Mónica da Rocha Zuzarte

Portuguese lavenders: evaluation of their potential use for health and agricultural purposes

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Portuguese lavenders: evaluation of their potential use for health and agricultural purposes

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Mónica da Rocha Zuzarte

A thesis presented to the Faculty of Sciences and Technology of the University of Coimbra in fulfillment of the requirements for the degree of Doctor of Philosophy in Biology (Speciality in Plant Physiology)

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Front cover: *Lavandula luisieri* growing in the wild at Serra do Caldeirão, Algarve, Portugal.

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"When you learn, teach, when you get, give"

Maya Angelou

I dedicate this thesis to my family and life companion, Rui, for their love, endless support and encouragement.

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- Zuzarte M., Gonçalves M.J., Cavaleiro C., Salgueiro L., Canhoto J.M., 2012. Essential oils from *Lavandula* spp. as potential food and crop preservatives. Food Chemistry.

- Zuzarte M., Gonçalves M.J., Cavaleiro C., Canhoto J.M., Cruz M.T., Salgueiro L. 2012. Cytotoxicity of *Lavandula* spp. essential oils. Phytomedicine.

ABSTRACT

Several species of the genus *Lavandula* L. are aromatic plants highly valued due to the economic potential of their essential oils mainly in the perfumery, cosmetic, food and pharmaceutical industries. Despite this popularity lavenders growing spontaneously in Portugal are poorly explored justifying the scientific-based study including taxonomical, chemical and biological features carried out in the present work.

Detailed morphological observations of several samples of fresh plants and herbarium specimens were performed allowing a better characterization of the species distribution in Portugal and confirming the high morphological variability, mainly in *L. luisieri* and *L pedunculata*, responsible for the controversial taxonomy in the section Stoechas.

Trichome analysis by scanning electron microscopy and light microscopy resulted in the identification of different types of glandular trichomes: peltate, capitate short-stalked (with a uni or bicellular head), capitate long-stalked, bifurcated and ramified mixed-type trichomes. An identification key based on trichomes analysis was proposed allowing the identification of the species based on these features. Furthermore, histochemical analysis showed that all types of trichomes, with exception of capitate short-stalked trichomes with a bicellular head, are involved in essential oil production.

Chemical analysis of the essential oils were performed using gas chromatography and gas chromatography/mass spectroscopy and a chemical profile defined for each species, as follows: *L. luisieri* has a distinct oil characterized by the presence of irregular monoterpenes (necrodane derivatives); *L. multifida* is rich in carvacrol and *cis*-*β*-ocimene; *L. pedunculata* has a high chemical polymorphism in the amounts of 1,8-cineole, fenchone and camphor; *L. viridis* showed high levels of 1,8-cineole. Results from the chemical analysis and taxonomical data pointed towards the classification of *L. luisieri* as an independent species instead of a subspecies of *L. stoechas*.

Several biological activities were performed in order to evaluate the potential application of the oils in the health and agricultural sectors. Regarding the health sector, the antifungal effects of the oils were evaluated against fungi involved in human and animal infections (*Candida* spp., *Cryptococcus neoformans*, dermatophytes and *Aspergillus* spp.). Minimal inhibitory concentrations (MICs) and minimal lethal concentrations (MLCs) indicated a significant antifungal activity mainly against

dermatophytes and *Cryptococcus neoformans*. Carvacrol proved to be the most active compound although its cytotoxicity limits its application. Moreover, significant germ tube inhibitions on *Candida* spp. showed a potential application of *L. luisieri, L. multifida* and *L. viridis* oils for the treatment of disseminative candidosis. Additional mechanisms of action on *Candida albicans*, using flow cytometry, were elucidated for *L. multifida* and *L. viridis* oils suggesting a mode of action starting on damage to mitochondrial membranes and leading to disruption of cytoplasmic membranes with cell death. The anti-inflammatory potential of the oils was also assessed on lipopolysaccharide-stimulated macrophages. *L. viridis* oil was able to inhibit NO production, through modulation of the selective pro-inflammatory signaling cascades (nuclear factor kappa B and extracellular-signal-regulated kinase, ERK 1/2) and through inhibitions at transcriptional and translational levels of pro-inflammatory mediators, namely nitric oxide synthase. Promising anti-inflammatory potential has also been pointed out in preliminary experiments performed with *L. luisieri* and *L. pedunculata* oils.

Considering the potential of the oils for a further application to human and/or animals, their safety profile was evaluated through cytotoxicity assays in different cell lines (keratinocytes, macrophages and alveolar epithelial cells), mimetizing the main ways of oil administration. The results allowed the determination of active safe concentrations with *L. viridis* showing the best results.

In what concerns agricultural application, the antifungal potential of the oil and its main compounds on ph ytopathogenic strains (*Alternaria alternata*, *Cladosporium cladosporioides*, *Fusarium circinatum*, *F. verticillioides* and *Penicillium itallicum*) was evaluated. MIC and MLC values showed interesting results against *A. alternata* and *C. cladosporioides*, being carvacrol identified as a potent active compound. The nematicidal activity against *Meloidogyne javanica* was also evaluated with *L. viridis* oil showing a potential application as a natural nematicide. Finally, the repellent capacity of the oils was evaluated on *Monochamus galloprovincialis*, the insect vector of pine-wilt disease, using the wind-tunnel assay. *L. luisieri* and *L. viridis* were the most effective causing major disturbance on t he insect behavior including flying away from the scent odor, demonstrating the repellent potential of the oils.

To avoid over-exploitation of lavenders growing in the field, *L. multifida*, a species with a restricted distribution in Portugal, was micropropagated through axillary shoot proliferation and an efficient protocol for the rapid multiplication of this species was

established allowing large scale propagation and contributing, in this way, for the preservation of natural resources.

In this way, the present work contributed to a better scientific understanding of the genus *Lavandula* in Portugal and the biological studies, evaluated for the first time in these species, confirmed the industrial potential of the oils. Further validations for the development of bio-products to be used in the health and agricultural sectors should be considered.

KEYWORDS: *Lavandula latifolia*; *L. luisieri*; *L. multifida*; *L. pedunculata*; *L. viridis*; essential oils; trichomes; chemotaxonomy; antifungal activity; anti-inflammatory activity; cytotoxicity; nematicidal activity; insect repellency; *in vitro* culture.

RESUMO

O género *Lavandula* L. engloba diversas plantas aromáticas altamente valorizadas devido ao potencial económico dos seus óleos essenciais com particular destaque em perfumaria, cosmética, indústrias alimentar e farmacêutica. Apesar desta popularidade as lavandulas espontâneas de Portugal encontram-se pouco exploradas justificando um estudo alargado, de base científica, com uma componente taxonómica, química e biológica como o desenvolvido no presente trabalho.

Observações morfológicas quer de material fresco quer de exemplares de herbário permitiram uma melhor identificação da distribuição das espécies em Portugal e confirmaram a elevada variabilidade morfológica principalmente em *L. pedunculata* e *L. luisieiri*, responsáveis pela taxonomia controversa da secção Stoechas.

A análise de tricomas recorrendo a microscopia electrónica de varrimento e a microscopia óptica permitiu a identificação de diversos tipos de tricomas glandulares: peltados, capitados de pedículo curto (com cabeça uni ou bicelular), capitados de pedículo longo, bifurcados e ramificados do tipo misto. Paralelamente uma chave dicotómica para a identificação das espécies baseada na presença dos diversos tipos de tricomas foi proposta. Os testes histoquímicos mostraram que todos os tricomas, à excepção dos capitados de pedículo curto de cabeça bicelular, estão envolvidos na produção de óleo essencial.

A análise química dos óleos essenciais por cromatografia gasosa e cromatografia gasosa acoplada à espectrometria de massa permitiu definir o perfil químico de cada *taxon*: *L. luisieri* caracteriza-se por ter uma composição única devido à presença de monoterpenos irregulares derivados do necrodano; *L. multifida* apresenta elevados teores de carvacrol e *cis-β*-ocimeno; *L. pedunculata* é altamente polimórfica particularmente no que diz respeito aos teores de 1,8-cineol, fenchona e cânfora; *L. viridis* caracteriza-se por apresentar sempre elevados teores de 1,8-cineol. Por outro lado, os dados da análise química dos óleos essenciais conjuntamente com dados taxonómicos apontam para que *L. luisieri* possa ser considerada uma espécie independente, e não uma subespécie de *L. stoechas*.

Diversas actividades biológicas foram contempladas de forma a avaliar o potencial dos óleos para uma futura aplicação na área da saúde e no sector agrícola. Para o primeiro caso, a actividade antifúngica dos óleos e dos seus constituintes maioritários foi avaliada em diversas estirpes patogénicas para o homem e outros animais (*Aspergillus* spp., *Candida* spp., *Cryptococcus neoformans* e dermatófitos) pela determinação das

concentrações mínimas inibitórias (CMIs) e as concentrações mínimas letais (CMLs). Os resultados mostraram que os óleos são efetivos particularmente contra dermatófitos e *C. neoformans*. O carvacrol mostrou ser o composto mais activo, mas a sua citotoxicidade limita a sua aplicação. Adicionalmente, os óleos de *L. luisieri*, *L. multifida* e *L. viridis* mostraram ser bastante promissores nos ensaios de inibição do tubo germinativo em *Candida* spp., sugerindo os seu potencial interesse no tratamento da candidíase disseminativa. O mecanismo de ação dos óleos de *L. multifida* e *L. viridis* foi também elucidado por citometria de fluxo, mostrando que os óleos provocam lesões da membrana mitocondrial muito antes de ocorrerem lesões a nível da membrana celular.

O potencial anti-inflamatório dos óleos foi também avaliado em macrófagos estimulados com lipopolissacarídeo bacteriano. O óleo de *L. viridis* inibiu a produção de óxido nítrico, modulando seletivamente as cascatas de sinalização pro-inflamatória (factor de transcrição nuclear kappa B e cinase regulada por sinais extracelulares, ERK 1/2) e inibindo os processos de transcrição e translação. Por outro lado, resultados promissores foram também evidenciados em ensaios preliminares com os óleos essenciais de *L. luisieri* e *L. pedunculata*.

Tendo em conta o potencial demonstrado pelos óleos para uma futura aplicação em humanos e/ou animais, a citotoxicidade dos mesmos foi avaliada em diferentes linhas celulares (queratinócitos, macrófagos e células alveolares epiteliais) de forma a mimetizar as principais vias de administração dos óleos. As concentrações activas e seguras foram determinadas e, particularmente, o óleo essencial de *L. viridis* mostrou ser um óleo bastante seguro.

Tendo por base uma aplicação agrícola, o potencial antifúngico dos óleos e dos seus compostos maioritários foi avaliado em estirpes fitopatogénicas (*Alternaria alternata*, *Cladosporium cladosporioides*, *Fusarium circinatum*, *F. verticillioides* e *Penicillium italicum*). As CMIs e CMLs determinadas comprovaram uma maior eficiência dos óleos contra *A. alternata* e *C. cladosporioides* e o composto isolado, carvacrol, revelou ser o mais activo. A actividade nematodicida contra *Meloidogyne javanica* foi também avaliada com sucesso para o óleo essencial de *L. viridis.* Por último, a acção repelente dos óleos em túnel de vento foi avaliada contra *Monochamus galloprovincialis*, o insecto vector do nemátode do pinheiro. Os óleos de *L. luisieri* e *L. viridis* provocaram mais distúrbios no comportamento dos insectos, que em casos extremos voaram para longe da fonte de odor, demostrando o potencial repelente destes óleos.

De forma a evitar a sobreexploração de algumas lavandulas, *L. multifida* com uma distribuição restrita em Portugal, foi micropropagada recorrendo à proliferação de meristemas axilares. Um protocolo eficiente para sua propagação em larga escala foi estabelecido, pela primeira vez para este *taxon* contribuindo, desta forma, para a preservação do seu património natural.

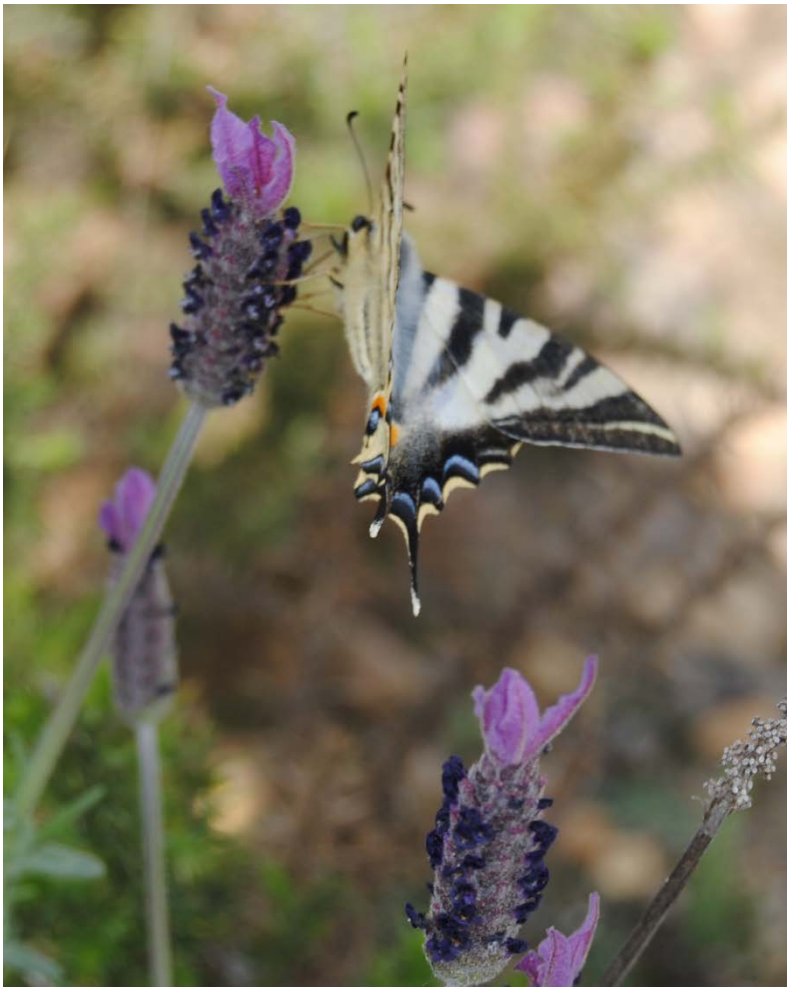
Tendo por base os resultados obtidos, o pr esente trabalho contribuiu para um melhor conhecimento científico do g énero *Lavandula* em Portugal. As actividades biológicas dos seus óleos essenciais, avaliadas pela primeira vez, demonstraram o eventual interesse industrial. Validações futuras de forma a complementar estes resultados deverão ser considerados para o desenvolvimento de bio-produtos com interesse nos sectores da saúde e agrícola.

PALAVRAS-CHAVE: *Lavandula latifolia*; *L. luisieri*; *L. multifida*; *L. pedunculata*; *L. viridis*; óleos essenciais; tricomas; quimiotaxonomia; actividade antifúngica; actividade anti-inflamatória; citotoxicidade; actividade nematodicida; repelência de insectos; cultura *in vitro*.

LIST OF ABBREVIATIONS

AmB - Amphotericin B
ATCC - American Type Culture Collection
BAP – Benzyladenine
CECT - Coleccion Espanhola de Cultivos Tipo
CFU – Colony Forming Units
CLSI - Clinical Laboratory Standard Institute
COI - Herbarium Universidade de Coimbra
COX - Cyclooxigenase
COX-2 -Inducible Cyclooxigenase
CR – Critically Endangered
DAI – Days After Inoculation
DMEM -Dulbecco´s Modified Eagle Medium
DMSO - Dimethyl Sulfoxide
ECF – Enhanced Chemifluorescence
EO - Essential Oil
EPA - Environmental Protection Agency
ERK - Extracellular Signal-Regulated Kinase
FDA - Food and Drug Administration
FEMA - Flavour Essence Manufacters Association
FID - Flame Ionization Detector
GC - Gas Chromatography
GC/MS - Gas Chromatography/Mass Spectrometry
GRAS - Generally Recognized As Safe
IBA – Indol-3-Butyric Acid
IFRA - International Flavour and Fragrance Association
IκBα – NF-κB inhibitor alpha
IL -Interleukin
iNOS - Inducible Nitrite Oxide Synthase
IPQ-CT - Instituto Português da Qualidade – Comissão Técnica
ISO – International Standards Organization
ISO-TC - International Standards Organization – Technical Committee
J1 – First stage juvenile
J2 – Second stage juvenile
JNK -c-Jun NH2-terminal Kinase (= SAPK)
LC – Least Concern
LISE - Herbarium Estação Agronómica Florestal
LISI - Herbarium Instituto Superior de Agronomia
LM - Light Microscopy
LPS - Lipopolysaccharide
MAPK - Mitogen Activated Protein Kinase
MAP2K - MAPK Kinase
MAP3K - MAPK Kinase Kinase
MEP/DOXP - Mevalonate Independent or 2C-Metil-D-Erythritol 4-Phosphate/-Deoxi-D-Xilulose-5-Phosphate Pathway
MIC - Minimal Inhibitory Concentration
MLC - Minimal Lethal Concentration
MS - Mass Spectrometry
MS medium - Murashige and Skoog medium
MTT - 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide
MVA - Mevalonate Dependent Pathway
NF-κB- Nuclear Transcription Factor *kappa* B
NMR - Nuclear Magnetic Resonance Spectroscopy
NO - Nitric Oxide
NOAEL - No Adverse Effect Level
NOS - Nitrite Oxide synthase
NRU - Neutral Red Uptake
NSAIDS – Non-Steroidal Anti-inflammatory Drugs
NTP – National Toxicology Program
NYP - N-Acetylglucosamine
PBS – Phosphate Buffered Solution
PCA - Principal Component Analysis
PCR- Polymerase Chain Reaction
PDA- Potato Dextrose Agar
PDVF – Polyvinylidene Difluoride
PGs - Prostaglandins
PI -Propidium Iodide
PO - Herbarium Universidade de Porto
PWD – Pine Wilt Disease
PWN –Pine-Wood Nematode
RI - Retention Indices
RIFM -Research Institute for Fragrance Materials
ROS - Reactive Oxygen Species
SAPK- Stress-Activated Protein Kinase
SD – Standard Deviation
SDA - Sabouraud Dextrose Agar
SEM - Scanning Electron Microscopy
SNAP- S-Nitroso-N-Acetyl-DL-Penicillamine
SPB-1- Polydimethylsiloxane
TBS – Tris-Buffered Saline
TNF-α -Tumour Necrose Factor alpha
ZEA - Zeatin

I. General Introduction and Objectives



1. General Introduction and Objectives

Medicinal plants play an important role in therapy with up to 80 % of the population, in developing countries, totally depending on plants for primary health care (Newmann, 2000). Despite the remarkable progress in synthetic chemistry during the last century, over 25 % of prescribed medicines in industrialized countries still derive, directly or indirectly, from plants and this percentage may reach 50 % when the over-the-counter market is considered (Newmann, 2000). A recent survey pointed out that 51 % of the 983 new chemical entities introduced as drugs during 1981-2006 were natural products, natural product derivatives or natural product mimics (Newman and Cragg, 2007). The great importance of natural products in drug discovery and the continuing threat to biodiversity with the on-going destruction of important ecosystems, justify the investigation of natural products as novel active agents (Hostettmann and Marston, 2002; Newman et al., 2003; Paterson and Anderson, 2005; Jones et al., 2007). In particular, plants represent an enormous reservoir of putative lead compounds since many species have still not been investigated for their pharmaceutical and pharmacological potential (Hostettmann and Marston, 2002).

During the last years there has been a growing demand for natural products with the global market of aromatic and medicinal plants gaining impact mainly in the health (Kong et al., 2003) and agricultural (Isman, 2006; Khater, 2012) sectors. An increasing number of people are also more amenable to accept such compounds as alternatives to more conventional drugs and pesticides. In fact, a long history of use and better patient tolerance, less side-effects as well as less environmental concerns also justify public acceptance (Waqar and Shaukat, 2006). Several industries have perceived this trend and are searching for bio-active products, biodegradable and without toxicity to humans and animals. A remarkable progression in the development of analytical methods has also contributed for a precise and more reliable characterization of plant metabolites (Philipson, 2007). Additionally, several screening assays to identify the most promising compounds, have been designed and adapted to different target organisms, including microorganisms (bacteria, fungi, viruses), invertebrates (insects, crustaceous, molluscs), animal and human cell cultures, isolated organs of vertebrates, isolated subcellular enzymes and receptors or whole animals (Hostettmann and Marston, 2002).

In recent years, research on aromatic plants and in particular their essential oils has also attracted many investigators (Bakkali et al., 2008). These products have long been used by local communities for their medicinal properties mainly as bactericidal, virucidal, fungicidal, anti-parasiticidal and insecticidal agents (Bakkali, 2008). However, many uses are based on old beliefs or faiths usually without any scientific support of their efficacy. This situation may cause severe problems and must be avoided through the identification of plant properties on a scientific-based manner. Moreover, once a plant is known to have some properties the local communities tend to overexploit it causing negative impacts on ecosystems. Many aromatic and medicinal plants are currently endangered due to inadequate culture practices, destruction of terrestrial ecosystems and overexploitation of natural resources. *In vitro* screening programs, based on ethnobotanical approaches, proved to be very efficient in validating traditional uses and searching for new active compounds (Alviano and Alviano, 2009; Knighorn et al., 2011). Many aromatic plants and their essential oils are currently valued in the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries (Buchbauer, 2000; Bakkali et al., 2008). Nevertheless, aromatic plants are well known for their high chemical variability mainly due to environmental and/or genetic factors that can affect their quality (Figueiredo et al., 2008). Therefore, it is necessary to develop methodologies that will allow a quality optimization, which guaranties a reliable final product with a well-defined chemical profile necessary to fulfill the very competitive markets in this sector. *In vitro* cultures have been applied for this purpose and provide effective alternatives to conventional propagation methods (Nalawade and Tsay, 2004; Debnath et al., 2006; Sidhu, 2010; Verma et al., 2012). Furthermore, although several studies have shown the huge potential of essential oils namely as antioxidant, antifungal and anti-inflammatory agents (e.g. Edris, 2007; Reichling et al., 2009), elucidation on t heir mechanism of action as well as safety and toxicological assays remain poorly explored. These steps are crucial for the development of new phytopharmaceuticals and should be considered in screening assays.

