min at 121°C.

As previously mentioned (Chapter 2), several species of the genus *Hypericum* have been shown to possess various biological activities, including the ability to produce antimicrobial constituents. Reports on the activity of *Hypericum* plant products against *Agrobacterium* have already been published. That is the case of essential oils of different species of the genus *Hypericum* including *H. perforatum*, which showed antimicrobial activity against *A. tumefaciens* (Saroglou *et al.*, 2007). Ethanolic extracts of *H. perforatum* showed also high antibacterial activity against *A. tumefaciens* (Milosevic *et al.*, 2007). Moreover, the co-culture of *H. perforatum* suspension cells with *Agrobacterium*, have already shown to induce a drastic reduction on the bacteria viability (Franklin *et al.*, 2008).

4- Conclusions

Although successful protocols of *H. perforatum* transformation have already been reported (Di Guardo *et al.*, 2003; Vinterhalter *et al.*, 2006), in this work several attempts to transform *H. perforatum*, *H. androsaemum* and *H. undulatum*, were not successful. The lack of hairy roots induction throughout the approaches here reported applied to the three *Hypericum* species, in which several parameters were tested (explants type and pre-culture period, methods of infection, co-cultivation period, addition of the inductor *vir* genes compound acetosyringone) seems to be related not to transfer and integration of T-DNA but with the effect of plant defence against *A. rhizogenes*. Similar conclusions were suggested by Franklin and co-workers (2007; 2008), who successfully transformed *H. perforatum* suspension cells when particle bombardment was used as the method of gene delivery with the same plasmid (pCAMBIA1301) that failed when transformation was mediated by *Agrobacterium*.

5- References

Banerjee, S., L. Rahman, G. C. Uniyal & P. S. Ahuja (1998). Enhanced production of valepotriates by *Agrobacterium rhizogenes* induced hairy root cultures of *Valeriana wallichii* DC. *Plant Science 131*(2), 203-208.

Bulgakov, V. P. (2008). Functions of *rol* genes in plant secondary metabolism. *Biotechnology Advances 26*(4), 318-324.

Bulgakov, V. P., M. V. Khodakovskaya, N. V. Labetskaya, G. K. Chernoded & Y. N. Zhuravlev (1998). The impact of plant *rolC* oncogene on ginsenoside production by ginseng hairy root cultures. *Phytochemistry 49*(7), 1929-1934.

Byrne, M. C., R. E. McDonnell, M. S. Wright & M. G. Carnes (1987). Strain and cultivar specificity in the *Agrobacterium*-soybean interaction. *Plant Cell, Tissue and Organ Culture 8*(1), 3-15.

Cangelosi, G., R. Ankenbauer & E. Nester (1990). Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. *Proc Natl Acad Sci U S A 87*(17), 6708–6712.

Canto-Canché, B. & V. Loyola-Vargas (1999). Chemicals from roots, hairy roots, and their application. *Chemicals via higher plant bioengineering*. Shahidi. NewYork, Kluwer Academic/Plenum Publishers. **23:** 5−244.

Cardarelli, M., D. Mariotti, M. Pomponi, L. Spanò, I. Capone & P. Costantino (1987). *Agrobacterium rhizogenes* T-DNA genes capable of inducing hairy root phenotype. *Molecular and General Genetics 209*(3), 475-480.

Casanova, E., M. I. Trillas, L. Moysset & A. Vainstein (2005). Influence of *rol* genes in floriculture. *Biotechnology Advances 23*(1), 3-39.

Chakrabarty, R., N. Viswakarma, S. Bhat, P. Kirti, B. Singh & V. Chopra (2002). *Agrobacterium*-mediated transformation of cauliflower: optimization of protocol and development of Bt-transgenic cauliflower. *J. Biosci 27*, 495—502.

Chateau, S., R. Sangwan & B. Sangwan-Norreel (2000). Competence of *Arabidopsis thaliana* genotypes and mutants for *Agrobacterium tumefaciens*-mediated gene transfer: role of phytohormones. *Journal of Experimental Botany 51*, 1961–1968.

Cho, H. J., J. M. Widholm, N. Tanaka, Y. Nakanishi & Y. Murooka (1998). *Agrobacterium rhizogenes*-mediated transformation and regeneration of the legume *Astragalus sinicus* (Chinese milk vetch). *Plant Science 138*, 53-65.

Christey, M. (2001). Use of ri-mediated transformation for production of transgenic plants. *In Vitro Cellular & Developmental Biology - Plant 37*(6), 687-700.

Das, D., M. Reddy, K. Upadhyaya & S. Sopory (2002). An efficient leaf-disk culture method for the regeneration via somatic embryogenesis and transformation of grape (*Vitis vinifera* L.). *Plant Cell Reports 20*, 999--1005.

De Kathen, A. & H.-J. Jacobsen (1990). *Agrobacterium tumefaciens*-mediated transformation of *Pisum sativum* L. using binary and cointegrate vectors. *Plant Cell Reports 9*(5), 276-279.

Deng, W., X.-A. Pu, R. Goodman, M. Gordon & E. Nester (1995). T-DNA genes responsible for inducing a necrotic response on grape vines. *Mol. Plant-Microbe Interact 8*(538--548).

Di Guardo, A., E. Cellàrovà, J. Koperdakova, L. Pistelli, B. Ruffoni, A. Allavena & A. Giovannini (2003). Hairy root induction and plant regeneration in *Hypericum perforatum* L. *J. Genet. Breed. 57*, 269–278.

Ditt, R. F., E. Nester & L. Comai (2005). The plant cell defense and *Agrobacterium tumefaciens*. *FEMS Microbiology Letters 247*(2), 207-213.

Ditt, R. F., E. W. Nester & L. Comai (2001). Plant gene expression response to *Agrobacterium tumefaciens*. *Proceedings of the National Academy of Sciences of the United States of America 98*(19), 10954-10959.

Doerr, B., R. Glomot, H. Kief, M. Kramer & T. Sakaguchi (1980). Toxicology of cefotaxime in comparison to other cephalosporins. *J Antimicrob Chemother. 6*(Suppl A), 79-82.

Dronne, S., S. Moja, F. Jullien, F. Berger & J.-C. Caissard (1999). *Agrobacterium*-mediated transformation of lavandin (*Lavandula* x *intermedia* Emeric ex Loiseleur). *Transgenic Research 8*, 335-347.

Fillipone, E. & R. P. R (1992). *Agrobacterium*-mediated gene transfer. *Biotechnology: Enhancing Research on Tropical Crops in Africa*. T. G, M. L, M. R. DR and M. AW, CTA/IITA co-publication. IITA, Ibadan**:** 197--202.

Finer, J. & K. Larkin (2008). *Genetic transformation of soybean*, CRC Press, Taylor & Frances Group.

Franklin, G., L. Conceição, E. Kombrink & A. Dias (2008). *Hypericum perforatum* plant cells reduce *Agrobacterium* viability during co-cultivation. *Planta 227*, 1401–1408.

Franklin, G., M. Oliveira & A. C. P. Dias (2007). Production of transgenic *Hypericum perforatum* plants via particle bombardment-mediated transformation of novel organogenic cell suspension cultures. *Plant Science 172*(6), 1193-1203.

Gilbert, M., Y. Zhang & Z. Punja (1996). Introduction and expression of chitinase encoding genes in carrot following *Agrobacterium* -mediated transformation. *In Vitro Cellular & Developmental Biology - Plant 32*(3), 171-178.

Giri, A., C. Giri, V. Dhingra & M. Narasu (2001). Enhanced Podophyllotoxin Production from *Agrobacterium rhizogenes* Transformed Cultures of *Podophyllum hexandrum*. *Natural Product Research 15*(4), 229-235.

Giri, A. & M. Narasu (2000). Transgenic hairy roots: recent trends and applications. *Biotechnology Advances 18*, 1–22.