The Lamiaceae is one of the families that comprise a higher number of aromatic plants with commercial interest. For example, the genus *Lavandula* includes several species highly valued due to their essential oils. This genus comprises 39 wild essential oil bearing species distributed from Macaronesia (Canary Islands, Madeira and Cape

Verde Islands) across northern Africa, the Mediterranean basin, South-West Asia, Arabian Peninsula and western Iran and also in India (Upson and Andrews, 2004). The genus is known for its complex taxonomy, many synonyms and frequent hybridizations, with classifications changing over the years according with new findings and contributions from more accurate taxonomical studies (Upson and Andrews, 2004).

The commercial products of the genus include essential oils, fresh and dried flowers and landscape plants, mainly of 4 s pecies: *L. stoechas, L. angustifolia*, *L. latifolia* and *L.* x *intermedia,* a hybrid between the two last species. The most economically important lavender is *L. angustifolia*, also known as common lavender, fine lavender or true lavender, widely used in fine perfumery. *Lavandula latifolia*, commonly known as spike lavender or spike is widely used in soap fragrances whereas *L.* x *intermedia*, commonly known as lavandin, is considerably cheaper and has been used in inexpensive fragrances, household and pharmaceutical products. *L. stoechas*, the common French or Spanish lavender is becoming increasingly popular as an ornamental species (Upson and Andrews, 2004).

Nowadays, lavender scent is very popular in pillows, bath care, home, and pet products and provides a unique taste to many beverages, sweets, jellies, jams, marmalades, honey and condiments, being the oils valuable products for the food (flavoring), perfumery and cosmetic industries, as well as in aromatherapy (Boelens, 1995; Upson and Andrews, 2004; Cavanagh, 2005). However, many other applications can be foreseen, as suggested in several reports on the biological activities of this genus. *Lavandula* oils have been referred for their sedative and antispasmodic properties (Cavanagh and Wilkinson, 2002) as well as acaricidal (Perrucci et al., 1996), antibacterial (e.g. Dadalioğlu and Evrendilek, 2004; Moon et al., 2006), antifungal (e.g. Angioni et al., 2006) and antioxidant activities (Matos et al., 2009). More recently, its application as a biopesticide has also been suggested (Ebadollahi et al., 2010; González-Coloma et al., 2011). *Lavandula* essential oils are valuable natural products, some of them regulated by international ISO standards (ISO TC 54 - ISO/CD 8902, 2007; ISO TC 54 N - ISO/WD 4719, 2009). Despite this popularity, native Portuguese lavenders remain mainly unexplored and have received poor recognition in markets. A lack of scientific-based studies, as well as the absence of guaranty of high-quality end-products is, at least, partially responsible for this situation.The Portuguese lavenders are included

in three distinct sections (Upson and Andrews, 2004): Section Lavandula (*L. latifolia*), Section Pterostoechas (*L. multifida*) and Section Stoechas (*L. luisieri*, *L. pedunculata* and *L. viridis*). These species have different distributions: *L. latifolia* occurs in a very limited region near Coimbra; *L. multifida* can be found in particular regions of Portugal, namely Arrábida, Sesimbra and Mértola, whereas species from section Stoechas are widely distributed, *L. viridis* occurring in the South and *L. pedunculata* together with *L. luisieri* distributed all over the country.

Taking into account the economic potential of lavenders and the sparse information on the species growing spontaneously in Portugal, a scientific-based study was carried out in order to evaluate the biological properties of the oils and to understand how they may exert their effects. Moreover, detailed morphological and chemical analyses were performed and taxonomical clarifications suggested mainly for the species of the section Stoechas. In the present study, *L. latifolia* essential oils were not analysed nor assessed for their biological activities due to limitations in spontaneous plant material and studies on *L. pedunculata* were based on previous results achieved during a Master course (Zuzarte, 2008) and further explored in the present study. In what concerns biological activities, *L. viridis* was selected to perform a great part of the experiments. This choice was based on the lack of information on the biological activities of this plant and its essential oil and by a practical motive related to the high essential oil yield, particularly interesting for industrial purposes.

The main objective of the present study was the valorization of lavender species growing spontaneously in Portugal towards a further economic exploitation. For this purpose several strategies were defined, namely:

1. taxonomical clarification particularly for *L. pedunculata* and *L. luisieiri*, based on morphology, distribution and chemical characteristics (sections 1-4, chapter II).
2. Chemical characterization and identification of the intraspecific variability of the oils, allowing the establishment of a well-defined chemical profiles (section 3, chapter II).

3. Evaluation of the bioactivity of the oils, for a further application in the health sector, by assessing their antifungal activity against pathogenic human and animal strains (*Candida* spp., *Cryptococcus neoformans*, dermatophytes and *Aspergillus* spp.; section 1, chapter III) and anti-inflammatory potential, by assessing the effect of the oil on several inflammatory mediators and signaling pathways using LPS-stimulated macrophages (section 2, chapter III); evaluation of a potential application of the oils in agriculture by assessing their antifungal effects against phytopathogenic fungi (*Alternaria* sp., *Cladosporium* sp., *Fusarium* spp. and *Penicillium* sp.; section 4, chapter III), nematicidal activity against the root-knot nematode (*Meloidogyne javanica*; section 5, chapter III) and insect repellency effects against *Monochamus galloprovincialis* (insect vector of pine–wilt disease; section 6, chapter III). A relationship between chemical composition and bioactivities allowing the identification of most active compounds and elucidation of the mechanisms of action involved in some biological activities was also considered.

4. Determination of the oils cytotoxicity in different cell lines (macrophages, keratinocytes, alveolar epithelial cells), an important step prior to their possible assessment for pharmaceutical and cosmetic purposes (section 3, chapter III).

5. Development of effective micropropagation protocols for the cloning of selected species/chemotypes and preservation of species collected in endangered habitats, through proliferation of meristems (chapter IV).

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II. Morphological and Chemical Characterization of *Lavandula* spp.

1. Taxonomy

1. Taxonomy

1.1 History

Since ancient times lavenders have been recognized as high-value plants. The earliest descriptions of these plants were found in the texts of Theophrastus (370-285 BC) (cited in Costas, 2005) followed by those of Dioscorides (40-90 AC) in his notable *De Materia Medica* (Dioscorides, 60 AC) in which the medicinal properties of *Lavandula stoechas* were first mentioned. During the Middle Age, botany books were rare, becoming more common during the Renaissance after the invention of printing. At this time, herb books became quite popular, due to the medicinal, fragrant and flavoring properties of these plants. During the sixteenth and seventeenth centuries the scientific interest in plants raised with the emergence of taxonomy. One of the most influent taxonomists of that time was Joseph Pitton de Tournefort (1656-1708) who divided lavenders into two genera: *Lavandula* and *Stoechas* (Tournefort, 1700, in Upson and Andrews, 2004). Later on, the well-known Carolus von Linnaeus (1707-1778) established the binomial classification and united these two genera, with several species being recognized (Linnaeus, 1753). Carl von Linnaeus (1741-1783), son of Carl von Linnaeus (1707-1778) was the first to write a dissertation on the genus *Lavandula* in which more species were considered (Linné, not published).

The second monograph on the genus (*Histoire Naturelle des Lavandes*) was written by Baron Gingins (1790-1863), who recognized the sections Spica, Stoechas and Pterostoechas based on floral and nutlet characters (Gingins, 1826).

A few years latter George Bentham (1800-1884) elaborated an extensive monograph on the family (*Labiatarum Genera et Species*) (Bentham, 1833, 1835). A fourth section, Chaetostachys, was recognized including species with single-flowered cymes and a spiral arrangement of the floral axis.

Latter on a new section, Subnudae, was established including the Arabian species from the section Chaetostachys (Chaytor, 1937). This separation was based on differences in habit, leaves, branching of the spike and nutlets features. Chaytor's monograph (*A Taxonomic Study of the Genus Lavandula*) is considered the fist most complete revision of the genus gathering many *taxa* previously identified by other

authors. Since then, several important studies have been performed, focusing on individual sections, geographic areas or cultivated *taxa*. For example, Arnaldo Rozeira revised the section Stoechas and divided it into two subsections, Dentata and Eu-Stoechas (Rozeira, 1949). The former included *L. dentata*, while the latter comprised *L. stoechas* and *L. viridis*. One of the most relevant aspects of this work was the classification of *L. pedunculata* as a subspecies of *L. stoechas*. This classification, although followed by many authors, remained controversial throughout the years. In more recent studies it was abandoned and was also not adopted in the present study.

In 1963, Devetak and Cenci organized the sections according to evolutionary relationships grouping in the same branch, Spica and Stoechas, and leaving Pterostoechas, Subnudae and Chaetostachys in another.

Miller (1985) revised taxonomically the genus in Arabia and North-East Africa and León-Arencibia (1984) in the Canary Islands. The native *taxa* of the Iberian Peninsula were studied using karyologic data (Suárez-Cervera, 1986), nutlets morphology (Suárez-Cervera, 1987) and pollen features (Suárez-Cervera and Seoane-Camba, 1986). One of the most relevant contributions of these works was the establishment of the Section Dentata, with only one species (*L. dentata*), previously included in Section Stoechas.

More recently, the chemotaxonomy of Iberian species was analysed (Garcia Vallejo, 1992) as well as the molecular phylogeny of the genus resulting in a provisional subgeneric classification of eight sections and three subgenera (Upson, 1997).

In 2004, Upson and Andrews collected all the information on wild and cultivated lavenders, providing a very comprehensive treatment of the genus and alerting to areas were more research was desirable (Upson and Andrews, 2004).

1.2 Features of the genus

Lavandula belongs to the Lamiaceae family, within the Nepetoideae (Dumortier) Luersson subfamily, and the Lavanduleae (Endl.) Boiss tribe. It comprises ca. 39 wild species widely distributed in Macaronesia and around the Mediterranean region, less frequent in north Africa, Arabian Peninsula and south Asia (Upson and Andrews, 2004).

Lavandula L., Sp. Pl.: 572 (1753)

Aromatic shrubs or subshrubs, woody-based perennials or short-lived herbs with a variable indumentum. Stems erect, rarely prostate, 4-angled, rarely 6-8-angled. Leaves opposite, entire, lobed or dissected, sessile or petiolated. Inflorescence a terminal spiciform thyrse, usually pedunculated with a simple or branched peduncle, occasionally with colored apical bracts, with verticillasters of cincinnus cymes, 2-9 flowered, with minute bracteoles or 1-flowered, usually without bracteoles, opposite or spirally arranged. Bracts subtending cymes, coriaceous, persistent, variable in form, reticulate or parallel veined. Calyx persistent, tubular, actinomorphic to 2-lipped, 5-lobed (3 lobes forming the upper lip and 2 ones the lower lip), lobes ± equal or posterior lip larger or modified into an appendage, (8-)13-or 15-veined, veins of the anterior lobes reaching the apex and sometimes fusing. Corolla weakly or strongly 2-lipped, 5-lobed (2 lobes forming the upper lip and 3 ones the lower lip), spreading, variable in size, corolla tube just exceeding or up to 3 x longer than calyx, blue-violet to purple, white or pink, rarely blackish purple or yellowish. Nectary disc large, lobes born opposite to the ovaries. Stamens 4, declinate usually didynamous, included, anterior pair longer; filaments typically glabrous, anthers kidney-shaped and synthecous. Stigma 2-lobed, complanate or capitate. Nutlets variable in shape, colour and size, with small basal abscission scar, sometimes a lateral scar (areole), 0.25 to 0.75 x the length of the nutlet, usually mucilaginous (Fig. 1).

Obs: Genus divided in 3 subgenera [**Lavandula** (cymes 3-9(-15)-flowered; nutlets with a basal scar); **Fabricia** (Adams.) Upson & Andrews (cymes single-flowered; nutlets with a basal and lateral scare; leaves ovate-lanceolate, dissected or lobed; calyx distinctly 2-lipped; corolla violet-blue or white, strongly 2-lipped); **Sabaudia** (Buscal. & Muschl.) Upson & S. Andrews (cymes single-flowered; nutlets with a basal and lateral scare; leaves simple, narrowly elliptic; calyx regular and lobes all equal; corolla yellow-brown, lobes equal)]. Subgenus **Lavandula** with 3 sections [Stoechas Ging., Dentatae Suarez-Cerv. & Seoane-Camba, Lavandula]; Subgenus

Fabricia with 4 sections [Pterostoechas Ging., Hasikenses Upson & S. Andrews, Subnudae Chaytor, Chaetostachys Benth.]; Subgenus **Sabaudia** with 1 section [Sabaudia].

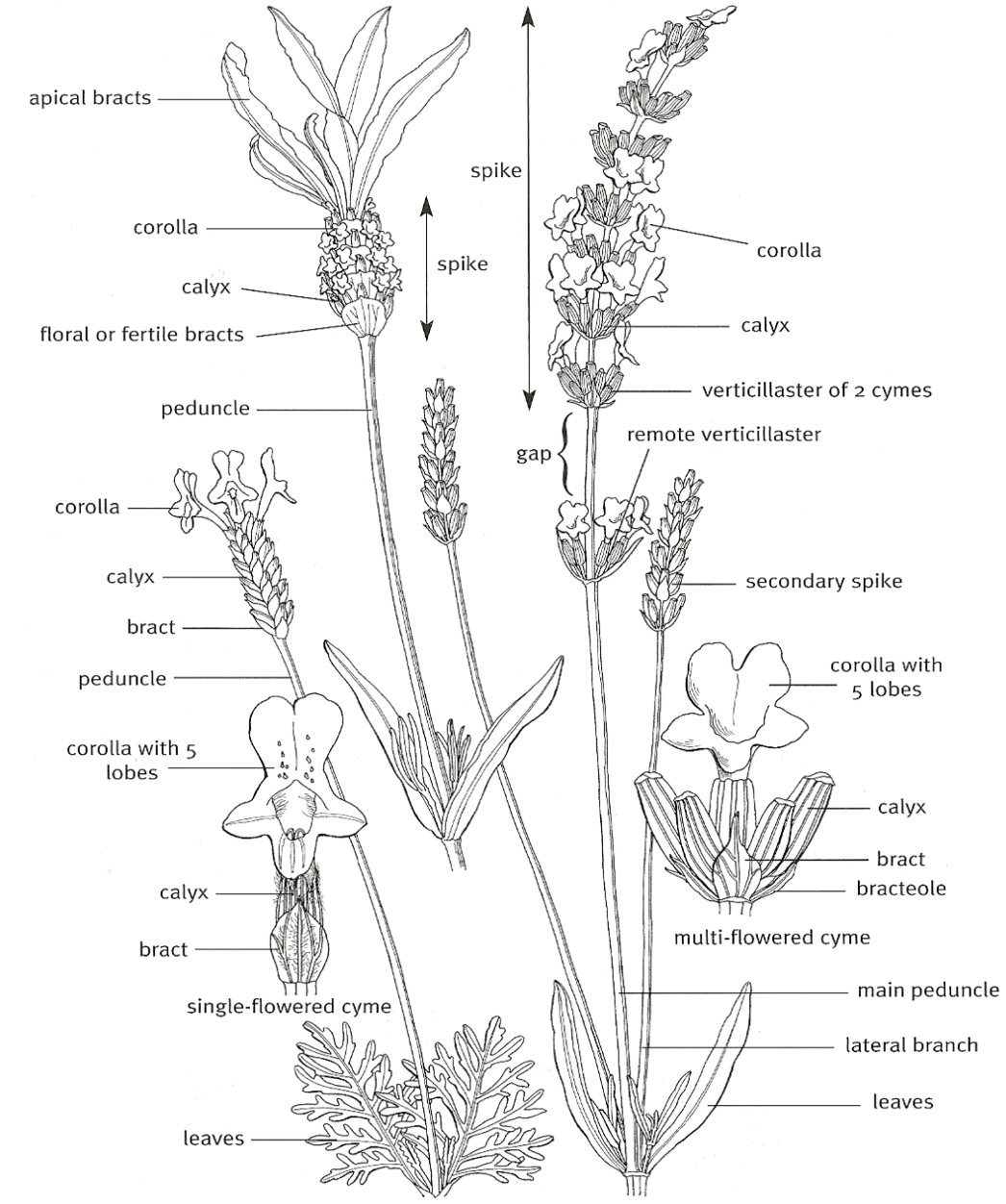

Figure 1. Flowering shoots of *Lavandula* spp. illustrating characteristic features of the genus. In Upson and Andrews (2004).

In the Volume XII of the Flora Iberica (Morales, 2010), one of the most actual floras, eight species of *Lavandula* were considered, but in Portugal only five spontaneous species occur.

Key to the species [adapted from Flora Iberica XII (Morales, 2010)]

1.	Leaves dentate sometimes deeply divided	***L. multifida***
	Leaves entire	2.
2.	Inflorescence compact with colored and larger apical bracts	3.
	Inflorescence with inferior verticillasters separated; without colored and larger apical bracts	***L. latifolia***
3.	Flowers and apical bracts greenish-white; fertile bracts 7.5-9.5 mm long	***L. viridis***
	Flowers and apical bracts violet or rarely white; fertile bracts 3.5-8 mm long	4.
4.	Peduncle long, more than 2x the inflorescence	***L. pedunculata***
	Peduncle up to 2x longer than the inflorescence	***L. luisieri***

1.3 The Portuguese Lavenders

Lavandula spp. have a very complex taxonomy with several *taxa* being identified under different names. In order to clarify some controversial classifications, namely in the section Stoechas (e.g. Morales, 2010 *vs* Rivas-Martinez, 1979), a detailed morphological characterization was performed on the five species growing wild in Portugal.

1.3.1 Material and methods

During the course of this research about 650 herbarium specimens from the four main Portuguese herbaria were studied: *Universidade de Coimbra* (COI), *Estação Agronómica Florestal* (LISE), *Instituto Superior de Agronomia* (LISI), and *Universidade do Porto* (PO). The main populations of *Lavandula* growing spontaneously throughout the country were identified (Fig. 2) and several field trips were made to harvest plants for the different activities contemplated in this study (Table 1). Voucher specimens were deposited at the COI herbarium.

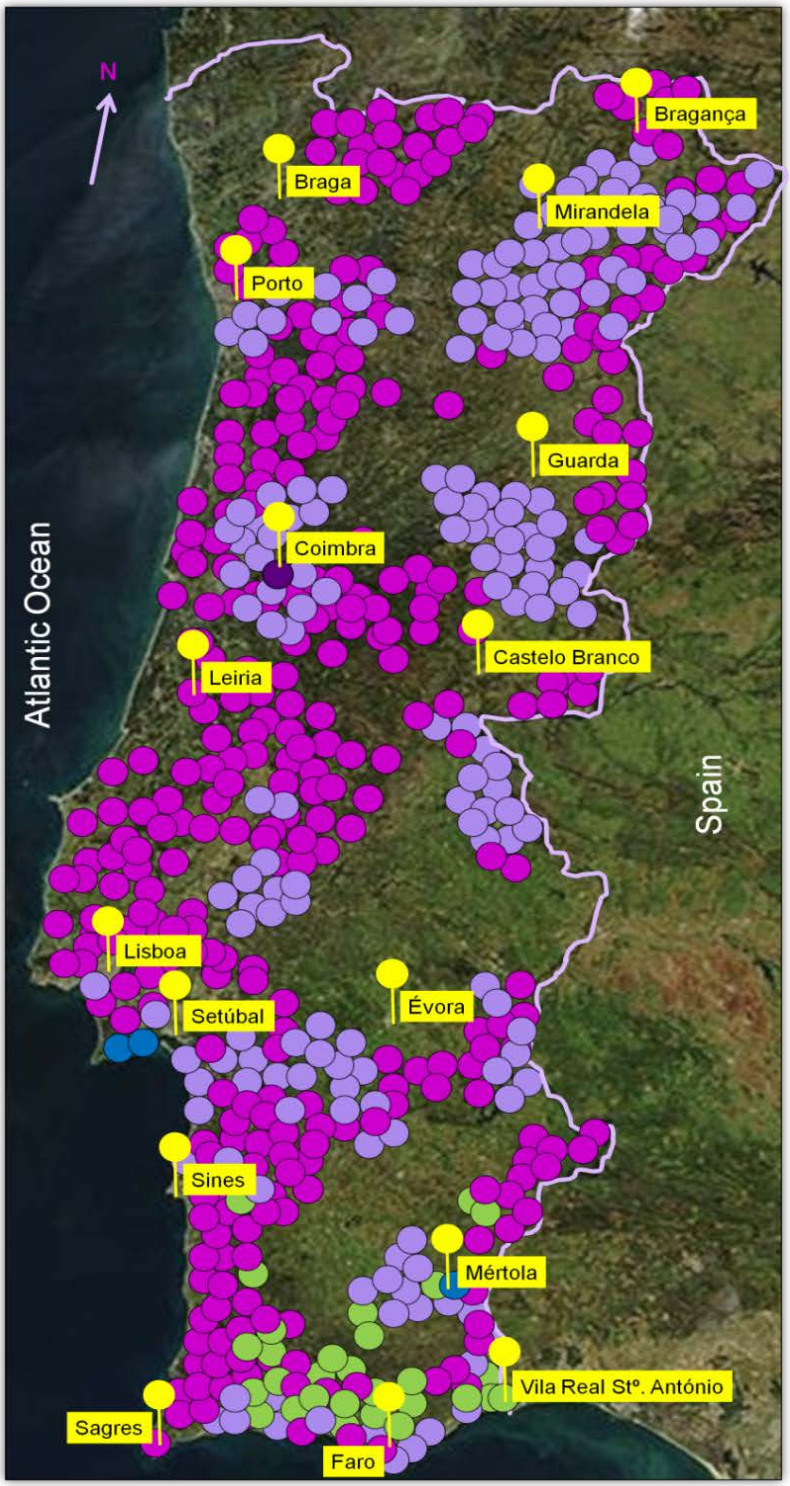

Figure 2. Distribution of spontaneous populations of *Lavandula* spp. throughout Portugal.