Goodman, R. & A. Novacky (1994). The hypersensitive reaction in plants to pathogens. A resistant phenomenon. St. Paul, Minnesota, APS PRESS**:** 244.

Hammerschlag, F., R. Zimmerman, U. Yadava, S. Hunsucker & P. Gercheva (1997). Effect of antibiotics and exposure to an acidified medium on the elimination of *Agrobacterium tumefaciens*

from apple leaf explants and on shoot regeneration. *J Am Soc Hortic Sci 122*, 758–763.

Hammond-Kosack, K. & J. Jones (1996). Resistance gene–dependent plant defense responses. *Plant Cell 8*, 1773—1791.

Handa, T. (1992). Genetic transformation of *Antirrhinum majus* L. and inheritance of altered phenotype induced by Ri T-DNA. *Plant Science 81*(2), 199-206.

Hansen, G. (2000). Evidence for *Agrobacterium*-Induced Apoptosis in Maize Cells. *Molecular Plant-Microbe Interactions 13*(6), 649-657.

Hiei, Y., T. Komari & T. Kubo (1997). Transformation of rice mediated by *Agrobacterium tumefaciens*. *Plant Mol Biol 35*, 205–218.

Hobbs, S., J. Kackson & J. Mahon (1989). Specificity of strain and genotype in the susceptibility of pea to *Agrobacterium tumefaciens*. *Plant Cell Reports 8*, 274-277.

Holford, P. & H. Newbury (1992). The effects of antibiotics and their breakdown products on the *in vitro* growth of *Antirrhinum Majas*. *Plant Cell Reports 11*, 93-96.

Hong, S.-B., C. Peebles, J. Shanks, K.-Y. San & S. Gibson (2006). Terpenoid indole alkaloid production by *Catharanthus roseus* hairy roots induced by *Agrobacterium tumefaciens* harboring *rol ABC* genes. *Biotechnology and Bioengineering 93*(2), 386-390.

Hu, Z. & A. Alfermann (1993). Diterpenoid production in hairy root cultures of *Salvia miltiorrhiza*. *Phytochemistry 32*, 699–703.

Jouhikainen, K., L. Lindgren, T. Jokelainen, R. Hiltunen, T. H. Teeri & K.-M. Oksman-Caldentey (1999). Enhancement of scopolamine production in *Hyoscyamus muticus* L. hairy root cultures by genetic engineering. *Planta 208*(4), 545-551.

Kornfeld, A., P. B. Kaufman, C. R. Lu, D. M. Gibson, S. F. Bolling, S. L. Warber, S. C. Chang & A. Kirakosyan (2007). The production of hypericins in two selected *Hypericum perforatum* shoot cultures is related to differences in black gland structure. *Plant Physiology and Biochemistry 45*(1), 24-32.

Królicka, A., I. Staniszewska, K. Bielawski, E. Maliñski, J. Szafranek & E. Lojkowska (2001). Establishment of hairy root cultures of *Ammi majus*. *Plant Science 160*, 259-264.

Kumar, V., B. Jones & M. R. Davey (1991). Transformation by *Agrobacterium rhizogenes* and regeneration of transgenic shoots of the wild soybean *Glycine argyrea*. *Plant Cell Reports 10*, 135–138.

Lièvre, K., A. Hehn, T. L. M. Tran, A. Gravot, B. Thomasset, F. Bourgaud & E. Gontier (2005). Genetic transformation of the medicinal plant *Ruta graveolens* L. by an *Agrobacterium tumefaciens*-mediated method. *Plant Science 168*(4), 883-888.

Maldonado-Mendoza, I. E., T. Ayora-Talavera & V. M. Loyola-Vargas (1992). Tropane alkaloid production in root cultures of *Datura stramonium*. *In Vitro Cell Dev. Biol. Plant 28*, 67-72.