L. latifolia *L. luisieri* *L. multifida* *L. pedunculata* *L. viridis*

Table 1. Site of collection of *Lavandula* spp.

Species	Date of collection	Region	Site of Collection	UTM Coordinates	Sample identification	Samples used for different activities				
						Voucher specimens (COI)	Morphological analyses	Essential oil analyses	Biological activities	*In vitro* culture
L. latifolia	May 2005	Coimbra	Almalaguês	29NE54	A		✓			✓
	August 2012			29NE54	B	✓	✓			
L. luisieri	May 2005	Coimbra	Fonte Coberta	29NE43	A		✓	✓	✓	✓
	May 2005	Castelo Branco	Sertã/Pedrogão Grande	29NE71	B		✓	✓		
	June 2005	Coimbra	Farelo, Penela	29NE52	C	✓	✓	✓		
	June 2005	Coimbra	Piódão	29NE95	D	✓	✓	✓	✓	
	May 2008	Setúbal	Sesimbra	29MC95	E		✓	✓		
	May 2008	Leiria	Espinheira	29MD86	F		✓	✓		
	May 2008	Alentejo	Zambujeira do Mar	29NB16	G	✓	✓	✓		
	May 2008	Algarve	Serra do Caldeirão	29NB91	H		✓	✓		
	May 2008	Algarve	Espargal	29NB71	I	✓	✓	✓		
	May 2008	Algarve	Guilhim	29NB90	J	✓	✓	✓		
	May 2009	Algarve	Salir	29NB82	K	✓	✓	✓		
	May 2009	Algarve	Cabo São Vicente	29NA09	L	✓	✓	✓	✓	
	May 2010	Algarve	C. São Vicente/Sagres	29NA09	L1	✓	✓	✓		
	June 2010	Algarve	C. São Vicente/Sagres	29NA09	L2	✓	✓	✓		
	June 2010	Algarve	C. São Vicente/Sagres	29NA09	L3	✓	✓	✓		
	June 2010	Algarve	C. São Vicente/Sagres	29NA09	L4	✓	✓	✓		
	June 2010	Alentejo	Arrifana	29NB12	M		✓	✓		

Species	Date of collection	Region	Site of Collection	UTM Coordinates	Sample identification	Samples used for different activities				
						Voucher specimens (COI)	Morphological analyses	Essential oil analyses	Biological activities	*In vitro* culture
L. multifida	May 2008	Setúbal	Sesimbra	29MC95	A	✓	✓	✓		
	May 2008	Setúbal	Portinho da Arrábida	29NC05	B		✓	✓		
	June 2008	Setúbal	Sesimbra	29MC95	AI		✓	✓	✓	✓
	May 2009	Alentejo	Mértola	29PB16	C	✓	✓	✓		
	June 2009	Setúbal	Sesimbra	29MC95	AII		✓	✓		
	May 2010	Setúbal	Sesimbra	29MC95	AIII		✓	✓		
	November 2010	Setúbal	Portinho da Arrábida	29NC05	BI		✓	✓		
	November 2011	Setúbal	Sesimbra	29MC95	AIV		✓	✓		
L. pedunculata	June 2005	Mirandela	Valbom dos Figos	29PG50	A0		✓	✓	✓	
	June 2005	Mirandela	Valbom dos Figos	29PG50	A1		✓	✓		
	June 2005	Mirandela	Quinta do Valongo	29PF59	A2		✓	✓		
	June 2005	Mirandela	Quinta do Valongo	29PF59	A3		✓	✓		
	June 2005	Mirandela	Valbom dos Figos	29PG50	A4		✓	✓		
	June 2005	Mirandela	Valbom dos Figos	29PG50	A5		✓	✓		
	July 2005	Bragança	Serra da Nogueira	29PG72	B0		✓	✓		
	July 2005	Bragança	Serra da Nogueira	29PG72	B1		✓	✓		
	July 2005	Bragança	Serra da Nogueira	29PG72	B2		✓	✓		
	July 2005	Bragança	Serra da Nogueira	29PG72	B3		✓	✓	✓	✓
	July 2005	Bragança	Serra da Nogueira	29PG72	B4		✓	✓		
	July 2005	Bragança	Serra da Nogueira	29PG72	B5		✓	✓		
	July 2005	Bragança	Carrazedo	29PG72	B6		✓	✓		
	July 2005	Bragança	Carrazedo	29PG72	B7		✓	✓		

Species	Date of collection	Region	Site of Collection	UTM Coordinates	Sample identification	Samples used for different activities				
						Voucher specimens (COI)	Morphological analyses	Essential oil analyses	Biological activities	*In vitro* culture
L. pedunculata	July 2005	Bragança	Carrazedo	29PG72	B8		✓	✓		
	July 2005	Bragança	Rebordãos	29PG82	B9		✓	✓		
	July 2005	Bragança	Rebordãos	29PG82	B10		✓	✓		
	July 2005	Bragança	Rebordãos	29PG82	B11		✓	✓		
	July 2005	Bragança	Rebordãos	29PG82	B12		✓	✓		
	July 2005	Bragança	Rebordãos	29PG82	B13		✓	✓		
	July 2005	Bragança	Rebordãos	29PG82	B14		✓	✓		
	July 2005	Guarda	Manteigas	29PE27	C0		✓	✓		
	July 2005	Guarda	Manteigas	29PE27	C1		✓	✓		
	July 2005	Guarda	Manteigas	29PE27	C2		✓	✓		
	July 2005	Guarda	Celorico da Beira	29PG72	C3		✓	✓		
	July 2005	Guarda	Celorico da Beira	29PG72	C4		✓	✓		
	July 2005	Guarda	Celorico da Beira	29PG72	C5		✓	✓		
	July 2005	Guarda	Celorico da Beira	29PG72	C6		✓	✓	✓	✓
	July 2005	Guarda	Celorico da Beira	29PG72	C7		✓	✓		
	July 2005	Guarda	Alvendre	29PE49	C8	✓	✓	✓		
	July 2005	Guarda	Alvendre	29PE49	C9	✓	✓	✓		
	July 2005	Guarda	Alvendre	29PE49	C10		✓	✓		
	July 2005	Coimbra	Foz de Arouce	29NE64	D0		✓	✓		
	July 2005	Coimbra	Foz de Arouce	29NE64	D1		✓	✓		
	July 2005	Coimbra	Foz de Arouce	29NE64	D2		✓	✓		
	July 2005	Coimbra	Foz de Arouce	29NE64	D3		✓	✓		
	July 2005	Coimbra	Foz de Arouce	29NE64	D4		✓	✓		

Species	Date of collection	Region	Site of Collection	UTM Coordinates	Sample identification	Samples used for different activities				
						Voucher specimens (COI)	Morphological analyses	Essential oil analyses	Biological activities	*In vitro* culture
L. pedunculata	July 2005	Coimbra	Foz de Arouce	29NE64	D5		✓	✓		
	July 2005	Coimbra	Foz de Arouce	29NE64	D6		✓	✓		
	July 2005	Coimbra	Foz de Arouce	29NE64	D7		✓	✓	✓	✓
	July 2005	Coimbra	Ceira	29NE54	D8		✓	✓		
	July 2005	Coimbra	Ceira	29NE54	D9		✓	✓		
	July 2005	Coimbra	Ceira	29NE54	D10		✓	✓		
	May 2008	Alentejo	Santiago do Cacém	29NC20	E	✓	✓	✓		
	June 2008	Vila Real	Alijó	29PF27	F		✓	✓		
L. viridis	May 2008	Algarve	Barranco do Velho	29NB92	A	✓	✓	✓	✓	✓
	May 2009	Algarve	Salir	29NB82	B	✓	✓	✓		
	May 2009	Algarve	Barranco do Velho	29NB92	AI		✓			
	May 2009	Algarve	Porto Nobre	29NB81	C	✓	✓	✓		
	May 2010	Algarve	Barranco do Velho	29NB92	AII		✓			
	May 2011	Algarve	Barranco do Velho	29NB92	AIII	✓	✓			

Note: Letters represent collective samples; letters and numbers refer to individual samples; letters with roman numerals refer to samples collected in different years at the same site of collection.

1.3.2 The species

1.3.2.1 ***Lavandula latifolia*** Medik., Bot. Beob. 1783: 135 (1784)

Woody dome-shaped shrub, (40-)50-70(-100) cm. Leaves oblanceolate to spathulate, 3-5(-6) x 0.6-0.8(-1.2) cm, margin revolute, grey-green to silver-grey, with dense felt-like indumentum. Spikes pedunculated [peduncles typically once-branched giving a 3-spiked flower head, sometimes branched further, 20-40(-50) cm long, (2-)4-8(-10) cm long, slender and often interrupted, or short, compact and blunt, occasionally with a remote verticillaster]. Bracts linear, apex acute, 1.0-1.5 cm x 0.2-0.3 mm, green to brown with a conspicuous middle vein. Bracteoles 0.3-0.5 mm long. Calyx 4.5-6.0 mm long, lobes rounded, appendage rotund, about 1 mm, indumentum of dense glandular hairs, often colored in upper quarter. Corolla 1.2-1.4 cm long, light violet-blue to deep violet-purple, often with a paler throat, upper lobes notched and about twice the size of the lower ones, triangular and reflexing. Nutlets 1.9-2.0 x 1.3-1.5 mm, ellipsoid, ± bright, dark brown color, with apical insertion. 2n= 24, 36, 50, 54, 60 (Fig. 3).

France: south-west and central; Spain: north-east, central and south-east; Portugal: Beira Litoral. Common names - English: spike lavender, broad-leaved lavender, common lavender; Portuguese: alfazema, alfazema-brava.

Obs.: L. latifolia is very rare in Portugal. Only a few individuals were found in a small area near Coimbra (Rio de Galinhas). It is not yet clear whether this species is native or has been naturalized (Pereira Coutinho, 1939; Franco, 1984).

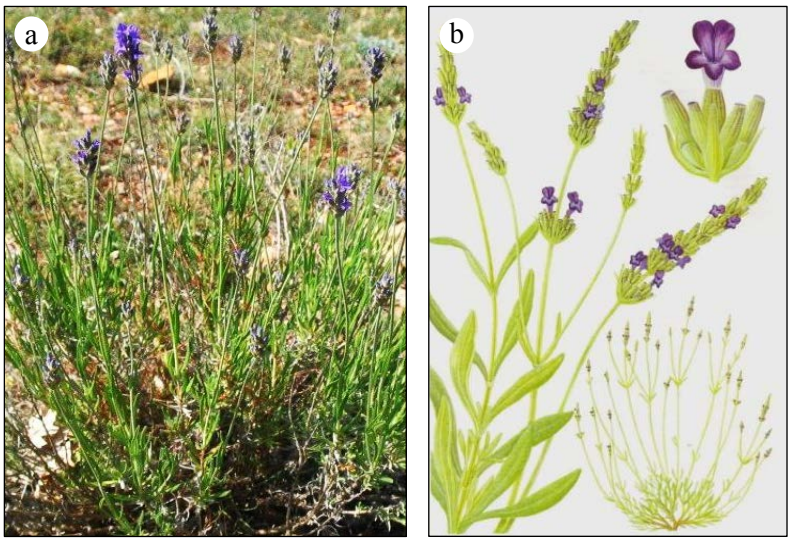

Figure 3. *Lavandula latifolia*. a) Field growing plant. b) Schematic drawing, in Upson and Andrews (2004).

1.3.2.2 ***Lavandula luisieri*** (Rozeira) Rivas Mart. in Lazaroa 1: 110 (1979)

Woody shrub, with erect stems 30-60(-200) cm tall. Leaves linear to narrowly elliptic, 2-3 x 0.3-0.5 cm, margin revolute, grey-green dense velvety indumentums of short white branched hairs. Spikes [with unbranched peduncles (2-)3-4(-6) cm], cylindrical, 3-5(-6) x 1-1.5 cm, secondary spikes smaller, 1.5-2.0 cm, long. Fertile bracts broadly ovate, apices acute to acuminate up the spike, 6-10 x 7-10 mm, slightly larger than the calyx, velvety indumentums of short branched hairs, particularly dense on the veins, often tinged purple-violet. Bracteoles minute, linear, ca.1 mm long. Apical bracts (2-)4(-6), narrowly elliptic to spathulate, 2.5-3.5 x 0.6 -0.8 cm, dark reddish purple. Calyx with the four anterior lobes triangular, the middle upper lobe a rotund appendage, indumentum of short white branched hairs, dense, becoming sparse on mature calyces. Corolla with a ring of simple hairs borne just inside the throat, corolla lobes rounded and sub-equal, very dark violet-purple. Nutlets 1.6-1.8 x 1.1-1.3 mm, ellipsoid, subtrigonous, sometimes flatened, with a convex face, smooth, shiny, often papillose at the apex, brown, 2n=3 (Fig. 4a).

Spain: southwest; Portugal: throughout the country. Common names – Portuguese: rosmaninho-menor.

Obs. Ramon Morales (Flora Ibérica XII, 2010) does not consider this *taxon* as a separated species. Instead, he considered two subspecies within *L. stoechas*: subsp. *stoechas* (Fig. 4b) - leaves uniform, white indumentum; inflorescence with peduncle shorter than cyme and subsp. *luisieri* (Fig. 4a) - leaves heterogeneous, normally axillar leaves lanceolate and bigger, grey indumentum; inflorescence with peduncle longer than cyme. Based on our chemical results, only subsp. *luisieri* was found in Portugal. Taking together morphological and chemical features we accept the classification adopted by Rivas-Martinez (1979) and Franco (1984), that considered *L. luisieri* as an independent species. More details about this decision are elucidated in section 4. Chemotaxonomy of the present chapter.

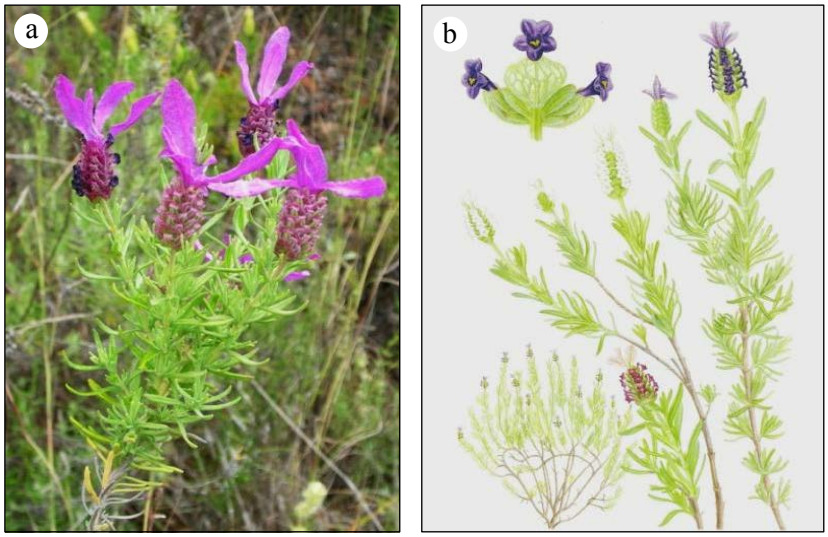

Figure 4. *Lavandula lusieiri*. a) Field growing plant. b) Schematic drawing, in Upson and Andrews (2004).

1.3.2.3 ***Lavandula multifida*** L., Sp. Pl. 572 (1753)

Woody-based perennial, 30-50 cm. Stems indumentum of long, white simple hairs, over short highly branched hairs. Leaves ovate, bipinnatisect, 2-4 cm (including petiole) x 15-30 mm. Spikes (with peduncles rarely branched, 7-15 cm), simple or once branched at the base, 30-50(-70) mm long, the cymes clearly twisting around the axis. Bracts broadly ovate with papery wings, apex sharply acute, typically with three dark main veins, occasionally the outer two veins branching, 0.75-1.0 x the length of the calyx, indumentum of short branched hairs, sparsely to densely pubescent. Calyx with the upper middle lobe broadly triangular and shorter than lateral ones, veins often coloured violet-blue. Corolla large, upper lip bilobed, twice as large as the lower lateral

ones, bicoloured, the lower lobes violet fading to blue-violet on the upper lobes, with darker violet guidelines. Nutlets 1.6-1.7 – 1.1-1.3 mm, subtrigonous, somewhat flatened, elliptic in outline, with the insert surface triangle of more than 1/3 than the total length, light brown. 2n=24 (Fig. 5).

Algeria; Morocco; Spain; Lybia; Italy; Egypt; Sudan: north; Portugal: Estremadura and Beira Baixa. Common names – English: fernleaf lavender; Portuguese: alfazema-de-folha-recortada.

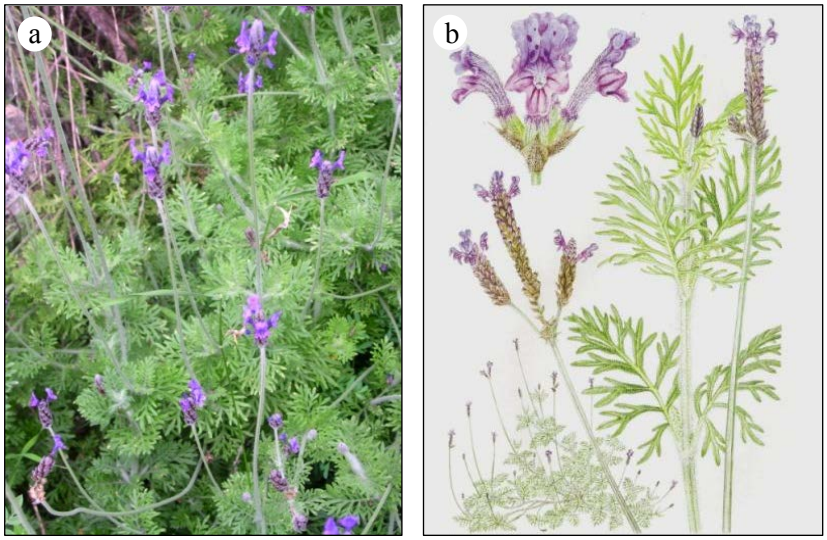

Figure 5. *Lavandula multifida*. a) Field growing plant. b) Schematic drawing, in Upson and Andrews (2004).

1.3.2.4 ***Lavandula pedunculata*** (Mill.) Cav., Descr. Pl.:70 (1802)

Woody shrub, with erect or ascending stems up to 50-80(-100) cm tall. Leaves linear, 2-4 cm long, those in axils 1.0-1.5 cm long, dense felt-like indumentum. Spikes compact, cylindrical, ovate, 2-4 x 1-1.5 cm, with unbranched peduncles, (10-)20-30 cm long, dense indumentum of highly branched hairs, the upper part flushed violet-pink. Fertile bracts broadly obovate-triangular, apex blunt with dentate edge, the upper pairs and rarely the lowest pair with a mucronate tip, 0.6-0.8(-1.0) cm long, covering cyme, sparse to dense felt like indumentum of highly branched hairs, upper part flushed with

violet-red. Bracteoles minute ca. 1 mm long, linear. Apical bracts (2-)4(-6), narrowly elliptic to obovate, erect, 2.0-3.5 x 0.5-0.6 cm, violet-pink. Nutlets 1.8-2.2 x 1.2-1.3 mm, ellipsoid ± subtrigonous, with the insert surface apical, light brown. 2n= 30 (Fig. 6).

Spain; Portugal: throughout the country. Common names – English: butterfly lavender, Portuguese or Spanish lavender; Portuguese: rosmaninho-maior.

Obs. Different authors have considered 3 subspecies for *L. pedunculata*: subsp. *lusitanica*, subsp. *pedunculata*, and subsp. *sampaiana* (e.g. Franco, 1984; Upson and Andrews, 2004). However, a great morphological variation is frequent in all populations, without any obvious geographical pattern. Therefore, throughout the study, infraspecific classifications were not considered. More details are elucidated in section 4. Chemotaxonomy of the present chapter.

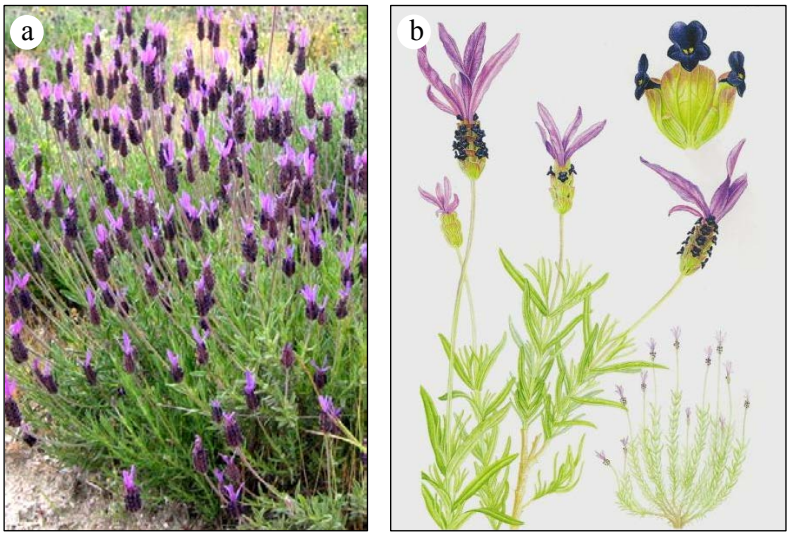

Figure 6. *Lavandula pedunculata*. a) Field growing plant. b) Schematic drawing, in Upson and Andrews (2004).

1.3.2.5 ***Lavandula viridis*** L'Hér., Sertum Anglicum:19, t. 21 (1789).

Highly aromatic and viscid woody shrub, 50-70(-100) cm tall. Leaves linear, 2.5-4.0 x 0.3-0.5 cm, entire with ± revolute margin, sessile, apex acuminate to blunt, indumentum of highly branched hairs with numerous glandular hairs. Spikes robust and compact, typically ± square in cross section, 2-4(-5) cm long, with unbranched peduncles, 5.0-10 cm long, frequently densely tomentose. Fertile bracts, rotund or

broadly ovate, 1.0-1.2 x 0.9-1.2 cm, apex acuminate, margins often crenulate, covering the cyme, venation reticulate and veins conspicuously raised. Bracteoles minute, green. Apical bracts oblanceolate or narrowly elliptic, short ca. 0.5-1.5 cm long, white to greenish white. Calyx 0.8-1.0 cm long, the four anterior lobes broadly triangular, reflexing in fruit, appendage circular as broad as calyx, sparse to dense indumentum. Corolla tube with a few hairs on lower half, widening at throat, upper lobes ca. 1.5 x size of the lower lobes, white; anthers conspicuously held at the throat of the tube, pollen orange. Nutlets 2.0 x 1.5-1.7 mm, somewhat flattened to ellipsoid, subtrigonous, smotth, with the insert surface apical; light brown. 2n=30 (Fig. 7).

Spain; Portugal: South, Azores and Madeira. Common names - English: madeira lavender, green lavender, lemon lavender. Portuguese: rosmaninho-verde.

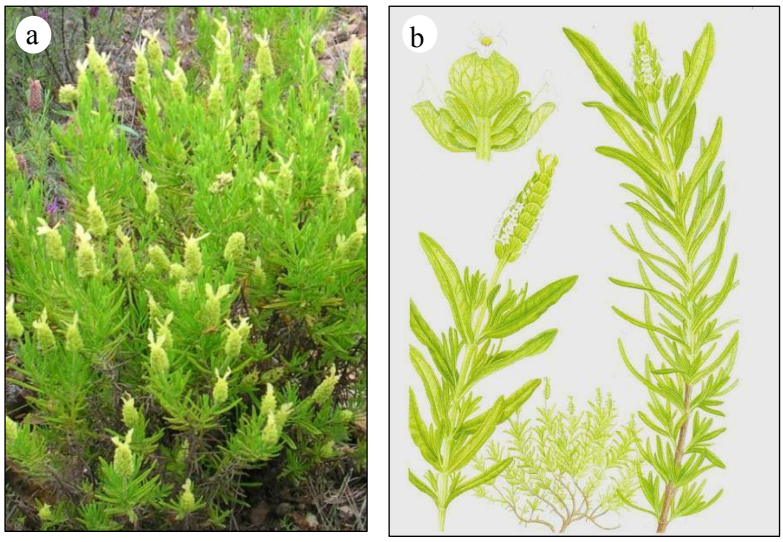

Figure 7. *Lavandula viridis*. a) Field growing plant. b) Schematic drawing, in Upson and Andrews (2004).

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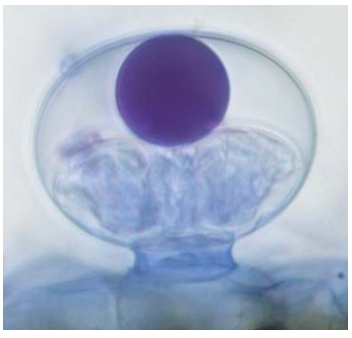


2. Trichomes

2. Trichomes

2.1 Introduction

Essential oils are produced and accumulated in specialized secretory structures that differ in morphology, structure, function and distribution. Therefore, these structures can be considered important taxonomical characters, mainly for the families in which they are most common such as Asteraceae, Apiaceae and Lamiaceae. These structures can be found on the surface of the plant organs or within the plant tissues, being classified as internal or external secretory structures, respectively (Svoboda and Svoboda, 2002). Internal secretory structures include secretory cells (often idioblasts), secretory cavities and secretory ducts whereas external ones include glandular trichomes, epidermal cells and osmophores. Glandular trichomes are very frequent and characteristic in the Lamiaceae (Werker et al., 1985) and consist of modified epidermal hairs that can be found in plant aerial organs. These trichomes contribute to control transpiration and temperature of the plant organs (Bosabalidis and Tsekos, 1982) and their essential oils are involved in plant defense and pollination (Bosabalidis and Tsekos, 1982; Weker, 1993).

The high diversity in size, form, constitution, and secretory process of the glandular trichomes makes their classification very difficult. In fact, different authors have adopted distinct terminologies (e.g Werker et al., 1985; Doaigey, 1992; Ascensão et al., 1999), making comparisons among the trichomes existing in different *taxa* very difficult. The most used terminology considers two main types of glandular trichomes: peltate and capitate. Peltate trichomes have a short one-celled stalk and a large flattened head, of about 60-90 μm in diameter, with eight secretory cells arranged in a single disk (e.g. Antunes and Sevinate-Pinto, 1991; Serrato-Valenti et al., 1997; Ascensão et al., 1999) or 12-18 cells arranged in two concentric circles, i.e., four central cells and eight or more peripheral cells (e.g. Weker et al., 1985; Bosabalidis, 1990). In these trichomes the secretory products are accumulated in large subcuticular spaces. On the other hand, capitate trichomes consist of rounded to pear-shaped heads formed by one or two cells supported by stalks of variable length (Ascensão et al., 1997). For the Lamiaceae species, Weker et al. (1985) considered three kinds of capitate trichomes (type I, type II and type III) according to their morphology and secretory process. Based on reports that

showed a higher diversity of capitate trichomes than those previously found, Ascensão et al. (1999) suggested the classification of short-stalked capitate trichomes and long-stalked capitate trichomes. The former correspond to capitate type I trichomes described by Werker et al (1985) with a short globoid to ovoid uni- or bicellular glandular head, with a diameter of about 20-25 μm, and a small subcuticular space. The latter resemble capitate type II and type III trichomes, described by Werker et al. (1985), although they may present some differences, mainly in the shape of the head. The long-stalked capitate trichomes exhibit uni- or bicellular stalks, a neck cell and a bulb-shaped to globular unicellular head with a very small subcuticular space. In order to avoid misleading classifications the terminology proposed by Ascensão et al. (1999) was adopted in the present work, i.e, capitate trichomes may be short-stalked with a unicellular head, short-stalked with a bicellular head or long stalked.

Studies on the morphology, ultrastructure and histochemistry of glandular trichomes have been carried out mainly in Asteraceae and Lamiaceae (e.g. Ascensão et al., 1995, 1999; Serrato-Valentini et al., 1997; Ascensão and Pais, 1998; Gersbach, 2002; Heinrich et al., 2002; Antunes et al., 2004; Marin et al., 2006; Mayekiso et al., 2008; Kaya et al., 2009; Perrini et al., 2009; Giulani et al., 2011; Kremer et al., 2012). In *Lavandula* spp., however, very few studies have been performed. Doaigey (1992) investigated the trichomes in *L. stricta* and *L. coronopifolia* and identified eight types of trichomes which included 17 forms of non-glandular and 13 forms of glandular trichomes. Iriti et al. (2006) analysed the morphology of glandular trichomes in *L. angustifolia* and Huang et al. (2008) studied the ultrastructure of the glandular trichomes in *L. pinnata*. In this latter species, the authors reported a different type of peltate trichomes than that reported for other lavender species, since the basal cell was highly elongated.

Concerning Portuguese lavender species, studies were performed only in *L. latifolia* (Mesquita and Santos Dias, 1988) and *L. stoechas* subsp. *pedunculata* (currently *L. pedunculata*) by Santos Dias and Mesquita (1990) and Mesquita et al. (1992). In these species the authors reported the presence of two types of glandular trichomes in the leaves, which they named capitate sessil glands and capitate-stalked glands (corresponding to the typical peltate and capitate short-stalked trichomes referred above). However, our preliminary studies (Zuzarte et al., 2008, 2009) showed a higher

diversity of trichomes than that previously reported, namely for *L. pedunculata*. This, together with the fact that trichomes morphology and structure is often referred as a useful character for systematic purposes, justified a more detailed investigation of the leaf trichomes existing in the five spontaneous lavenders growing in Portugal (*L. latifolia*, *L. luisieri*, *L. multifida*, *L. pedunculata* and *L. viridis*). Therefore, a detailed morphological and structural study using light microscopy (LM) and scanning electron microscopy (SEM) was performed together with histochemical tests to identify the chemical nature of the secretions in glandular trichomes.

2.2 Material and methods

Leaves of five lavender species (*L. latifolia*, *L. luisieri*, *L. multifida*, *L. pedunculata* and *L. viridis*), at various stages of maturation, were selected and processed for LM and SEM to elucidate the morphological and structural characteristics of the non-glandular and glandular trichomes. Moreover, to elucidate which types of trichomes are involved in the production of the secretion and to identify their chemical nature, several histochemical tests were performed.

2.2.1 Light microscopy

Sections (ca. 50-80 μm) of fresh leaves were obtained using razor blades. The sections were used either fresh or fixed for 4 h, at room temperature, with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) supplemented with 1 mM/L calcium chloride (Glauert and Lewis, 1998). After rinsing with the same buffer, the samples were post-fixed in 1 % buffered osmium tetroxide for 2 h, rinsed again and post-fixed in 1 % aqueous uranyl acetate. After rinsing in distilled water, the samples were dehydrated in a graded acetone series (70-100 %) and impregnated and embedded in a low viscosity resin (Spurr, 1969). Thin sections (0.5-1.5 μm) were cut on a LKB Ultratome NOVA ultramicrotome equipped with a glass knife. The sections were mounted on glass slides and stained with 1 % aqueous toluidine blue (Knight and Lewis, 1992) during 45 min. A few sections of fresh leaves were also mounted on glass slides using the histomount mounting solution and observed unstained. Observations

were made with a Nikon Eclipse E400 light microscope equipped with a Nikon Digital Sight DS-U1 photographic camera, using the Act-2U software.

2.2.2 Scanning electron microscopy

Samples of leaves were fixed as referred above. Following dehydration in a graded acetone series, the samples were critical point dried (CPD 020, B alzers) and sputter coated with a 20 nm layer of gold-palladium at 1200 V and 6 mA, during 10 min (JEOL JFC-1100). Observations were carried out on a JEOL JSM-5400 at 15 kV.

2.2.3 Histochemistry

Histochemical tests were performed in 30-50 μm thick sections of fresh leaves, obtained with a Sorvall® tissue sectioner. To identify which types of trichomes are involved in the production of the secretion as well as the main classes of metabolites in the secreted material, the following histochemical tests were performed: total lipids were localized by staining leaf sections with both Sudan black B and Sudan red B (Pearse, 1968); neutral and acidic lipids were detected using Nile blue A (Cain, 1947); terpenes were identified using NADI reagent (David and Carde, 1964); polysaccharides were identified through the periodic acid-Schiff (PAS) reaction (Jensen, 1962). Controls were performed using the same treatments after lipid extraction with methanol:chlorophormium (1V:1V) at 45 ºC for 24 h . For PAS reaction controls consisted of sections treated in the same way for polysaccharides identification but without periodic acid oxidation. After each test, the leaf sections were mounted on glass slides using glycerine as a mounting medium. Observations were made in a Nikon Eclipse E400 light microscope equipped with a Nikon Digital Sight DS-U1 photographic camera, using the Act-2U software.

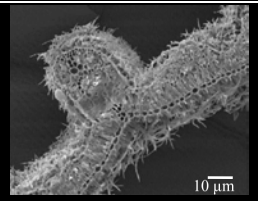
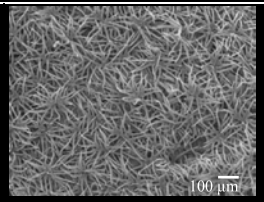
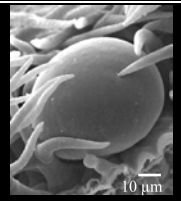
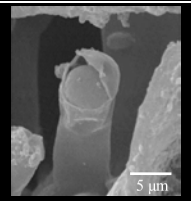
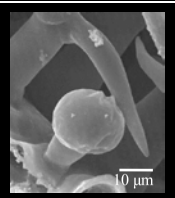
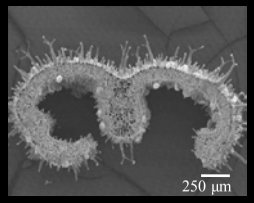
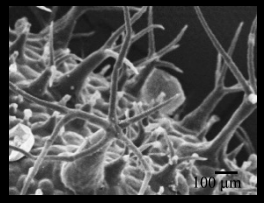
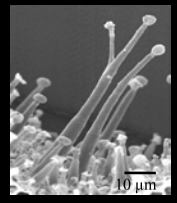
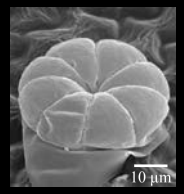
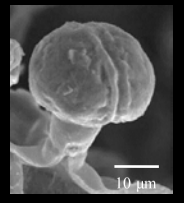
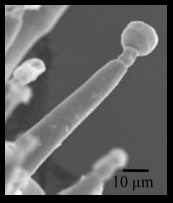
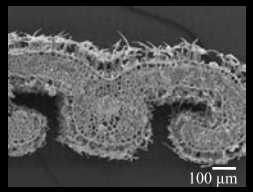
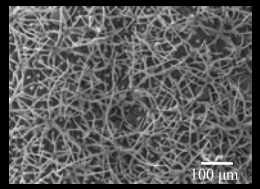
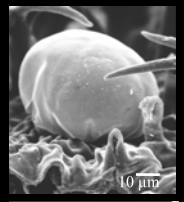
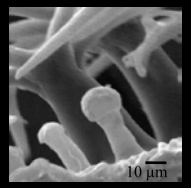
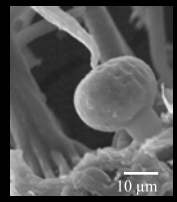
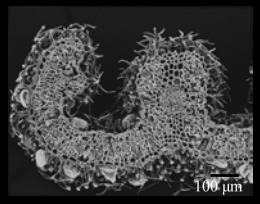
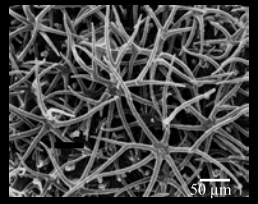
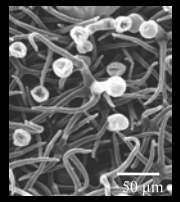
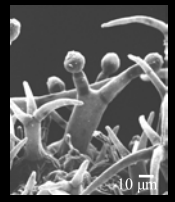
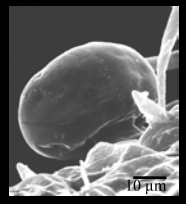
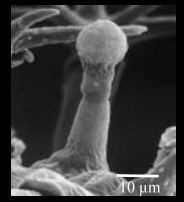
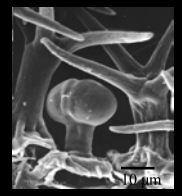
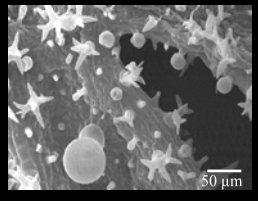
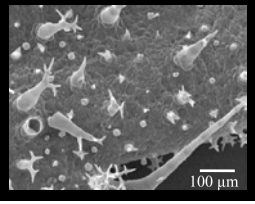
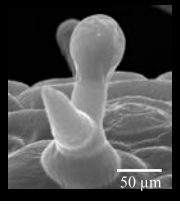
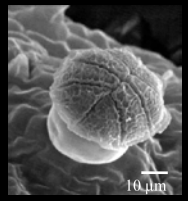
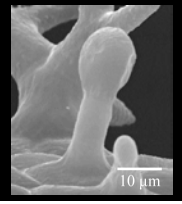
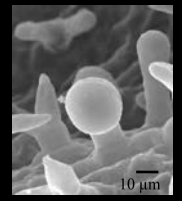
2. 3 Results and discussion

2.3.1 Trichomes diversity

The present study revealed a higher diversity of trichomes in *L. latifolia* and *L. pedunculata* than that previously reported (Mesquita and Dias, 1988; Mesquita et al.,

1992). Besides, the trichomes existing in *L. luisieri*, *L. multifida* and *L. viridis* were characterized for the first time. In all the species examined, LM and SEM observations revealed a very heterogeneous leaf indumentum which included non-glandular and glandular trichomes of different types: peltate, capitate (short-stalked with a unicellular head, short-stalked with a bicellular head and long-stalked) and bifurcated glandular trichomes. Also, a mixed type of trichomes with characteristics of both non-glandular and glandular trichomes was found in some species, which were named ramified mixed type. Table 1 summarizes the different types of trichomes existing in the leaves of all the lavender species analysed.

Table 1. Non-glandular and glandular trichomes on the leaves of lavenders growing wild in Portugal.

Species	General view	Non-glandular trichomes	Ramified mixed type trichomes	Bifurcated trichomes	Peltate trichomes	Short-stalked capitate trichomes		Long-stalked capitate trichomes
						Unicellular head	Bicellular head	
L.latifolia			—	—				—
L. viridis			—			—		
L. luisieri			—	—				—
L. pedunculata								—
L. multifida								—

The morphological and structural characterization of each type of trichome is described below.

Non-glandular trichomes:

Non-glandular trichomes were present in all the species investigated although they varied greatly in size and density (Table 1; Fig. 1). In *L. latifolia* these trichomes were very abundant (Fig. 1a) whereas in *L. viridis* they were rare, occurring occasionally in the curved borders of some of the revolute leaves (Fig. 1b). Non-glandular trichomes in *L. latifolia*, *L. luisieri*, *L. pedunculata* and *L. viridis* were unisseriate stellate hairs, normally with 3-6 projections emerging from the same point (Fig. 1b-1d). Only in *L. multifida* the non-glandular trichomes showed a d ifferent arrangement with the stalk being formed by one or two cells and being always bifurcated at the top into two arms reasonably ramified (Fig 1e and 1f).

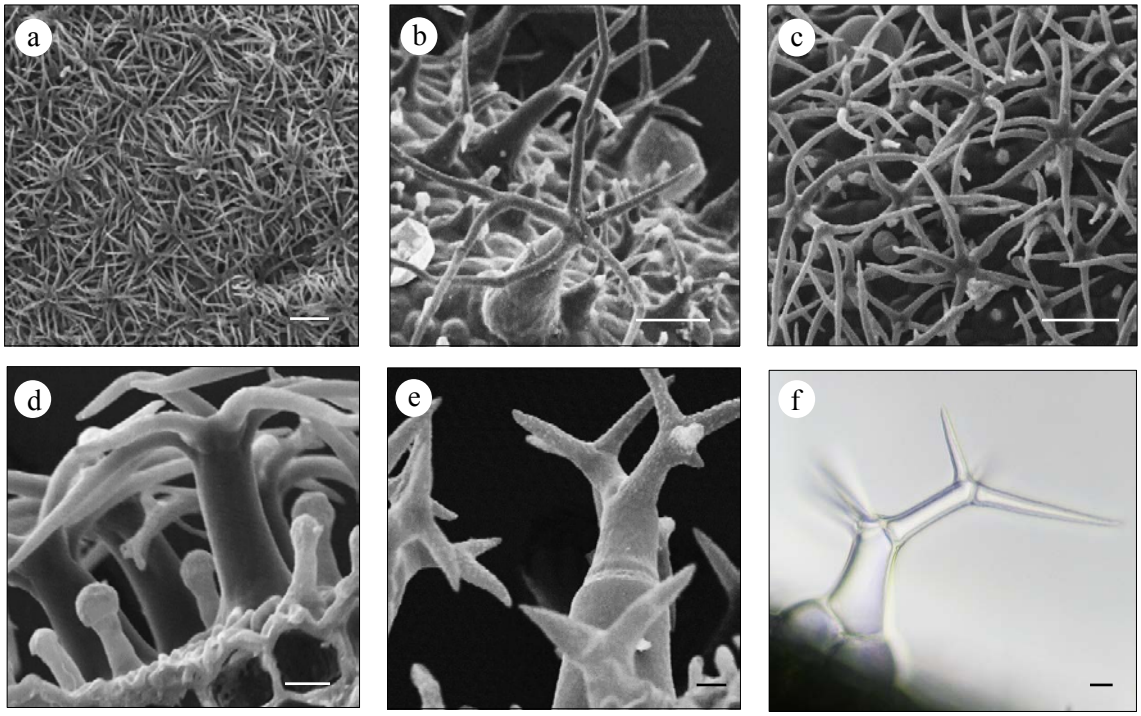


Figure 1. SEM (a - e) and LM (f) micrographs of non-glandular trichomes in leaves of *Lavandula* spp. a) Leaf adaxial surface of *L. latifolia*, bar = 100 µm. b) Non-glandular trichomes in *L.viridis*, bar = 50 µm. c) Leaf adaxial surface of *L. luisieri*, bar = 50 µm. d) Detail of a stellate-shaped trichome in *L. luisieri*, bar = 10 µm. e) Trichome of *L. multifida*, showing the bicellular stalk and the ramified arms at the top, bar = 10 µm. f) Detail of a trichome in *L. multifida*, bar = 10 µm.