Malekzadeh, F., H. Ehsanifar, M. Shahamat, M. Levin & R. R. Colwell (2001). Antibacterial activity of black myrobalan (*Terminalia chebula* Retz) against *Helicobacter pylori*. *International Journal of Antimicrobial Agents 18*(1), 85-88.

Mathias, R. & L. Boyd (1986). The effect of cefotaxime on the growth and regeneration of callus from four varieties of barley (*Hordeum vulgare* L.). *Plant Science 46*, 217-23.

Mercuri, A., L. Benedetti, G. Burchi & T. Schiva (2000). *Agrobacterium*-mediated transformation of African violet. *Plant Cell Tissue and Organ culture 60*, 39—46.

Meyer, A., J. Tempé & P. Costantino (2000). Hairy root: A molecular overview. Functional analysis of *Agrobacterium rhizogenes* T-DNA genes. *Plant-microbe interactions*. G. Stacey and N. T. Keen. St. Paul, APS Press**:** 93–139.

Milosevic, T., S. Solujic & S. Sukdolak (2007). *In Vitro* Study of Ethanolic Extract of *Hypericum perforatum* L. on Growth and Sporulation of Some Bacteria and Fungi. *Turk J Biol 31*, 237-241.

Nakano, M. & M. Mii (1993). Antibiotics stimulate somatic embryogenesis without plant growth regulators in several *Dianthus* cultivars. *J. Plant Physiol. 141*, 721-725.

Nauerby, B., K. Billing & R. Wyndaele (1997). Influence of the antibiotic timentin on plant regeneration compared to carbenicillin and cefotaxime in concentrations suitable for elimination of *Agrobacterium tumefaciens*. *Plant Science 123*, 169-177.

Nguyen, C., F. Bourgaud, P. Forlot & A. Guckert (1992). Establishment of hairy root cultures of *Psoralea* species. *Plant Cell Reports 11*, 42-47.

Oksman-Caldentey, K.-M., O. Kivelä & R. Hiltunen (1991). Spontaneous shoot organogenesis and plant regeneration from hairy root cultures of *Hyoscyamus muticus*. *Plant Science 78*(1), 129-136.

Owens, L. & D. Cress (1985). Genotypic variability of soybean response to *Agrobacterium* strains harboring Ti or Ri plasmids. *Plant Physiology 77*, 87.

Palazón, J., R. Cusidó, C. Roig & M. Piñol (1997). Effect of *rol* genes from *Agrobacterium rhizogenes* TL-DNA on nicotine production in tobacco root cultures. *Plant Physiol Biochem 35*, 155–162.

Palazón, J., R. Cusidó, C. Roig & M. Piñol (1998). Expression of the *rolC* gene and nicotine production in transgenic roots and their regenerated plants. *Plant Cell Reports 17*, 384–390.

Parrott, D. L., A. J. Anderson & J. G. Carman (2002). *Agrobacterium* induces plant cell death in wheat (*Triticum aestivum* L.). *Physiological and Molecular Plant Pathology 60*(2), 59-69.

Pellegrineschi, A., J.-P. Damon, N. Valtorta, N. Paillard & D. Tapfer (1994). Improvement of Ornamental Characters and Fragrance Production in Lemon-scented Geranium Through Genetic Transformation by *Agrobacterium rhizogenes*. *Nat Biotech 12*(1), 64-68.

Pellegrineschi, A. & O. Davolio-Mariani (1997). *Agrobacterium rhizogenes*-mediated transformation of scented geranium. *Plant Cell, Tissue and Organ Culture 47*(1), 79-86.

Pu, X.-A. & R. Goodman (1992). Induction of necrosis by *Agrobacterium tumefaciens* on grape explants. *Physiol. Mol. Plant Pathol. 41*, 245-254.

Rao, M. & G. Rao (2007). *Agrobacterium*-mediated transformation of indica rice under Acetosyringone-free conditions. *Plant Biotechnology 24*, 507–511.

Richter, T. & P. Ronald (2000). The evolution of disease resistance genes. *Plant Mol. Biol. 42*, 195–204.

Sales, E., J. Segura & I. Arrillaga (2003). *Agrobacterium tumefaciens*-Mediated Genetic Transformation of the Cardenolide-Producing Plant *Digitalis minor* L. *Planta Med 69*(02), 143-147.

Sarma, K. S., N. E. Evans & C. Selby (1995). Effect of carbenicillin and cefotaxime on somatic embryogenesis of Sitka spruce (*Picea sitchensis* (Bong.) Carr.). *J. Exp. Bot. 46*(11), 1779-1781.

Saroglou, V., P. D. Marin, A. Rancic, M. Veljic & H. Skaltsa (2007). Composition and antimicrobial activity of the essential oil of six *Hypericum* species from Serbia. *Biochemical Systematics and Ecology 35*(3), 146-152.

Shackelford, N. & C. Chlan (1996). Identification of antibiotics that are effective in eliminating *Agrobacterium tumefaciens*. *Plant Molecular Biology Reporter 14*(1), 50-57.

Spanò, L., D. Mariotti, M. Cardarelli, C. Branca & P. Costantino (1988). Morphogenesis and auxin sensitivity of transgenic tobacco with different complements of Ri T-DNA. *Plant Physiology 87* 479–483.

Spena, A., T. Schmulling, C. Koncz & J. Schell (1987). Independent and synergistic activity of *rolA, B* and *C* loci in stimulating abnormal growth in plants. *EMBO J 6*, 3891-3899.

Stachel, S. E., E. Messens, M. Van Montagu & P. Zambryski (1985). Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature 318*(6047), 624-629.

Tang, W. (2003). Additional virulence genes and sonication enhance *Agrobacterium tumefaciens*-mediated loblolly pine transformation. *Plant Cell Reports 21*(6), 555-562.

Tao, J. & L. Li (2006). Genetic transformation of *Torenia fournieri* L. mediated by *Agrobacterium rhizogenes*. *South African Journal of Botany 72*(2), 211-216.

Tepfer, D. (1984). Transformation of several species of higher plants by *Agrobacterium rhizogenes*: Sexual transmission of the transformed genotype and phenotype. *Cellular Microbiology 37*, 959–67.

Tepfer, D. (1990). Genetic transformation using *Agrobacterium rhizogenes*. *Physiol. Plantarum 79*, 140-146.

Tepfer, M. & F. Casse-Delbart (1987). *Agrobacterium rhizogenes* as a vector for transforming higher plants. *Microbiological Sciences 4*(1), 24-28.

Trick, H. N. & J. J. Finer (1997). SAAT: sonication-assisted *Agrobacterium*-mediated transformation. *Transgenic Research 6*(5), 329-336.

Tzvi Tzfira, C. S. J. A. V. A. A. (1997). Transformation and regeneration of transgenic aspen plants via shoot formation from stem explants. *Physiologia Plantarum 99*(4), 554-561.

van Altvorst, A. C., R. J. Bino, A. J. van Dijk, A. M. J. Lamers, W. H. Lindhout, F. van der Mark & J. J. M. Dons (1992). Effects of the introduction of *Agrobacterium rhizogenes rol* genes on tomato plant and flower development. *Plant Science 83*(1), 77-85.

Vanhala, L., R. Hiltunen & K. M. Oksman-Caldentey (1995). Virulence of different *Agrobacterium* strains on hairy root formation of *Hyoscyamus muticus*. *Plant Cell Reports 14*, 236-240.

Veluthambi, K., A. Gupta & A. Sharma (2003). The current status of plant transformation technologies. *Current Science 84*(3), 368-380.

Villemont, E., F. Dubois, R. S. Sangwan, G. Vasseur, Y. Bourgeois & B. S. Sangwan-Norreel (1997). Role of the host cell cycle in the *Agrobacterium*-mediated genetic transformation of *Petunia*: Evidence of an S-phase control mechanism for T-DNA transfer. *Planta 201*(2), 160-172.