Glandular trichomes:

a) Peltate trichomes:

Peltate trichomes were found on both leaf surfaces of all the species examined (Table 1; Fig. 2). These trichomes consisted of one basal cell, a very short and wide unicellular stalk with highly cutinized lateral walls, and a round multicellular head (Fig. 2a and 2b). At the final stage of maturation, the head comprised eight secretory cells arranged in a circle (Fig. 2c and 2d) and, as a result of the separation of the cuticle, a large space developed above the secretory cells, allowing the accumulation of the secretion (Fig 2e and 2f). It is known that the secretion remains trapped in this space and that under natural conditions, factors such as high temperature and low air humidity (Ascensão et al., 1995) or contact with predators (Werker, 1993) can cause the cuticle to burst, releasing the oil. During SEM observations cuticle rupture was often observed. This rupture usually occurred following a horizontal line of apparent fragility located in the diametrical region of the head (Fig. 2c and 2h). Except for *L. multifida*, the peltate trichomes identified in all the lavenders studied are very similar in morphology and structure to those found in other *Lamiaceae*, as for example *Leonotis leonurus* (Ascensão et al., 1995), *Plectranthus ornatus* (Ascensão et al., 1999) and *Salvia aurea* (Serrato-Valentini et al., 1997), although in a few species of this family, peltate trichomes with a higher number of secretory cells arranged in two concentric circles can occur (e.g. Bosabalidis, 1990; Weker et al., 1985).

Particularly in *L. multifida*, peltate trichomes showed a very long stalk and a distinct arrangement of the eight secretory cells in the head (Fig. 2g-2i). Similar trichomes were found in *Salvia officinalis* (Corsi and Botega, 1999), *Prasimun majus*, *Scutellaria galericulata*, *Stachys germanica* (Giuliani and Bini, 2008) and *Sideritis itallica* (Giuliani et al., 2011). In these species however, the authors considered that it was the elongation of the basal cell that caused the elevation of the trichome. In *L. multifida*, the elongation of the basal cell was not clear and the presence of a long stalk was seen (Fig. 2i).

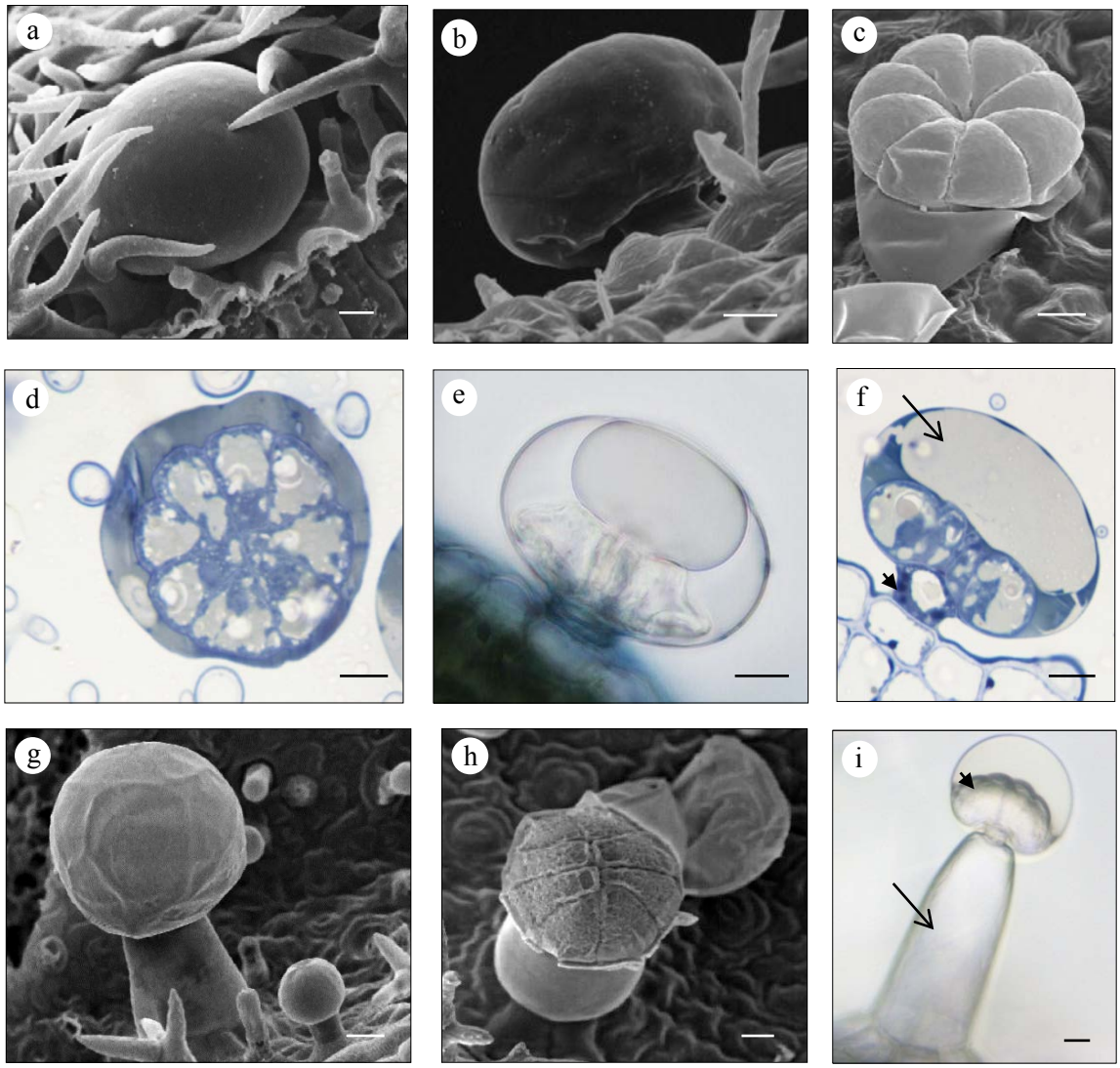


Figure 2. SEM (a-c, g and h) and LM (d-f and i) micrographs of peltate trichomes in *Lavandula* spp. a) Trichome in *L. latifolia* surrounded by non-glandular trichomes. b) Trichome in *L. pedunculata* showing a short wide stalk. c) Detail of the secretory cells arrangement in *L. viridis.* d) Paradermal section showing the eight secretory cells in the final stage of maturation. e) Trichome in a fresh leaf section of *L. viridis* showing the accumulation of secretion in the subcuticular space. f) Cross-section of a trichome showing the highly cutinized lateral walls (arrowhead) of the stalk, the secretory cells and a large drop of secretory material (arrow). g) Trichome detail in *L. multifida*. h) Secretory cell arrangement in the trichomes of *L. multifida*. i) Detail of a trichome in *L. multifida* showing the long stalk (arrow) subtending the secretory head (arrowhead). Bars = 10 µm.

b) Capitate trichomes:

In general, capitate trichomes were found on both the adaxial and abaxial surfaces of the leaves of all the species examined (Table 1; Fig. 3). They were more abundant than peltate trichomes (Fig. 3a) and, as mentioned above, were of two main types: short-stalked and long-stalked. Short-stalked capitate trichomes, corresponding to capitate type I trichomes described by Werker et al. (1985), consisted of one basal cell, a short unicellular stalk and a bicellular (Fig 3b and 3c) or a unicellular head (Fig. 3d). Long-stalked capitate trichomes consisted of a basal cell, a unicellular or bicellular stalk, a neck cell and a unicellular head (Fig. 3e – 3h).

Capitate trichomes are widespread in the *Lamiaceae*, but they vary greatly in structure, size and function. Short-stalked capitate trichomes seem to be the only type of capitate trichomes common to all the *Lamiaceae* species examined so far, although in some studies they have been named capitate type I (Werker et al., 1985) or type B short capitate trichomes (Giuliani et al., 2011).

In the present study, long-stalked capitate trichomes were identified only in *L. viridis*, in which they were particularly very abundant and displayed some variation in stalk length (Fig. 3f *vs* Fig. 3g). Also, Giuliani et al. (2011) identified similar capitate trichomes in *Sideritis italica* but in other species of Lamiaceae different types of capitate trichomes were found. For example, Corsi and Botega (1999) identified four different types of capitate trichomes (type I, II, III and IV) on the leaves of *Salvia officinalis*. Each type had a different structure, namely in what concerns the number of stalk cells and the head shape, secreting different combinations, or different proportions, of lipophilic and hydrophilic material.

As in peltate trichomes, the secretion in capitate trichomes is also accumulated in the subcuticular space (Fig 3c and 3d), being secreted when the cuticle ruptures (Fig. 3d) or through pores, which were sometimes visible on SEM images (data not shown).

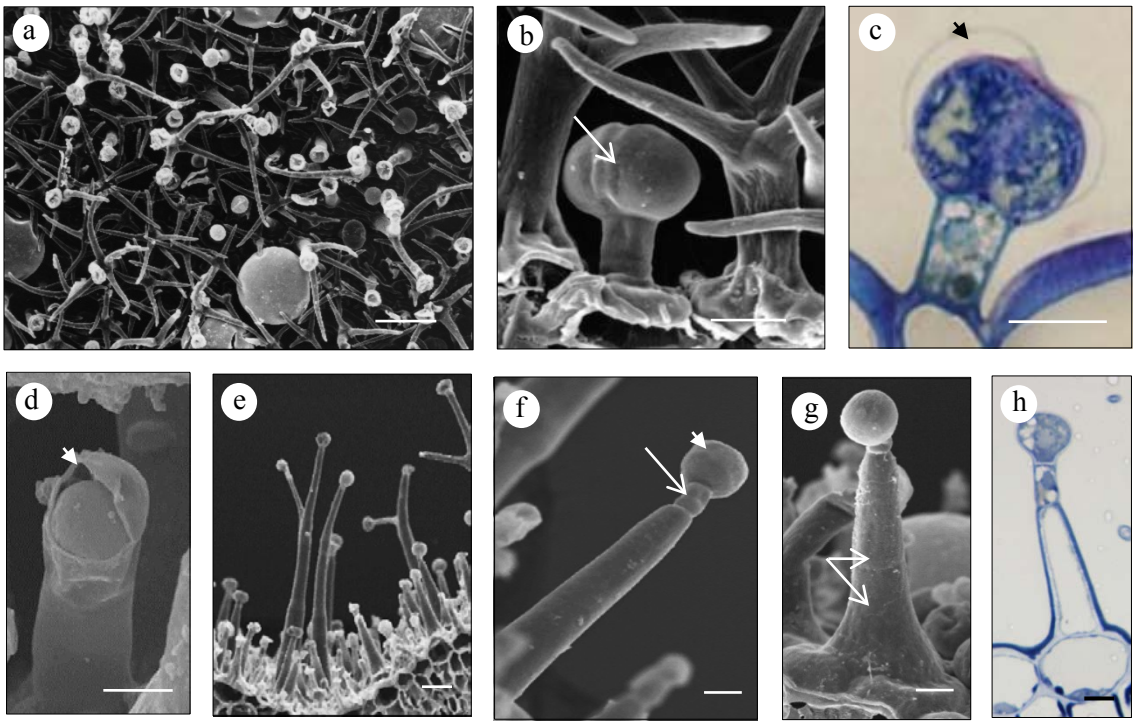


Figure 3. SEM (a-b, d-g) and LM (c and h) micrographs of capitate trichomes in *Lavandula* spp. a-d) Short-stalked capitate trichomes. e-h) Long-stalked capitate trichomes. a) General aspect of the leaf adaxial surface of *L. pedunculata*, showing abundant capitate trichomes in comparison to peltate trichomes, bar = 10 µm. b) Trichome detail in *L. peduculata* showing the bicellular head (arrow), bar = 10 µm. c) Cross section of a trichome with a visible small subcuticular space (arrowhead), bar = 10 µm. d) Trichome detail in *L. peduculata* showing the unicellular head and cuticle disruption (arrowhead), bar = 10 µm. e) Leaf adaxial surface of *L. viridis* showing a high density of long-stalked capitate trichomes, bar = 100 µm. f) Trichome detail in *L. viridis* showing the long one celled stalk, a neck cell (arrow) and the unicellular secretory head (arrowhead), bar = 10 µm. g) Trichome detail in *L. viridis* showing a bicellualr stalk (arrows), bar = 10 µm. h) Cross section of a trichome in *L. viridis* (toluidine blue), bar = 10 µm.

c) Bifurcated trichomes:

In *L. multifida*, *L. pedunculata* and *L. viridis* an interesting type of trichomes was found which, as far as we know, have not been reported before in other species or have been disregarded. These trichomes, which we have named bifurcated trichomes, consisted of a unicellular stalk subtending two individual glandular structures very similar in appearance to long-stalked capitate trichomes (Fig. 4a-d) even though significant differences were found mainly on the length of the stalk. Particularly in *L. viridis* the stalk of bifurcated trichomes was very long (ca. 200 µm) (Fig. 4a) whereas in *L. multifida* it was much shorter (ca. 10 µm) (Fig. 4d).

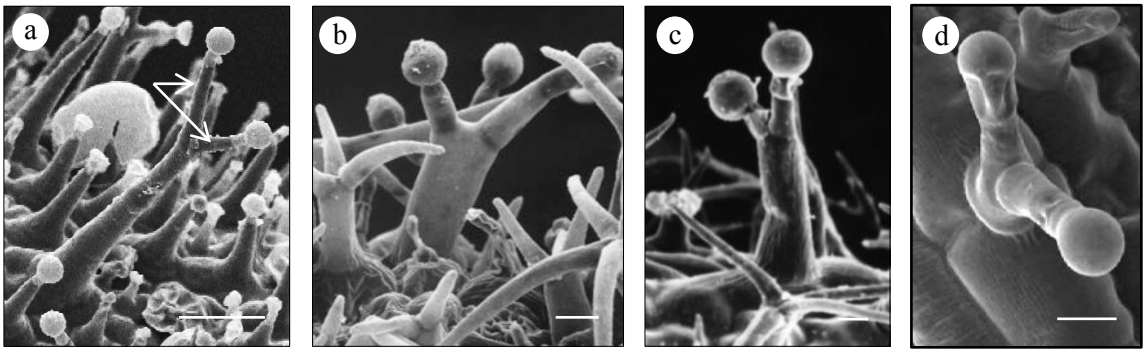


Figure 4. SEM micrographs of bifurcated trichomes in *Lavandula* spp. a) Trichomes in *L. viridis*, showing the very long stalk, and the similarity of the glandular structures with long-stalked capitate trichomes (arrows). b and c) Trichomes detail in *L pedunculata*. d) Trichome detail in *L. multifida*, showing a very short stalk. Bars = 10 µm.

d) Ramified mixed type trichomes:

The ramified mixed trichomes resembled the stellate non-glandular trichomes except for one, or two, of the 2-6 ramifications which were very similar to capitate trichomes (Fig. 5). These trichomes, displaying characteristics of both non-glandular and glandular trichomes, were present only in *L. pedunculata* (Fig. 5a-5b) and *L. multifida* (Fig. 5c-5e). As far as we know mixed trichomes have not been reported before in the *Lamiaceae* or have been disregarded.

Previously, Bonzani et al. (2003) identified mixed trichomes in *Chenopodium carinatum* and *C. purnilio*, but no histochemical tests were performed to elucidate their role in essential oil production.

Although the significance of these trichomes is still unknown, we suggest that they may be related to the evolution of the non-glandular trichomes to glandular ones. Further research is needed to evaluate the importance of these trichomes in taxonomic and/or evolutive studies.

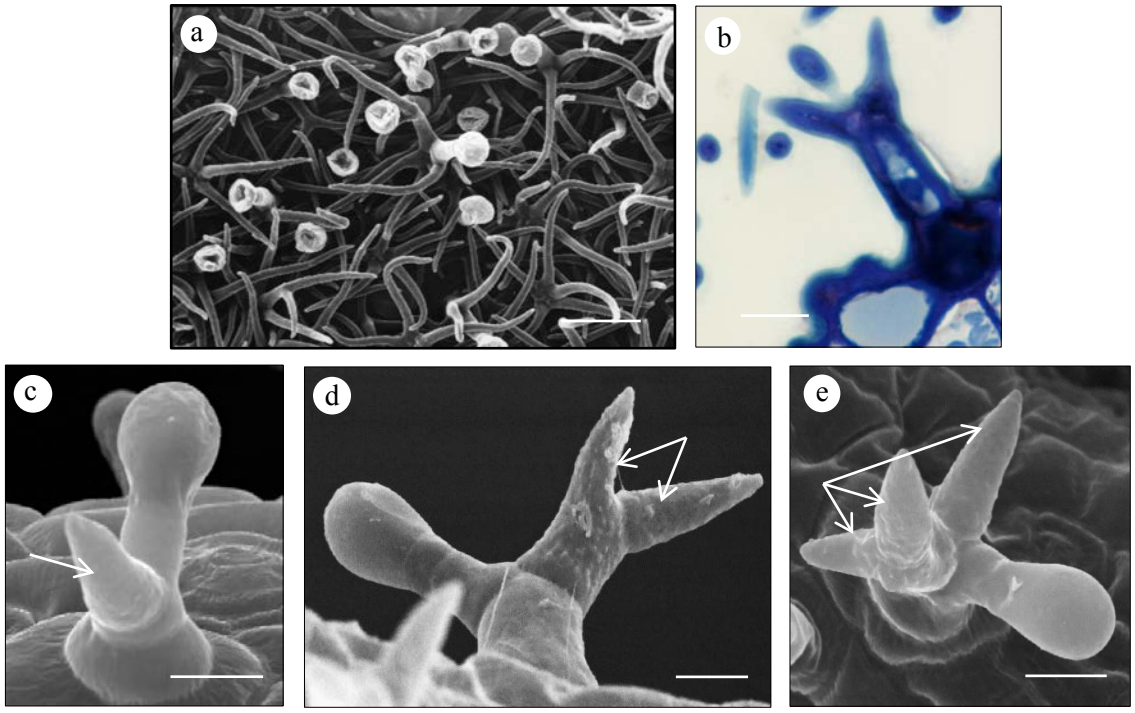


Figure 5. SEM (a, c-e) and LM (b) micrographs of ramified mixed trichomes in *Lavandula* spp. a) Detail in *L. pedunculata*, bar = 50 µm. b) Trichome cross-section in *L. pedunculata* (toluidine blue), bar = 50 µm. c-e) Trichome details in *L. multifida,* showing one (c), two (d) and three (e) ramifications (arrows), bars = 10 µm.

2.3.2 Histochemistry

In the present study, the histochemical analysis of the glandular secretion was performed through several staining methods (Fig. 6). Staining for total lipids provided a positive reaction (black and red staining for Sudan black B and Sudan red B, respectively) in the peltate trichomes (Fig. 6a and 6e), short-stalked capitate trichomes with a unicellular head (Fig. 6b-6d and 6f), long-stalked capitate trichomes, bifurcated trichomes and ramified mixed trichomes (Fig. 6g). Also, staining for neutral lipids produced a positive reaction (pink or blue staining) in the peltate (Fig. 6h and 6i), short-stalked capitate trichomes with a unicellular head (Fig. 6j and 6k), long-stalked capitate, bifurcated and ramified mixed trichomes. For terpenoid localization, the NADI reagent gave a violet staining of the secretion also in the peltate (Fig.6l-6n), short-stalked capitate with a unicellular head (Fig. 6o), long-stalked capitate (Fig. 6p and 6q), bifurcated (Fig. 6r) and ramified mixed trichomes.

Short-stalked capitate trichomes with a bicellular head were the only glandular trichomes that reacted negatively to all the staining tests for lipids and essential oils detection. Thus, the glandular secretion in lavenders growing wild in Portugal is rich in lipidic and terpenic compounds and, with the only exception of the short-stalked capitate trichomes with a bicellular head, all types of trichomes are involved in the essential oil production. Short-stalked capitate trichomes with a bicellular head are not involved in the production of essential oils, being presumably implicated in other roles. In the present study, these trichomes were positively stained following the PAS reaction which reveals the presence of polysaccharides in the secretion (Fig. 6s). According to a number of authors (e.g. Schnepf, 1972; Heinrich, 1977), these trichomes are considered trichome-hydathodes.

The controls were also negative for all the tests performed (data not shown).