Vinterhalter, B., S. Ninkovic, A. Cingel & D. Vinterhalter (2006). Shoot and root culture of *Hypericum perforatum* L. transformed with *Agrobacterium rhizogenes* A4M70GUS. *Biologia Plantarum 50*(4), 767-770.

Weber, S., W. Friedt, N. Landes, J. Molinier, C. Himber, P. Rousselin, G. Hahne & R. Horn (2003). Improved *Agrobacterium* -mediated transformation of sunflower (*Helianthus annuus* L.): assessment of macerating enzymes and sonication. *Plant Cell Reports 21*(5), 475-482.

Wise, R., T. Rollason, M. Logan, J. M. Andrews & K. A. Bedford (1978). HR 756, a Highly Active Cephalosporin: Comparison with Cefazolin and Carbenicillin. *Antimicrob. Agents Chemother. 14*(6), 807-811.

Yepes, L. M. & H. S. Aldwinekle (1994). Factors that effect leaf regeneration efficiency in apple, and effect of antibiotics in morphogenesis. *Plant Cell, Tissue and Organ Culture 37*(3), 257-269.

Young, P., A. Hutchins & M. Canfield (1984). Use of antibiotics to control bacteria in shoot cultures of woody plants. *Plant Sci Lett 34*, 203–209.

Yu, T.-A., S.-D. Yeh & J.-S. Yang (2001). Effects of carbenicillin and cefotaxime on callus growth and somatic embryogenesis from adventitious roots of papaya. *Bot. Bull. Acad. Sin 42*, 281-286.

Yu, Y. & Z. M. Wei (2008). Influences of cefotaxime and carbenicillin on plant regeneration from wheat mature embryos. *Biologia Plantarum 52*(3), 553-556.

Zehra, M., S. Banerjee, A. A. Naqavi & S. Kumar (1998). Variation in the growth and alkaloid production capability of the hairy root of *Hyoscyamus albus, H. muticus* and their somatic hybrid. *Plant Science 136*, 93-99.

Chapter 7

FINAL REMARKS AND FUTURE PERSPECTIVES

For thousands of years, plants have had an important role in the Human life, either as a food supply or as a source of important medicines. In fact, during the last decades there has been, all over the world, an increase in the search on plant based pharmaceuticals. The research for natural products, such as essential oils (EO), has involved several type of industries such as food, and flavour and fragrance industries. Consequently, nowadays an increase in the global trade for aromatic and medicinal plants is occurring. However, some of these useful plant species can be difficult to regenerate by conventional methods or are in danger of extinction. Plant cell and tissue culture is seen as a useful alternative for multiplying and conserving plant species and is being used in the commercial propagation of some medicinal plants (Rout *et al.*, 2000).

Several species of the *Hypericum* genus have a long traditional value as medicinal plants, being used in ailments as knowledge-based medicine in several countries, including Portugal, where *H. androsaemum*, *H. perforatum*, and *H. undulatum*, are the most used species of the genus. The main objective of this thesis was to increase our knowledge about the essential oils profiles produced by the above mentioned *Hypericum* species (*H. androsaemum*, *H. perforatum* and *H. undulatum*). Although several reports on *H. perforatum* essential oils have been published, information on the essential oils of the other two species is scant. As far as we know, the most detailed lists of essential oils constituents of *H. androsaemum* and *H. undulatum* are herein reported for the first time. Besides the contribution for a deeper knowledge on the biochemical characteristics of these species, this work can be a basis for definition of the biochemical markers for quality control of natural products based in essential oils of these *Hypericum* species. Even though the three studied species are known for their therapeutic properties, in many cases there is no information on the identity of the constituents responsible for the attributed biological activities. Therefore, the detailed characterization of the essential oils herein described is crucial for the subsequent identification of constituents responsible for the putative biological activities.

This study showed that the essential oils of *H. androsaemum* and *H. perforatum* plants are characterized by the predominance of the sesquiterpene hydrocarbons, while *H. undulatum* essential oils are majority constituted by *n*-alkanes.