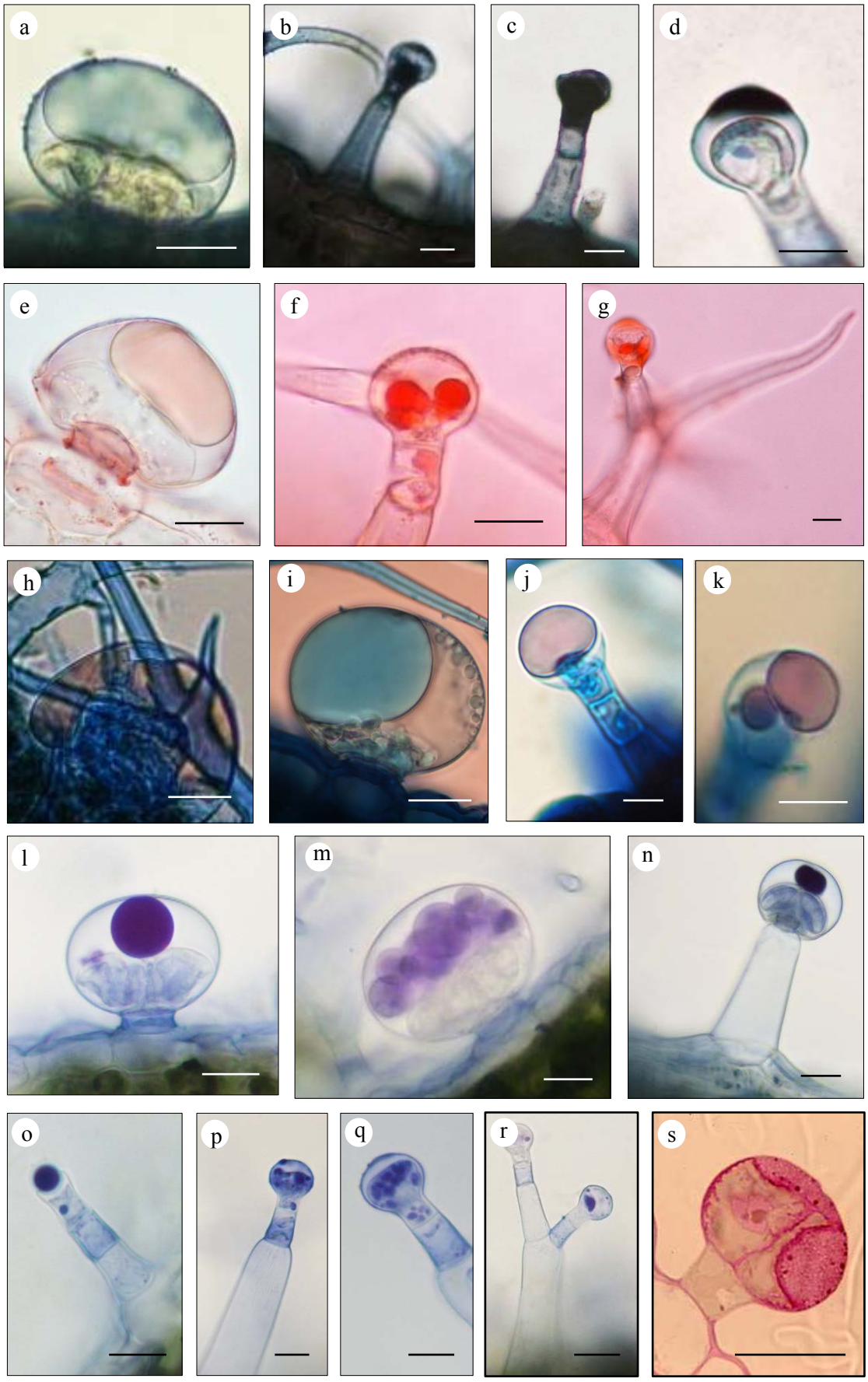

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Figure 6. Histochemistry of leaf trichomes of *Lavandula* spp. a-d) Sudan black B staining positively the secretion in the secretory head of peltate trichomes (a) and short-stalked capitate trichomes with a unicellular head (b-d). e-g) Sudan red B staining red the secretion in peltate trichomes (e), short-stalked capitate trichomes with a u nicellular head (f) and ramified mixed type trichomes (g). h-k) Nile blue A staining pink neutral lipids in peltate trichomes (h), and short-stalked capitate trichomes with a unicellular head (j and k) and showing a blue stain for acidic lipids in peltate trichomes (i); l-r) Nadi reagent identifying terpenes in typical peltate trichomes (l and m), and characteristic peltate trichomes found in *L. multifida* (n), short-stalked capitate trichomes with a unicellular head (o), long-stalked capitate trichomes of *L. viridis* (p and q) and bifurcated trichomes (r). s) PAS reaction revealing a positive stain in short-stalked capitate trichomes with a bicellular head. Bars = 20 μm.

Trichomes diversity may be important for taxonomical purposes and for quality control in the market of aromatic and medicinal plants as they allow, in some cases, to verify the authenticity of plant material and detect adulterations (Svoboda and Svoboda, 2000). The differences pointed out in the present study concerning the trichomes of lavenders growing wild in Portugal (Table 1) enables species separation based on trichomes analysis. Taking into account the present results, *L. viridis* was the most distinct species since it was the only one with a very high density of glandular trichomes on the leaves, being the non-glandular trichomes very scarce; also, it was the only lavender species bearing long-stalked capitate trichomes (up to 100 μm long). On the other hand, *L. multifida* showed a very low density of both glandular and non-glandular trichomes. In this species a distinct peltate trichome was found, which presents a visible long stalk. *L. pedunculata* can be separated from *L. luisieri* and *L. latifolia* due to the presence of both ramified mixed trichomes and bifurcated trichomes. Most similarities were found between *L. latifolia* and *L. luisieri*, since both have the same type of glandular trichomes (peltate, short-stalked capitate with a unicellular head and short-stalked capitate with a bicellular head). However, *L. latifolia* showed a higher density of non-glandular trichomes than *L. luisieri* and these were shorter and more uniform in size (see Table 1). A dichotomous key based on trichomes characterization is proposed below.

Dichotomous key based on trichome morphology to lavender species in Portugal

1. Low density of non-glandular trichomes on the leaves ……2
   High density of non-glandular trichomes on the leaves …….3

2. Low density of glandular trichomes; peltate trichomes with a long stalk; long-stalked capitate trichomes absent …….....*L. multifida*
   High density of glandular trichomes; long-stalked capitate trichomes present …..... *L. viridis*

3. Ramified mixed trichomes and bifurcated glandular trichomes absent…….....4
   Ramified mixed trichomes and bifurcated glandular trichomes present……....*L. pedunulata*

4. Short and regularly ramified non-glandular trichomes, covering totally the leaf surface……...*L. latifolia*
   Long and irregularly ramified non-glandular trichomes, not covering totally the leaf surface …...…….....*L. luisieri*

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3. Essential Oils

3. Essential Oils

3.1 Introduction

Essential oils, also known as essences, volatile oils, etheric oils or aetheroleum are natural products formed by several volatile compounds, mainly terpenic ones (Sangwan et al., 2001; Baser and Demirci, 2007). According to the International Standard Organization on Essential Oils (ISO TC – ISO 9235, 1997) and the European Pharmacopoeia (Council of Europe, 2007) an essential oil is defined as the product obtained from plant raw material by hydrodistillation, steam distillation or dry distillation or by a suitable mechanical process without heating (for *Citrus* fruits). This definition excludes other aromatic/volatile products obtained by different extractive techniques like extraction with solvents, supercritical fluid extraction and headspace.

In nature essential oils play very important roles in plants defense and signaling processes (Harborne, 1993; Bowsher et al., 2008; Taiz and Zeiger, 2010). For example, essential oils are involved in plant defense against microorganisms, insects and herbivores, attraction of pollinating insects and fruit-dispersing animals, water regulation and allelopathic interactions (Fahn, 1979; Harborne, 1993; Pichersky and Gershenzon, 2002; Bakkali et al., 2008). Also, they are valuable natural products used as raw materials in many fields, like pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries (Buchbauer, 2000). According to Lawrence (1993), table 1 s ummarizes the main essential oils produced worldwide, being the most commercialized identified as Top 20.

Essential oils can be found in various plant organs (flowers, fruits, seeds, leaves, stems, and roots) being produced and stored in secretory structures (previous section).

Table 1. Main essential oils produced worldwide (adapted from Lawrence, 1993).

Plant Family	Essential Oils	Species	Top 20
Apiaceae	Anise	*Pimpinella anisum* L.	
	Ajowan	*Trachyspermum copticum* (L.) Link	
	Bitter fennel	*Foeniculum vulgare* Mill. var. *vulgare*	
	Caraway	*Carum carvi* L.	
	Celery seed	*Apium graveolens* L.	
	Coriander	*Coriandrum sativum* L.	18
	Cumin	*Cuminum cyminum* L.	
	Dill weed	*Anethum graveolens* L.	
	Indian dill seed	*Anethum sowa* Roxb. ex Flem.	
	Sweet fennel	*Foeniculum vulgare* Mill. var. *dulce*	
Asteraceae	Armoise	*Artemisia herba-alba* Asso	
	Blue chamomile	*Chamomilla recutita* (L.) Rauschert	
	Davana	*Artemisia pallens* Wall. ex DC	
	Muhuhu	*Brachylaena hutchinsii* Hutch.	
	Roman chamomile	*Anthemis nobilis* L.	
	Sea wormwood	*Artemisia maritima* L.	
	Tarragon	*Artemisia dracunculus* L.	
	Tagetes	*Tagetes minuta* L.	
	Wild chamomile	*Ormenis mixta* Dumort. and *O. multicaulis* Braun-Blanq & Maire	
	Wormwood	*Artemisia absinthum* L.	
Cupressaceae	Cedarwood (Chinese)	*Chamaecyparis funebris* (Endl.) Franco	14
	Cedarwood (USA)	*Juniperus virginiana* L. and *J. ashei* Buchholz	9
Gramineae	Citronella	*Cymbopogon winterianus* Jowitt and *C. nardus* (L.) Rendle	4
Lamiaceae	Basil	*Ocimum basilicum* L.	
	Clary sage	*Salvia sclarea* L.	
	Cornmint	*Mentha arvensis* L. f. *piperascens* Malinv. ex Holmes	2
	Lavandin	*Lavandula* x *intermedia* Emeric ex Loisel	15
	Lavender	*Lavandula angustifolia* Mill.	
	Marjoram	*Origanum majorana* L.	
	Native spearmint	*Mentha spicata* L.	13
	Ocimum	*Ocimum gratissimum* L. *gratissimum*	
	Patchouli	*Pogostemon cablin* (Blanco) Benth.	20
	Peppermint	*Mentha* x *piperita* L.	5
	Rosemary	*Rosmarinus officinalis* L.	
	Sage	*Salvia officinalis* L.	
	Scotch spearmint	*Mentha gracilis* Sole	
	Spike lavender	*Lavandula latifolia* Medik.	
	Thyme	*Thymus zygis* L. and *T. vulgaris* L.	
Lauraceae	Camphor	*Cinnamomum camphora* (L.) J. Presl.	17
	Litsea cubeba	*Litsea cubeba* (Lour.) Pers.	10
	Sassafras (Brazil)	*Ocotea pretiosa* (Nees) Benth.	11
	Sassafras (Chinese)	*Cinnamomum micranthum* (Hayata) Hayata	16
Myrtaceae	Eucalyptus cineole-type	*Eucalyptus globulus* Labill., *E. polybractea* R.T. Baker and other *Eucalyptus* species	3
	Eucalyptus citronellal-type	*Eucalyptus citriodora* Hook.	7
	Clove leaf	*Syzygium aromaticum* (L.) Merr. and L.M. Perry	8
Rutaceae	Grapefruit	*Citrus paradisi* Macfady	19
	Lemon	*Citrus limon* (L.) N.L. Burm.	6
	Lime distilled	*Citrus aurantifolia* (Christm. & Panz.) Swingle	12
	Orange	*Citrus sinensis* (L.) Osbeck	1

Terpenes are one of the main groups of compounds found in essential oils. However, some oils may contain high amounts of other compounds namely, phenylpropanoids, fatty acids and their esters and, more rarely, nitrogen and sulphur derivatives (Baser and Demirci, 2007; Bakkali et al., 2008).

Terpenes result from the condensation of a pentacarbonate unit (isoprene) and therefore are many times called isoprenoides. These are classified according to the number of isoprene units in their structure. Monoterpenes (2 units of isoprene) and sesquiterpenes (3 units of isoprene) are the main compounds found in essential oils although diterpenes (4 units of isoprene) may also occur (Bakkali et al., 2008). In higher plants terpenes are biosynthesized through the mevalonate dependent (MVA) and mevalonate independent or 2C-metil-D-erythritol 4-phosphate/1-deoxi-D-xilulose-5-phosphate (MEP/DOXP) pathways, schematized in figure 1. The former takes place in the cytosol while the later occurs in the chloroplasts (Bouwmeester, 2006).

Phenylpropanoids are synthetized via the shikimic acid pathway, being their main precursors the cinnamic acid and *p*-hydroxycinnamic acid, originated from the aromatic amino acids phenylalanine and tyrosine, respectively (Sangwan et al., 2001; Dewick, 2002).

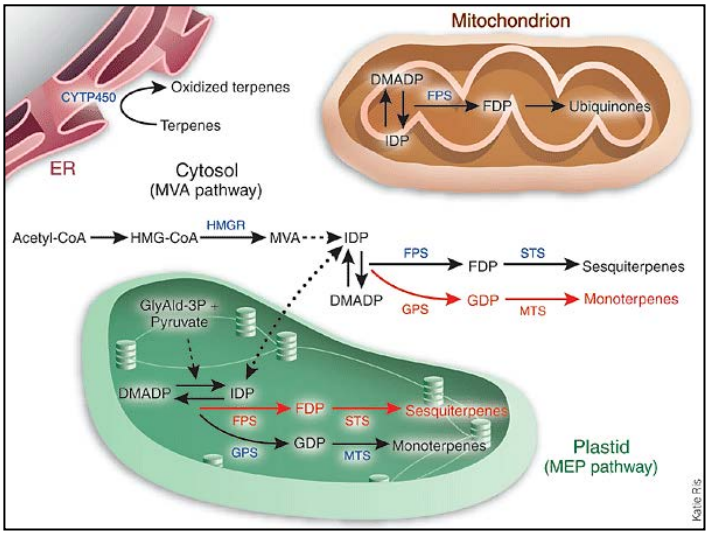

Figure 1.Terpenoid biosynthesis in plants. Solid and dashed arrows represent single and multiple enzymatic steps, respectively. DMADP, dimethylallyl diphosphate; FDP, farnesyl diphosphate; GDP, geranyl diphosphate; GlyAld-3P, glyceraldehyde-3-phosphate; HMG-CoA, hydroxymethylglutaryl-CoA; IDP, isopentenyl diphosphate; MVA, mevalonic acid; MEP, methyl erythritol phosphate; CYTP450, cytochrome P450 hydroxylase; FPS, FDP synthase; GPS, GDP synthase; HMGR, HMG-CoA reductase; MTS, monoterpene synthase; STS, sesquiterpene synthase. In Bouwmeester (2006).

Essential oils are characterized as volatile liquids, usually with a strong odor, rarely colored, soluble in organic solvents and insoluble in water. Most of the methods applied in the analysis of essential oils rely on chromatographic procedures, which enable component separation and identification. However, additional confirmatory evidence is required for reliable identification, avoiding equivocated characterizations. The most used methods for essential oil analysis are gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) (Lahou, 2004; Rubiolo et al., 2010). Identification of the compounds is made by the comparison of both the GC retention indices and mass spectra data with those of authentic samples included in home-made libraries and with literature data (e.g. Adams, 1995, 2007; Joulain and König, 1998). It is advised to confirm retention indices on two columns of different polarity in order to avoid misleading identifications. Moreover, for complex mixtures, several fractionating techniques should be considered and to identify compounds that are not easily separated by GC or essential oils with similar stereo-isomeric compounds, other methodologies should be used as for example, Nuclear Magnetic Resonance Spectroscopy (^{13}C-NMR) (Tomi et al., 1995).

In aromatic plants the composition of the essential oils usually varies considerably due to both intrinsic (sexual, seasonal, ontogenetic and genetic variations) and extrinsic (ecological and environmental aspects) factors (Figueiredo et al., 2008; Tiaz & Zeiger, 2010). Genetic variations may result in the expression of different metabolic pathways and consequently, quantitative and qualitative variations in essential oil composition may occur. When significant differences are found, a intraspecific category (chemotype) is identified (Salgueiro, 1994; Figueiredo et al., 2008). In general, the industries choose the chemotypes that have most commercial interest, in order to obtain high quality end products as well as efficient biological activities. Therefore, essential oil quality strongly depends upon all these factors that may interfere and also limit plant yield. Analytical guidelines published by several institutions such as European Pharmacopoeia, ISO and World Health Organization are available and must be followed to assure the good quality of the commercialized essential oils and of the plants from which they are obtained.

Several plant families, namely Apiaceae, Asteraceae, Cupressaceae, Hypericaceae, Lamiaceae, Lauraceae, Myrtaceae, Pinaceae and Rutaceae comprise

well-known aromatic species, many of them included on the Generally Recognized as Safe (GRAS) list fully approved by the US Food and Drug Administration (FDA) and Environmental Protection Agency (EPA, USA) for addition to food and beverages. In particular, *Lavandula* species, mainly *L. angustifolia*, *L. latifolia*, *L.* x *intermedia* and *L. stoechas*, are explored for their essential oils with high economic interest in fragrance, pharmaceutical, food and flavour industries (Boelens, 1995; Upson and Andrews, 2004). These lavender scents are very popular in pillows, bath care, home and pet products, and provide a unique taste to many beverages, sweets, jellies, jams, marmalades, honey and condiments. Some lavender essential oils are strictly regulated by international ISO standards (e.g. ISO TC 54 - ISO/CD 8902, 2007; ISO TC 54 N - ISO/WD 4719, 2009) and, during the last few years, the economic exploitation of native species has increased due to a renewed interest in the use of these naturally derived compounds. However, Portuguese species are poorly explored, being mainly used in traditional remedies by local communities or for ornamental purposes (Upson and Andrews, 2004).

In the present study a characterization of Portuguese lavenders essential oils was performed, contributing to a better knowledge and valorization of these species.

3.2 Material and methods

3.2.1 Plant material

Several samples of *Lavandula* spp. (*L. luiseri, L. pedunculata, L. multifida and L. viridis*) were collected at different regions of Portugal for essential oil isolation and characterization (see table 1, section1, chapter II). From all the samples analysed, only the chemical composition of representative oils or those with higher variability were selected, avoiding a fastidious results section. The site of collection of the selected samples is summarized in table 2.

For *L. pedunculata* a more detailed study, including 43 individual samples was performed due to the high variability reported for this species, with several authors identifying different subspecies (Franco, 1984; Upson and Andrews, 2004; Morales, 2010) and varieties (Morales, 2010) in Portugal. Also, the essential oils of *L. stoechas*

collected in two Mediterranean regions (Barcelona and Sardinia) were included in this study for chemotaxonomic purposes.

Table 2. Site of collection of *Lavandula* spp.

Species	**Region**	**Site of Collection**	**Sample**
L. luisieri	Coimbra	Fonte Coberta	A
		Piódão	D
	Castelo Branco	Sertã	B
	Algarve	Cabo de São Vicente	L
L. multifida	Setúbal	Sesimbra	AI
		Portinho da Arrábida	B
	Alentejo	Mértola	D
L. pedunculata	Mirandela	Valbom dos Figos	A0
	Bragança	Serra da Nogueira	B3
	Guarda	Celorico da Beira	C6
	Coimbra	Foz de Arouce	D7
	Alentejo	Santiago do Cacém	E
L. viridis	Algarve	Barranco do Velho	A
		Salir	B
		Porto Nobre	C
L. stoechas	Barcelona	Anoia, El Bruc	A
	Sardinia	Cagliari	B

3.2.2 Isolation of the essential oils

Aerial parts of the plants were submitted to a hydrodistillation for 3 h, using a *Clevenger*-type apparatus (Fig. 2) according to the European Pharmacopoeia (Council of Europe, 2007). The oils were preserved in a s ealed vial at 4 ºC until further experiments.

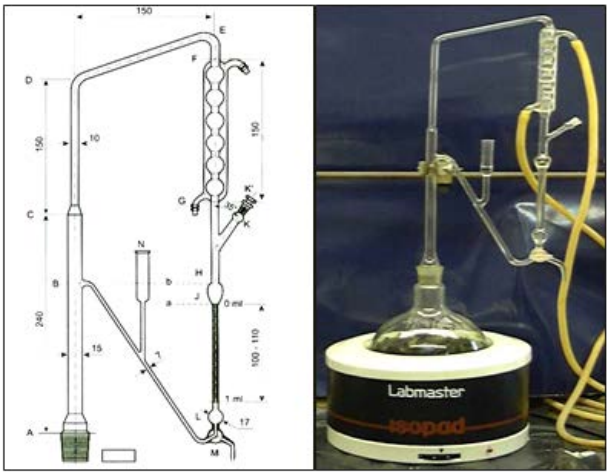

Figure 2. *Clevenger* apparatus for essential oil extraction at a lab scale.

3.2.3 Qualitative and quantitative analyses

Analytical gas chromatography (GC) was carried out on a Hewlett Packard 6890 gas chromatograph (Agilent Technologies, Palo Alto, California, USA) with a HP GC ChemStation Rev. A.05.04 data handling system, equipped with a single injector and two flame ionization detectors (FID). A graphpak divider (Agilent Technologies, Part Number 5021-7148) was used for simultaneous sampling in two Supelco (Supelco Inc., Bellefont, PA, USA) fused silica capillary columns with different stationary phases: SPB-1 (polydimethylsiloxane; 30 m × 0.20 mm i.d., film thickness 0.20 μm), and SupelcoWax 10 (polyethylene glycol; 30 m × 0.20 mm i.d., film thickness 0.20 μm). Oven temperature program: 70 – 220 ºC (3 Cº/min), 220 ºC (15 min); injector temperature: 250 ºC; detector carrier gas: He, adjusted to a linear velocity of 30 cm/s; splitting ratio 1:40; detector temperature: 250 ºC. Gas chromatography-mass spectrometry (GC/MS) analyses were performed on a Hewlett Packard 6890 g as chromatograph fitted with a HP1 fused silica column (polydimethylsiloxane; 30 m × 0.25 mm i.d., film thickness 0.25 μm), interfaced with an Hewlett Packard Mass Selective Detector 5973 (Agilent Technologies, Palo Alto, CA, USA) operated by HP Enhanced ChemStation software, version A.03.00. GC parameters as above; interface temperature: 250 ºC; MS source temperature: 230 ºC; MS quadrupole temperature: 150 ºC; ionization energy: 70 eV; ionization current: 60 μA; scan range: 35 – 350 u; scans/s: 4.51.

3.2.4 Compound identification

The volatile compounds were identified by both their retention indices and their mass spectra. Retention indices (RI), calculated by linear interpolation relative to retention times of a series of n-alkanes, were compared with those of authenticated samples from the database of the Laboratory of Pharmacognosy, Faculty of Pharmacy, University of Coimbra. Mass spectra were compared with reference spectra from a home-made library or from literature data (Adams, 1995, 2007; Joulain and König, 1998). Relative amounts of individual components were calculated based on GC peak areas without FID response factor correction.