Essential oils production and/or accumulation is known to be modulated by several factors, either intrinsic or extrinsic to the plant (Figueiredo *et al.*, 2008). Indeed, the exhaustive work here reported based in periodic sampling and chromatographic analysis of the essential oils from the three *Hypericum* species showed that the respective production and/or

accumulation is influenced by harvest time and season, geographical distribution, growing conditions (*in vitro* and *in vivo*) and plant organ.

In both *H. androsaemum* and *H. undulatum*, the lowest essential oil contents were detected in the spring. In the essential oils of vegetative aerial parts of plants of *H. perforatum* and *H. perforatum* cv. Topaz it was shown a decrease in the relative content of sesquiterpene hydrocarbons during the spring and summer, increasing afterwards to its maximum in the winter, in coincidence with the lowest contents of the essential oils in the plants. In the essential oils of *H. androsaemum* the minimum content of sesquiterpene hydrocarbons was registered later in the summer. Generally, over the year, a pattern of inverse variation in the relative contents of sesquiterpene hydrocarbons and monoterpene hydrocarbons was evident in the essential oils of the three *Hypericum* species here studied.

The different plant organs of the three target *Hypericum* species showed different essential oil contents and composition profiles. The highest contents of essential oils were found in ripened seed capsules of *H. androsaemum*, in flowers of *H. perforatum* and leaves of *H. undulatum*. The stems of *H. androsaemum* and *H. undulatum* were the organs with the lowest essential oils content. Variations in essential oils composition depending of the growth site were also found in the studies performed with *H. androsaemum* and *H. perforatum*.

Because *in vitro* micropropagation can be used as a way to produce plant material and valuable secondary metabolites, under environmental and nutritional controlled conditions, *in vitro* shoot cultures of *H. androsaemum*, *H. perforatum*, and *H. undulatum* were established from nodal segments. The essential oils composition of *in vitro* shoots of the *H. androsaemum*, *H. perforatum*, and *H. undulatum* was reported in this work, for the first time. Likewise results obtained in other plant species (Charlwood *et al.*, 1988; Santos-Gomes *et al.*, 2003), essential oils from *in vitro* shoots of *H. androsaemum*, *H. perforatum*, and *H. undulatum* were different from those of the *in vivo* plants. Less complex essential oils were found in the *in vitro* shoots of these species comparing to those produced by *in vivo* plants. As the first results obtained on the essential oils produced by *in vitro* shoots of *H. androsaemum*, *H. perforatum*, and *H. undulatum*, this work opened up a field of investigation involving *in vitro* cultures in order to modulate the essential oils composition through culture media manipulation and culture growth conditions. This type of cultures can also be used in future works, as model systems in the elucidation of biosynthetic pathways of the respective essential oil constituents through the isolation and characterisation of enzymes involved in these biosynthetic routes.

An interesting result demonstrated in this work was the predominance of the *n*-alkanes group in the essential oils from plants, *in vitro* shoots, and micropropagated and acclimatized

plants of *H. undulatum*. *n*-Nonane was the major compound in all the essential oils obtained from *H. undulatum* plant material. Its function in the essential oils however remains unclear. As high contents of *n*-nonane were found in the essential oils of *in vitro* shoots of this species, this type of cultures might be a good system in the attempt of elucidating, not only its biosynthetic pathway, but also its function as a constituent of the essential oils. The results herein reported might be the start point for further studies, in order to increase the biotechnological production of this compound, which is usually used as a fuel additive and in biodegradable detergent. Extraction methods can also be optimized to increase the content of *n*-nonane extracted from *H. undulatum* plant material, not only in essential oils but also in other type of extracts (*e.g.* lipidic extracts).

Several strategies have been developed to enhance production of secondary metabolites in plant cultures, including plant transformation. Gene transfer by *Agrobacterium* is the method of choice for the genetic transformation of most plant species. However, a successful protocol of transformation depends on the establishment of a reliable plant regeneration system and an efficient interaction of *Agrobacterium*-plant. Aware of the fact that several factors affect this bacteria-plant interaction, in this work some of them were evaluated (explants pre-culture, bacterial density, type of explants, explants wounding, addition of acetosyringone to the bacterial suspension and co-culture medium, and co-culture period) on the several attempts of transformation of *H. androsaemum, H. perforatum* and *H. undulatum* mediated by *A. rhizogenes* A4. However, hairy roots production was not achieved in any of the plant species tested, probably due to the trigger of a plant defence mechanism against *A. rhizogenes*. Recently, a reduction on the *Agrobacterium* viability during co-cultivation with *H. perforatum* was reported (Franklin *et al.*, 2008). The authors suggested that it might be caused by the release of antimicrobial substances from the plant cells. Antibacterial activity of essential oils from different species of *Hypericum* genus against *A. tumefaciens* has already been reported (Saroglou *et al.*, 2007). Our results together with those reported on the literature rise the need of new approaches and molecular studies using this agroinfection system to understand the possible defence mechanisms underlying this phenomenon, as well as antibacterial assays of several extracts of the studied *Hypericum* species (*e.g.* crude extracts, essential oils) to evaluate their activity against *Agrobacterium*. Even though successful protocols of *Agrobacterium*-mediated transformation of *H. perforatum* have been reported (Di Guardo *et al.*, 2003; Vinterhalter *et al.*, 2006), the lack of hairy roots induction herein reported and the results from other authors (Franklin *et al.*, 2007) show the need in finding alternative ways such as that of particle bombardment as it was used in the transformation of *H. perforatum*. Hence, in order to

try the manipulation of essential oils pathways through plant transformation, more work should to be done in the optimization of a particle bombardment protocol of the studied *Hypericum* species.

References

Charlwood, B. & C. Moustou (1988). Essential oil accumulation in shoot-proliferation cultures of *Pelargonium* ssp. *Manipulating Secondary Metabolism in Cultures*. R. Robins and M. Rhodes. Cambridge, U. K., Cambridge University Press.

Di Guardo, A., E. Cellàrovà, J. Koperdakova, L. Pistelli, B. Ruffoni, A. Allavena & A. Giovannini (2003). Hairy root induction and plant regeneration in *Hypericum perforatum* L. *J. Genet. Breed. 57*, 269–278.

Figueiredo, A., J. Barroso, L. Pedro & J. Scheffer (2008). Factors affecting secondary metabolite production in plants: volatile components and essential oils. *Flavour and Fragrance Journal 23*, 213–226.

Franklin, G., L. Conceição, E. Kombrink & A. Dias (2008). *Hypericum perforatum* plant cells reduce *Agrobacterium* viability during co-cultivation. *Planta 227*, 1401–1408.

Franklin, G., M. Oliveira & A. C. P. Dias (2007). Production of transgenic *Hypericum perforatum* plants via particle bombardment-mediated transformation of novel organogenic cell suspension cultures. *Plant Science 172*(6), 1193-1203.

Rout, G. R., S. Samantaray & P. Das (2000). *In vitro* manipulation and propagation of medicinal plants. *Biotechnology Advances 18*(2), 91-120.

Santos-Gomes, P. & M. Fernandes-Ferreira (2003). Essential Oils Produced by *in Vitro* Shoots of Sage (*Salvia officinalis* L.). *Journal of Agricultural and Food Chemistry 51*, 2260-2266.

Saroglou, V., P. D. Marin, A. Rancic, M. Veljic & H. Skaltsa (2007). Composition and antimicrobial activity of the essential oil of six *Hypericum* species from Serbia. *Biochemical Systematics and Ecology 35*(3), 146-152.

Vinterhalter, B., S. Ninkovic, A. Cingel & D. Vinterhalter (2006). Shoot and root culture of *Hypericum perforatum* L. transformed with *Agrobacterium rhizogenes* A4M70GUS. *Biologia Plantarum 50*(4), 767-770.