3.2.5 Statistical analyses

For the essential oils of *L. pedunculata* the quantitative data of the 43 individual samples were submitted to a multivariate statistical analysis using a SPSS 13.0 statistical package (SPSS Inc., USA). Sixteen constituents over 1.0 % were used as variables for analysis. Cases were classified by a hierarchical clustering using Ward linkage with square Euclidean distance measure. Factor analyses using the Principal Components extraction method was used to achieve the most relevant variables in the total variance. The aptitude of the complete correlation matrix was checked by the Kaiser Meyer-Olkin criterion (KMO = 0.447).

3.3 Results and discussion

3.3.1 *Lavandula luisieri*

Constituents of the essential oils of four representative samples of *L. luisieri* (samples A, B, D, and L) collected in different regions of Portugal (Fonte Coberta, Sertã, Piódão and Cabo de São Vicente) are listed in Table 3. The essential oil yield varied from 0.5 to 1.10 % (v/w) with a total of 76 compounds identified. All samples were characterized by high amounts of oxygenated monoterpenes and several irregular monoterpenes, namely: 3,4,4-trimethyl-2-cyclohexen-1-one; *trans*-*α*-necrodol; 1,1,2,3-tetramethyl-4-hidroximethyl-2-cyclopentene; 2,3,4,4-tetramethyl-5-methylene-

cyclopent-2-enone, *trans*-*α*-necrodyl acetate and *cis*-*α*-necrodyl acetate (Table 3). Significant quantitative differences were found among the samples concerning the amounts of the three main constituents: 1,8-cineole (1.5 - 33.9 %), fenchone (0.0 - 18.2 %) and trans-*α*-necrodyl acetate (3.2 – 17.4 %) (Table 3).

Table 3. Composition of the essential oils of *Lavandula luisieri* from four regions of Portugal.

IR SPB-1	IR SW-10	Compound	Fonte Coberta (A)	Sertã (B)	Piódão (D)	Cabo de São Vicente (L)
930	1029	*α*-pinene	2.1	2.5	3.2	1.1
943	1075	camphene	0.3	0.2	-	0.5
946	1129	verbenene	-	0.2	-	0.2
964	1127	sabinene	0.3	0.2	-	0.4
970	1127	*β*-pinene	0.3	0.3	1.6	0.7
977	1161	myrcene	-	0.2	-	0.3
1006	1151	Δ-3-carene	0.3	0.5	1.8	-
1012	1272	*p*-cymene	0.2	0.1	0.4	0.3
1020	1205	limonene	0.3	0.3	0.7	0.9
1020	**1214**	**1,8-cineole**	**10.5**	**1.5**	**6.4**	**33.9**
1024	1232	*cis*-*β*-ocimene	0.4	0.5	0.5	0.9
1033	1247	*trans*-*β*-ocimene	-	0.2	-	0.2
1047	1247	*γ*-terpinene	-	t	-	0.3
1055	1447	3,4,4-trimethyl-2-cyclohexen-1-one	1.2	0.3	0.8	0.7
1055	1439	*cis*-linalool oxyde	0.9	0.2	0.9	0.5
1066	**1401**	**fenchone**	**-**	**-**	**-**	**18.2**
1070	1467	*trans*-linalool oxyde	0.5	0.2	0.7	0.4
1078	1285	terpinolene	0.1	0.3	-	-
1082	1542	linalool	5.3	3.2	6.2	3.0
1102	1489	*α*-campholenal	0.2	0.2	0.1	-
1103	1575	nopinone	-	t	-	0.1
1105	1556	*cis*-*p*-2-menthen-1-ol	-	t	-	0.1
1118	1513	camphor	1.5	0.5	2.5	2.2
1120	1649	*cis*-verbenol	0.2	0.1	0.2	-
1121	1648	*trans*-pinocarveol	0.2	0.1	0.2	0.3
1130	**1657**	*trans*-*α*-necrodol	8.9	7.5	7.1	4.5
1135	1563	pinocarvone	t	0.2	-	0.4
1142	1720	*p*-mentha-1,5-dien-8-ol	0.4	t	-	-
1144		teresantalol	t	1.8	1.7	1.3
1147	1668	lavandulol	0.4	0.2	0.3	0.5
1154	1712	1,1,2,3-tetramethyl-4-hidroximethyl-2-cyclopentene	2.1	1.0	2.0	1.1

1159	1599	terpinen-4-ol	0.6	0.2	-	1.2
1159	1645	2,3,4,4-tetramethyl-5-methylene-cyclopent-2-enone	3.3	2.5	2.8	0.3
1166	1622	myrtenal	0.2	t	0.3	-
1169	1689	*α*-terpineol	0.2	0.2	0.2	1.2
1176	1781	myrtenol	t	t	-	0.8
1177	1701	verbenone	0.5	0.2	0.4	-
1204	1467	fenchyl acetate	-	-	-	0.6
1212	1731	carvone	-	-	-	0.2
1265	**1590**	***trans*-*α*-necrodyl acetate**	**17.1**	**16.2**	**17.4**	**3.2**
1269	1602	lavandulyl acetate	5.7	5.5	7.6	2.2
1269	1657	lyratyl acetate	3.0	4.1	2.4	0.3
1281	**1611**	*cis*-*α*-necrodyl acetate	1.3	1.2	1.8	0.4
1301	1683	myrtenyl acetate	-	0.1	-	2.0
1340	1719	neryl acetate	0.7	t	0.6	0.1
1342	1459	*α*-cubebene	t	t	0.2	-
1361	1479	cyclo-sativene	t	0.2	1.4	-
1370	1489	*α*-copaene	0.3	0.4	0.3	-
1375	1521	*β*-bourbunene	0.1	0.1	0.1	-
1382	1533	*β* -cubebene	t	0.2	0.4	-
1403	1525	*α* -gurjunene	t	0.7	1.4	-
1409	1590	*E*-*β*-caryophyllene	0.4	0.8	0.9	0.2
1442	1664	*α*-humulene	0.2	0.2	-	-
1451	1638	*allo*-aromadendrene	0.3	0.4	0.7	-
1462	1682	*γ*-muurolene	0.2	0.2	-	-
1468	1696	germacrene D	0.1	0.2	-	-
1473	1719	*β*-salinene	0.3	0.5	0.6	0.2
1478		bicyclosesquiphellandrene	t	0.3	0.2	-
1484	1715	*α*-selinene	0.2	t	1.0	-
1488	1721	*α*-muurolene	0.5	0.8	0.2	-
1500	1749	*γ*-cadinene	t	0.3	0.7	-
1503	1827	*cis*-calamelene	t	0.2	0.5	-
1508	1749	*δ*-cadinene	0.9	3.2	1.3	0.8
1521	1904	*α*-calacorene	0.3	0.8	-	-
1529	1773	selina-3,7(11)-diene	t	1.8	1.4	0.3
1541	2073	*cis*-*α*-copaene-8-ol	0.8	2.3	1.0	0.7
1548	1913	palustrol	0.2	0.2	-	-
1559	1967	caryophyllene-oxyde	0.5	0.9	0.8	0.3
1571	2068	viridiflorol	3.3	6.2	2.1	1.4
1583	2013	ledol	0.6	2.0	1.3	0.3
1583	2022	humulene oxide	0.2	0.1	-	0.1
1618	2156	T-cadinol	0.1	0.1	0.9	-
1618	2172	T-muurolol	0.2	0.8	-	0.2
1621	2185	*α*-muurolol	-	-	-	0.2
1631	2216	*α* -cadinol	0.1	0.2	0.7	-

Monoterpene hydrocarbons	4.3	5.6	8.2	5.8
Oxygen containing monoterpenes	31.2	18.8	30.7	69.5
Sesquiterpene hydrocarbons	4.3	11.6	11.3	1.5
Oxygen containing sesquiterpenes	6.0	12.8	6.8	3.2
Others	33.9	28.7	31.9	3.2
Total identified	**79.7**	**77.5**	**88.9**	**90.2**

Compounds listed in order of elution from the *SPB-1* column.
RI SPB-1: GC-retention indices relative to C9 – C23 *n*-alkanes on the *SPB-1* column.
RI SW 10: GC-retention indices relative to C9 – C23 *n*-alkanes on the *SupelcoWax-10* column.
t= traces (≤ 0.05 %).

L. luisieri essential oils are very distinct from other *Lavandula* species due to the presence of irregular monoterpenes, namely necrodane derivatives. Although significant quantitative differences may occur in the amounts of these compounds, they are always present in this species. As far as it is known, these compounds are unique in the plant kingdom and therefore can be considered a chemotaxonomic marker of this species. Garcia-Vallejo (1992) was the first to identify these compounds. Later on, different authors found other uncommon monoterpenes, hence confirming the peculiarity of *L. luisieri* essential oils (Lavoine-Hanneguelle et al., 2004; Sanz et al., 2004; Baldovini et al., 2005; González-Coloma et al. 2011). In the present study a significant quantitative variability of these compounds depending on plant origin was detected. Moreover, the essential oils from plants harvested in Cabo de São Vicente region were the most distinct. The oil was the only one with high amounts of fenchone (18.2 %) and 1,8-cineol (33.9 %). The presence of high amounts of these compounds is characteristic of *L. pedunculata* (see table 5) and *L. stoechas* essential oils (see table 7). Also necrodane derivatives were present in very low quantities in comparison to the other samples analysed. At this region the plants showed a typical dwarf shape characteristic of coastal regions and it is possible that essential oil composition presents some variation due to edaphic and climatic influences. Eventually a hybrid between *L. luisieri* and *L. pedunculata* could be suggested but morphological characteristics were not conclusive for this matter. Also, it is known that the flowering period of these two species in slightly lagged. Therefore, it seems more reasonable that an ecotype in Cabo de São Vicente region may occur.

3.3.2 *Lavandula multifida*

The essential oil composition of three representative oils of *L. multifida* (samples AI, B and D) collected in different regions of Portugal (Sesimbra, Portinho da Arrábida and Mértola) is shown in Table 4. *L. multifida* showed a very low essential oil yield, up to 0.13 % (v/w). A total of 37 compounds were identified, representing more than 90 % of the total volatile oils. The oils from all samples were characterized by high contents of oxygen-containing monoterpenes (41.8 - 61.6 %), being the main constituents of the oils carvacrol (41.5 - 59.0 %) and *cis*-*β*-ocimene (3.0 - 27.4 %). The latter was identified for the first time in this species. The oil from Portinho da Arrabida was the only one with low amounts of this compound; all the other samples were quite rich in *cis*-*β*-ocimene. Sesquiterpenic compounds attained up t o 15.9 % of the oil's composition. *L. multifida* occurs in small quantities in the three regions previously referred, being more frequent in Sesimbra. In order to evaluate eventual chemical variations throughout the years, plants were harvested at this region from 2008 to 2011 (AI, AII, AIII, AIV, see section 1, chapter II). The chemical composition of the oils AII, AIII and AIV (data not shown) was similar to that reported in table 4 for the sample from the same region, collected in 2008 (AI), showing a high homogeneity of the oils growing at the same region.

Table 4. Composition of the essential oils of *Lavandula multifida* from three regions of Portugal.

RI SPB-1	RI SW-10	Compound	Sesimbra (AI)	Portinho da Arrábida (B)	Mértola (D)
931	1029	*α*-pinene	0.8	2.3	0.6
961	1253	3-octanone	0.4	0.6	0.3
961	1441	oct-1-en-3-ol	0.6	0.9	0.5
971	1118	*β*-pinene	-	t	-
982	1161	myrcene	5.7	1.9	5.5
1000	1169	*α*-phellandrene	0.4	0.2	0.2
1007	1152	Δ-3-carene	0.5	0.3	0.3
1010	1184	*α*-terpinene	0.6	0.2	0.6
1013	1272	*p*-cymene	0.3	0.2	0.2
1022	1205	limonene	0.6	0.4	0.3
1027	**1233**	***cis*-*β*-ocimene**	**27.4**	**3.0**	**27.0**

1037	1249	*trans*-*β*-ocimene	1.7	0.6	1.5
1048	1253	*γ*-terpinene	0.2	0.4	0.1
1067	1397	fenchone	-	t	-
1073	1435	cymenene	0.1	0.4	0.1
1079	1285	terpinolene	3.1	2.7	2.1
1084	1539	linalool	0.3	0.3	0.2
1100	1581	α-fenchol	-	t	-
1118		*allo*-ocimene	-	t	-
1224	1598	carvacrol metyl ether	0.1	2.2	0.1
1281	**2201**	**carvacrol**	**42.8**	**59.0**	**41.5**
1411	1591	*E*-*β*-caryophyllene	0.8	0.8	0.9
1431	1598	aromadendrene	0.1	0.6	0.1
1445	1661	*α*-humulene	0.1	0.3	0.1
1451	1637	*allo*-aromadendrene	t	0.1	0.1
1451	1661	*trans*-*α*-farnesene	t	0.1	0.1
1469	1699	germacrene-D	0.5	0.6	0.3
1481	1687	ledene	0.1	0.3	0.1
1484	1723	*α*-murolene	0.7	0.8	0.8
1495	1743	*E,E*-*α*-farnesene	2.6	2.5	2.8
1498	1720	*β*-bisabolene	5.6	5.4	5.0
1510	1748	*δ*-cadinene	0.2	0.3	0.2
1556	2107	spathulenol	0.6	1.7	0.8
1560	1966	caryophyllene-oxyde	0.3	1.6	0.2
1619	2170	T-murolol	0.2	0.3	0.3
1632	2216	*α*-cadinol	0.2	0.2	0.3
1662	2211	*α*-bisabolol	0.2	0.3	0.2
Monoterpene hydrocarbons			41.4	12.7	38.5
Oxygen containing monoterpenes			43.2	61.6	41.8
Sesquiterpene hydrocarbons			10.8	11.8	10.5
Oxygen containing sesquiterpenes			1.5	4.1	1.8
Others			1.0	1.5	0.8
Total identified			**97.9**	**91.7**	**93.4**

Compounds listed in order of elution from the *SPB-1* column.
RI SPB-1: GC-retention indices relative to C9 – C23 *n*-alkanes on the *SPB-1* column.
RI SW 10: GC-retention indices relative to C9 – C23 *n*-alkanes on the *SupelcoWax-10* column.
t= traces (≤ 0.05 %).

In a previous study carried out by Garcia Vallejo (Garcia Vallejo, 1992) samples of *L. multifida* from the Iberian Peninsula were analysed. The essential oil composition reported differs from the present results in what concerns to the main compounds. *cis*-*β*-ocimene, the second main compound of Portuguese oils was absent in that study. Also a recent study on the chemical composition of the aromatic extracts obtained with hexane

from Tunisian plants showed the presence of carvacrol, *β*-bisabolene and acrylic acid dodecyl ester in high amounts and the absence of *cis*-*β*-ocimene (Chograni et al., 2010). Thus, our results report, for the first time, the presence of this compound in *L. multifida* essential oils, which may occur in high amounts (ca. 27 %). This is a very peculiar feature since *L. multifida* is the only known lavender with high amounts of this compound. In other lavenders *cis*-*β*-ocimene is a minor compound (Garcia-Vallejo, 1992). The high amount of carvacrol is also an interesting finding, since phenolic compounds are not frequent in *Lavandula* spp. In the present study, the essential oils from Portinho da Arrábida region showed a higher concentration of carvacrol (59.0 %) and lower amounts of *cis*-*β*-ocimene in comparison to the other samples analysed. These plants grow very close to the seashore, with a high Atlantic influence, that can affect essential oil composition as previously reported for other species (Figueiredo et al., 2008).

The high amounts of carvacrol are a very interesting point considering the limited occurrence of this phenol in nature. It was previously identified as an important compound in *Thymbra capitata* (Salgueiro et al., 2004) and in some *Thymus* (Gonçalves et al., 2010) and *Origanum* species (Salgueiro et al., 2003) being responsible for the high antifungal activity reported for these species. Furthermore, the very low amounts of trichomes found in this species (see previous section) justify its low oil yield.

3.3.3 *Lavandula pedunculata*

L. pedunculata is widely distributed throughout Portugal, being taxonomically very controversial, with several authors reporting the presence on s ubspecies and varieties, as previously referred. In order to clarify this issue, 43 individual samples were analysed with yields ranging from 0.5-1.1 % (v/w). Considering all the samples analyzed oxygenated monoterpenes were the main group of constituents (69.0 - 89.0 %) followed by monoterpene hydrocarbons (4.5 - 22.5 %). Nevertheless, important quantitative differences were found within the populations analyzed, particularly in the amounts of the main constituents: fenchone (1.3 – 59.7 %), 1,8-cineole (2.4 – 55.5 %) and camphor (3.6 – 48.0 %). The chemical composition of 5 s elected oils of *L. pedunculata* (samples A0, B3, C6, D7 and E) collected in different regions of Portugal

(Valbom dos Figos, Serra da Nogueira, Celorico da Beira, Foz de Arouce and Santiago do Cacém) is listed in Table 5.

Table 5. Composition of the essential oils of *Lavandula pedunculata* from five regions of Portugal.

RI SPB-1	RI SW 10	Compounds	Valbom dos Figos (A0)	Serra da Nogueira (B3)	Celorico da Beira (C6)	Foz de Arouce (D7)	Santiago do Cacém (E)
921	1021	tricyclene	-	t	0.7	t	0.4
930	1031	*α*-pinene	2.4	2.5	3.8	8.0	5.7
943	1074	camphene	3.5	0.8	6.1	1.8	4.6
959	1444	oct-1-en-3-ol	-	t	0.2	t	-
965	1124	sabinene	-	0.1	0.4	0.6	-
970	1115	*β* -pinene	0.4	9	1.4	1.4	0.3
981	1161	myrcene	-	0.1	0.4	0.4	0.3
1001	1169	*α*-phellandrene	-	0.1	0.1	0.6	-
1005	1152	Δ-3-carene	-	-	-	-	-
1008	1184	*α* -terpinene	-	-	-	-	-
1012	1273	*p*-cimene	-	t	0.5	0.3	0.4
1019	1205	limonene	0.5	1.0	1.5	1.0	-
1019	**1214**	**1,8-cineole**	**17.1**	**34.3**	**25.1**	**5.1**	**13.7**
1024	1233	*cis*-*β*-ocimene	-	0.8	0.3	0.4	0.7
1035	1251	*trans*-*β*-ocimene	-	0.7	0.1	0.2	-
1046	1247	*γ*-terpinene	-	0.1	0.2	0.7	0.2
1055	1439	*cis*-linalool oxide	-	-	-	-	-
1055	1459	camphelinone	-	t	t	0.4	0.5
1065	**1400**	**fenchone**	**14.9**	**7.6**	**6.2**	**44.5**	**41.6**
1069	1467	*trans*-linalool oxide	-	0.3	0.2	t	-
1076	1286	terpinolene	-	0.1	0.3	0.2	0.4
1081	1542	linalool	3.2	3.8	1.2	0.5	1.4
1091	1376	oct-1-en-3-yl acetate	-	0.1	0.2	t	-
1097	1577	*α*-fenchol	0.8	1.8	0.3	0.1	1.1
1102	1489	*α*-campholenal	-	0.1	0.1	0.1	0.3
1118	**1515**	**camphor**	**41.5**	**9.9**	**34.0**	**8.7**	**20.6**
1122	1648	*cis*-verbenol	1.1	2.8	0.2	1.0	-
1125	1670	*trans*-verbenol	0.2	1.1	2.0	1.5	0.5
1135	1563	pinocarvone	-	0.9	0.5	0.5	-
1140	1723	*p*-mentha-1,5-dien-8-ol	-	-	-	-	0.1
1145	1692	borneol	0.1	3.4	0.6	1.1	1.5

1158	1841	*p*-cymene-8-ol	-	t	0.1	0.1	-
1158	1596	terpinen-4-ol	0.2	1.2	0.8	1.7	0.8
1166	1622	myrtenal	0.4	2.4	0.8	1.2	-
1169	1689	α-terpineol	-	0.9	0.1	0.4	0.4
1177	1695	verbenone	-	0.5	0.2	0.5	0.3
1196	1826	*trans*-carveol	-	0.1	0.1	0.2	-
1205	1467	fenchyl acetate	-	0.1	0.1	0.5	-
1213	1726	carvone	-	0.1	0.1	t	0.2
1266	1575	bornyl acetate	1.7	0.9	1.3	3.5	0.8
1271	1603	lavandulyl acetate	-	0.1	0.8	0.4	-
1469	1697	germacrene D	-	0.1	t	t	-
1476	1719	eremophilene	-	0.1	0.2	0.4	0.3
1502	1825	*cis*-calamenene	-	-	-	-	-
1509	1749	γ-cadinene	-	0.9	0.3	0.5	-
1521	1904	α-calacorene	-	0.9	0.1	0.1	-
1560	1967	caryophyllene oxide	-	0.6	0.1	0.2	-
1631	2216	α-cadinol	0.1	3.1	0.2	2.5	-
Monoterpene hydrocarbons			6.8	15.4	15.8	15.6	13.0
Oxygen-containing monoterpenes			81.2	72.4	74.9	72.1	83.8
Sesquiterpene hydrocarbons			0.0	2.0	0.6	1.1	0.3
Oxygen-containing sesquiterpenes			0.1	3.7	0.3	2.7	0.0
Others			0.0	0.2	0.4	0.1	0.0
Total identified			**88.1**	**93.7**	**92.0**	**91.6**	**97.1**

Compounds listed in order of elution from the *SPB-1* column.
RI SPB-1: GC-retention indices relative to C9 – C23 *n*-alkanes on the *SPB-1* column.
RI SW 10: GC-retention indices relative to C9 – C23 *n*-alkanes on the *SupelcoWax-10* column.
t= traces (≤ 0.05 %).

Regarding the statistical analysis applied to *L. pedunculata* oils, the hierarchical clustering phenogram, based on the original variables, divided the 43 individual samples of *L. pedunculata* into two significant clusters (*Cluster I* and *Cluster II*; Fig.3). Principal component analysis (PCA) reduced the 16 variables to two principal components, representing 92.24 % of the total variance, with the variables fenchone, 1,8-cineole, and camphor scoring the highest component coefficients. Statistical analysis differentiated two main types of essential oils, one characterized by the predominance of fenchone and the other one by the predominance of 1,8-cineole. Oils of *Cluster I* (37.2 % of the samples) were characterized by fenchone (average = 3 6.5 %, S.D. = 12.9 %), 1,8-

cineole (average = 11.4 %, S.D. = 5.2 %), and camphor (average = 18.8 %, S.D. = 8.5 %). All the samples collected at Coimbra region (*D0 – D10*) were grouped in this cluster together with 45.4 % of the samples collected at Guarda region (*C0*, *C1*, and *C8 – C10*). Oils of *Cluster II* (62.8 % of the samples) were characterized by the presence of 1,8-cineole (average = 28.5 %, S.D. = 10.1 %), camphor (average = 23.7 %, S.D. = 13.9 %) and fenchone (average = 14.2 %, S.D. = 9.3 %). Within this cluster two subgroups (*IIa* and *IIb*) were well defined showing distinct compositions when taking into account the percentages of 1,8-cineole (20.9 ± 8.4 % *vs*. 32.9 ± 8.3 %) and camphor (37.5 ± 8.1 % *vs*. 15.6 ± 9.4 %). All the samples collected at Mirandela (*A0 – A5*), 20.0 % of the samples collected at Bragança (*B6*, *B8*, and *B14*), and 18.2 % of the samples collected at Guarda (C2 and *C6*) regions were grouped in *Cluster IIa*. The remaining samples collected at Bragança (80.0 %; *B0 – B5*, *B7*, and *B9 – B13*) and at Guarda (36.4 %; *C3 – C5* and *C7*) regions were included in *Cluster IIb*. Plants growing at Guarda region (*C0 – C10*) were the most polymorphic, being present in all clusters, *i.e.*, 45.4 % in *Cluster I*, 18.2 % in *Cluster IIa* and 36.4 % in *Cluster IIb*.

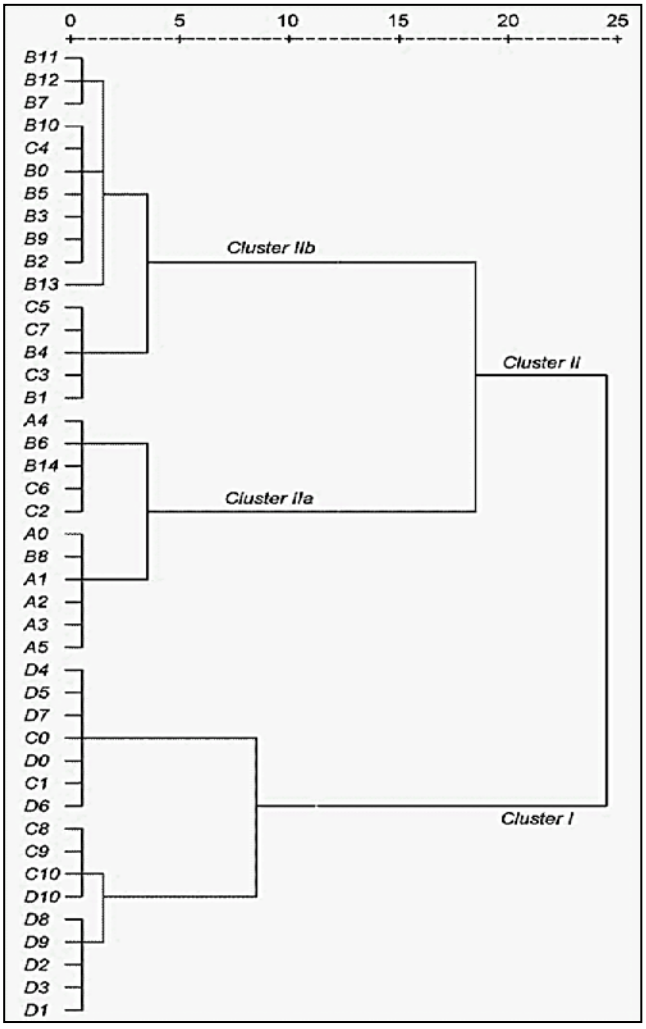

Figure 3. Phenogram from the hierarchical cluster analysis of the individual samples of *L. pedunculata* based on the original variables. Individual samples from Mirandela (A), Bragança (B), Guarda (C) and Coimbra (D).

Considering that the above different chemical types were found in plants at the same developmental stage and in plants from the same or very close localities, sharing similar ecological and edaphic features, it seems that the chemical polymorphism observed in the essential oils of *L. pedunculata* is mainly due to genetic factors.

The essential oil composition of *L. pedunculata* from Portugal shows similarities with that reported for *L. stoechas* from other Mediterranean regions, namely Spain (Garcia Vallejo, 1989), Cyprus (Valentini et al., 1993), Greece (Kokkalou, 1988; Skoula et al., 1996), Corse (Ristorcelli et al., 1998) and Turkey (Dadalioğlu and Evrendilek, 2004). Also the essential oil of *L. stoechas* from Barcelona and Sardinia, analysed in the present study (see table 7), showed similarities with that of *L. pedunculata*.

3.3.4 *Lavandula viridis*

The composition of 3 representative oils of *L. viridis* (samples A, B and C) from different regions of Portugal (Barranco do Velho, Salir, Porto Nobre) are listed in Table 6. The oil showed a yield varying from 1.5-1.8 % (v/w) and making this species very interesting under an industrial point of view. A total of 51 compounds were identified, representing more than 90 % of the total volatile oils. The oils were characterized by high contents of oxygen-containing monoterpenes (69.3 - 75.3 %), followed by monoterpenic hydrocarbons (15.5 – 21.8 %). The main constituents of the oils were 1,8-cineole (34.5 - 42.2 %), camphor (12.8 - 13.4 %), *α*-pinene (9.0 – 14.1 %), and linalool (6.0 – 7.9 %).

L. viridis only occurs at the south of Portugal. In order to evaluate eventual chemical variations throughout the years, plants from Barranco do Velho region were harvested, during the flowering period, from 2008 to 2011 (A, AI, AII, AIII, see section 1 of the present chapter) and their essential oils composition compared. The chemical composition of the oils (data not shown) was similar to that reported for the samples from the same region, collected in 2008 (A, Table 6).

Table 6. Composition of the essential oils of *Lavandula viridis* from three regions of Portugal

RI SPB-1	RI SW 10	Compound	Barranco do Velho (A)	Salir (B)	Porto Nobre (C)
920	1020	tricyclene	0.3	0.3	0.4
930	**1030**	***α*-pinene**	**9.0**	**9.0**	**14.1**
942	1075	camphene	3.3	3.7	4.3
942	1129	verbenene	0.3	0.4	0.7
951	1303	1-octen-3-one	1.0	1.4	2.0
960	1337	hept-5-ene-6-methyl-2-one	0.2	0.3	0.1
963	1253	3-octanone	0.1	0.2	0.1
964	1127	sabinene	0.3	0.1	0.1
969	1116	*β*-pinene	1.1	0.5	0.5
977	1388	3-octanol	0.2	t	t
980	1161	myrcene	0.2	0.1	0.1
1008	1185	*α*-terpinene	0.2	0.1	0.2
1012	1272	*p*-cymene	0.3	0.1	0.1
1020	**1215**	**1,8-cineole**	**34.5**	**42.2**	**40.5**
1021	1205	limonene	0.8	0.6	0.5
1027	1233	*cis*-*β*-ocimene	0.7	0.4	0.6

1035	1249	*trans*-*β*-ocimene	0.4	0.1	0.1
1047	1249	*γ*-terpinene	0.2	0.1	0.1
1051	1459	*trans*-sabinene hydrate	0.2	0.1	0.1
1055	1439	*cis*-linalool oxyde	0.9	1.2	0.7
1070	1467	*trans*-linalool oxyde	1.0	1.0	0.6
1082	**1542**	**linalool**	**7.9**	**6.7**	**6.0**
1098		chrysanthenone	0.2	0.2	0.2
1102	1489	*α*-campholenal	0.3	0.4	0.4
1104	1574	nopinone	0.2	0.2	0.2
1118	**1514**	**camphor**	**13.4**	**13.4**	**12.8**
1121	1645	*cis*-verbenol	0.8	0.5	0.6
1134	1563	pinocarvone	0.4	0.3	0.4
1143	1721	*p*-mentha-1,5-dien-8-ol	1.2	1.2	1.0
1146	1667	isoborneol	2.8	3.1	3.1
1149		3-methylacetophenone	0.2	t	t
1158	1595	terpinen-4-ol	0.9	1.0	0.6
1165	1621	myrtenal	0.4	0.2	0.1
1169	1689	*α*-terpineol	0.9	1.2	0.7
1176	1694	verbenone	1.3	0.9	1.0
1196	1829	*trans*-carveol	0.3	0.4	0.4
1212	1728	carvone	0.2	0.2	0.2
1237	1553	linalyl acetate	0.2	0.1	0.1
1264	1574	bornyl acetate	0.2	0.2	0.2
1301	1683	myrtenyl acetate	0.2	0.1	0.3
1342	1459	*α*-cubebene	t	t	t
1357	1750	geranyl acetate	0.7	0.4	0.2
1456		phenyl-2-methyl-butirate	0.5	0.1	0.1
1467	1699	germacrene-D	0.7	0.6	0.2
1473	1716	*β*-salinene	0.3	0.1	0.1
1490	1721	*β*-bisabolene	1.1	0.9	0.4
1521	1773	selina-4(15),7(11)-diene	0.5	0.1	0.1
1529	1773	selina-3,7(11)-diene	0.9	0.2	0.1
1529	1769	*α*-bisabolene	0.6	0.1	0.1
1628	2208	*α*-eudesmol	0.3	0.1	0.1
1667	1709	*β*-bisabolol	0.3	0.1	0.1
Monoterpene hydrocarbons			17.1	15.5	21.8
Oxygen containing monoterpenes			69.3	75.3	70.4
Sesquiterpene hydrocarbons			4.2	2.0	1.1
Oxygen containing sesquiterpenes			0.6	0.2	0.2
Others			2.0	2.1	2.4
Total identified			**93.2**	**95.1**	**95.9**

Compounds listed in order of elution from the *SPB-1* column.
RI SPB-1: GC-retention indices relative to C9 – C23 *n*-alkanes on the *SPB-1* column.
RI SW 10: GC-retention indices relative to C9 – C23 *n*-alkanes on the *SupelcoWax-10* column.
t= traces (≤ 0.05 %).

In a previous study carried out by Garcia Vallejo (1992) some individual samples of *L. viridis* from the South of Portugal and Spain were analyzed. The chemical composition of these samples was very similar to that of our collective samples, 1,8-cineol being the major component in all samples. This fact points to a very high homogeneity in the composition of the essential oils of *L. viridis* from Portugal and Spain. Furthermore, the high density o glandular trichomes observed in this species (see previous section) justify its high essential oil yield.

3.3.5 *Lavandula stoechas*

The composition of the essential oil of *L. stoechas* from Sardinia and Barcelona is shown in Table 7. The analysis of this species was included in the present study for taxonomical purposes. The oils composition was compared to that of *L. pedunculata* and *L. luisieri* since controversial classifications for these three species are very common, as previously referred. The oils of *L. stoechas* were characterized by high contents of oxygenated monoterpenes (87.4 % and 81.9 %) being the main compounds identified in both samples, fenchone (37.0 % and 27.6 %), camphor (27.3 % and 42.8 %) and 1,8-cineole (6.0 % and 8.6 %).

Table 7. Composition of the essential oils of *Lavandula stoechas* from Sardinia and Barcelona.

RI SPB-1	RI SW 10	Compound	Sardinia (A)	Barcelona (B)
921	1021	tricyclene	0.2	0.1
930	1031	*α*-pinene	0.4	2.6
943	1074	camphene	2.8	2.8
965	1124	sabinene	-	0.2
970	1115	*β*-pinene	-	0.2
981	1161	myrcene	-	0.1
1012	1273	*p*-cymene	0.4	0.1
1020	1206	limonene	0.2	0.5
1019	**1214**	**1,8-cineole**	**6.0**	**8.6**
1055	1459	camphenilone	0.3	0.1
1065	**1400**	**fenchone**	**37.0**	**27.6**
1076	1286	terpinolene	-	0.3
1081	1542	linalool	-	0.3
1097	1577	*α*-fenchol	0.4	0.1
1118	**1515**	**camphor**	**27.3**	**42.8**
1140	1723	*p*-mentha-1,5-dien-8-ol	0.4	0.2
1145	1692	borneol	0.8	0.2
1158	1596	terpinen-4-ol	-	t
1176	1781	myrtenol	0.5	0.1
1177	1695	verbenone	0.3	0.2
1266	1575	bornyl acetate	6.2	1.4
1271	2183	thymol	3.1	t
1281	2201	carvacrol	3.4	-
1301	1683	myrtenyl acetate	1.7	0.2
1409	1590	*E*-*β*-caryophyllene	-	t
1509	1749	δ-cadinene	0.3	0.1
1560	1967	caryophyllene oxide	-	t
1571	2068	viridiflorol	2.6	0.7
1574	2079	guaiol	1.7	0.2
1615	2172	T-muurolol	0.4	0.1
Monoterpene hydrocarbons			4.0	6.9
Oxygen containing monoterpenes			87.4	81.9
Sesquiterpene hydrocarbons			0.3	0.2
Oxygen containing sesquiterpenes			4.7	1.0
Others			-	-
Total			**96.4**	**90.0**

Compounds listed in order of elution from the *SPB-1* column.
RI SPB-1: GC-retention indices relative to C9 – C23 *n*-alkanes on the *SPB-1* column.
RI SW 10: GC-retention indices relative to C9 – C23 *n*-alkanes on the *SupelcoWax-10* column.
t= traces (≤ 0.05 %).

This species has been object of several phytochemical studies that have pointed out its high chemical variability. The essential oils are characterized by significant variations in the amounts of fenchone, camphor and 1,8-cineole, being the fenchone/camphor chemotype the most commonly identified (Dadalioğlu and Evrendilek, 2004; Angioni et al., 2006; Dob et al., 2006; Benabdelkader, 2011; Messaoud et al., 2011). In the present study, the chemical composition of *L. stoechas* essential oils is in accordance to that reported previously by these authors, since high amounts of fenchone and camphor were also present. Phenolic compounds are not usual in this species. However, in the essential oils from Sardinia thymol (3.1 %) and carvacrol (3.4 %) were detected.

The essential oil composition of *L. stoechas* shows similarities with that reported for *L. pedunculata* from Portugal (see table 5). In fact, the essential oils of both *taxa* consist of the same major components and the same chemical polymorphism, being very difficult to distinguish the species based on essential oil composition. *L. stoechas* is one of the 4 main lavender species widely commercialized while *L. pedunculata* is very poorly recognized. However, due to the chemical similarity of their oils, *L. pedunculata* from Portugal may be used for the same purposes as *L. stoechas* from other Mediterranean regions.

When comparisons are made between the essential oil composition of *L. stoechas* and *L. luisieri* (see table 3), significant differences are found. Although these species are morphologically very similar, the later has a very unique and distinctive chemical profile. A more detailed analysis on the chemotaxonomy of these species in presented in the following chapter.

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4. Chemotaxonomy

4.1 Chemotaxonomy

The genus *Lavandula* is known for its complex taxonomy, many synonyms and frequent hybridizations. Currently, the lavenders growing wild in Portugal are included in three distinct sections: Section Lavandula (*L. latifolia*), Section Pterostoechas (*L. multifida*) and Section Stoechas (*L. luisieri*, *L.pedunculata* and *L. viridis*) (Upson and Andrews, 2004).

The complex taxonomy of the genera, mainly in section Stoechas, with several authors in some European floras (e.g. Pereira Coutinho, 1939; Tutin, 1972; Franco, 1984; Morales, 2010) implementing different classifications for *L. pedunculata*, *L. lusieri* and *L. stoechas* (names used in the present study), justified the detailed morphological examination (section 1, chapter II), trichomes characterization (section 2, Chapter II) and essential oil analyses (section 3, chapter II) carried out in the present study. Moreover, *L. stoechas* from Barcelona and Sardinia were included in this study in order to clarify the taxonomy of the section Stoechas. The main conclusions and a taxonomical clarification are presented below.

a) Section Stoechas

The distinct large sterile and colored apical bracts at the apex of the flower spikes allow the immediate identification of the species included in section Stoechas (Upson and Andrews, 2004). As previously referred, in Portugal three species occur: *L. luisieri*, *L. pedunculata* and *L. viridis*.

Lavandula luisieri is endemic to the Iberian Peninsula, being very common throughout Portugal and in the southwest of Spain (Franco, 1984; Upson and Andrews, 2004). It has been considered as a subspecies of *L. stoechas* due to similarities in flower morphology, namely the presence of a ring of hairs in the throat of the corolla tube and the length (short) of the peduncle of the inflorescence (Upson and Andrews, 2004; Morales, 2010). According to Upson and Andrews (2004) the two subspecies are easily differentiated by the indumentums of the calyx:

Calyx indumentum woolly of long, simple to once-branched hairs ….. subsp. *stoechas*
Calyx indumentum velvety to short-branched hairs ……... subsp. *luisieri*

Morales (2010), on the other hand, refers other features to separate the two subspecies, as translated below:

Leaves uniform in size, with white indumentum, inflorescence with peduncle shorter than spike……	subsp. *stoechas*
Leaves heterogeneous in size, generally with axillary lanceolated and larger leaves, greyish indumentum, inflorescence with peduncle longer than spike …….	subsp. *luisieri*

In the present work, besides morphological studies, the chemical composition of the essential oils was also considered for quimiotaxonomic purposes.

Regarding *L. luisieri* essential oils, a p eculiar composition (presence of necrodane derivatives), very distinct from the other lavender oils, has been reported (Garcia-Vallejo, 1992; Lavoine-Hanneguelle et al., 2004; Sanz et al., 2004; Baldovini et al., 2005; González-Coloma et al., 2011). In the present study, significant quantitative variations among samples were also found, namely in the amounts of the main constituents: 1,8-cineole, fenchone and *trans*-α-necrodyl acetate. However, necrodane derivatives were present in all the samples analysed (see section 3 of this chapter).

Considering *L. stoechas*, the essential oils are characterized by significant variations in the amounts of the main compounds fenchone, camphor and 1,8-cineole (Dadalioğlu and Evrendilek, 2004; Angioni et al., 2006; Dob et al., 2006; Benabdelkader, 2011; Messaoud et al., 2011). In the present study, high amounts of fenchone and camphor were also identified in the samples from Barcelona and Sardinia but necrodane derivatives were absent. In fact, these compounds have never been reported for *L. stoechas* and, as far as it is known, are unique in the plant kingdom and therefore can be considered a ch emotaxonomic mark of *L. luisieri* (Garcia-Vallejo, 1992). For this reason, it seems reasonable to consider *L. luisieri* as a separated species. This classification has already been suggested by Rivas-Martinez (1979) and Franco (1984) however, a recent work by Morales (2010) did not consider the separation of this *taxon*.

Lavandula pedunculata is unevenly distributed from the west Mediterranean region (Portugal, Spain and Morocco) until Turkey. Particularly in Portugal it is very frequent throughout the country. This species has undergone several classifications over

the years (e.g. Franco, 1984; Upson and Andrews, 2004; Morales, 2010). Rozeira (1949) was the first to consider *L. pedunculata* as a subspecies of *L. stoechas*. Nowadays, this classification is rarely adopted and *L. pedunculata* is considered an independent species (Upson and Andrews, 2004; Morales, 2010). In fact, *L. pedunculata* is easily distinguished by differences in flower morphology, namely the lack of a ring of hairs in the throat of the corolla tube as well as the distinct long inflorescence peduncle compared to the short peduncle in *L. stoechas* (Upson and Andrews, 2004; Morales, 2010). However, infraspecific divisions in this species remain controversial, due to the similarities among the *taxa*, with some plants showing intermediate characteristics (Upson and Andrews, 2004). Different authors have considered 3 subspecies for *L. pedunculata*: subsp. *lusitanica*, subsp. *pedunculata* and subsp. *sampaiana* (e.g. Franco, 1984; Upson and Andrews, 2004). In the present study a detailed essential oils analysis, including several individual and collective samples of *L. pedunculata* from distinct regions of Portugal was performed, in order to identify eventual chemical variations. Plant observations showed a great morphological variation mainly in the length of the inflorescence peduncles and shape and size of bracts. The variations occurred in plants from the same population without any geographical pattern. Also the different chemical types (see section 3 of the present chapter) were found both in plants at the same developmental stage and in plants from the same or very close localities, sharing similar ecological and edaphic features. Therefore, the polymorphism observed in *L. pedunculata* do not seem enough to sustain the existence of subspecies being more plausible to explain the differences observed as a result of intraspecific variation which can be caused by many factors. The chemical variability was not correlated to the morphological variations observed, and therefore, in our opinion, it doesn´t seem reasonable to consider the aforementioned division in several subspecies. Moreover, hybridization between *L. pedunculata* and other species may occur, making identification of the *taxa* more complex and difficult.

Comparing the essential oil composition of *L. pedunculata* with that reported for *L. luisieri*, important differences were detected. As referred above, *L. luisieri* has a very peculiar chemical composition. Also, trichomes differ in these two species, since only in *L. pedunculata* ramified mixed-type trichomes and bifurcated trichomes were found (see section 2 of this chapter).

The essential oil composition of *L. pedunculata* from Portugal shows similarities with that reported for *L. stoechas* from the Mediterranean region, namely Spain (Garcia Vallejo et al., 1989), Chipre (Valentini et al., 1993), Greece (Kokkalou, 1988; Skoula et al., 1996), Corse (Ristorcelli et al., 1998) and Turkey (Dadalioğlu e Evrendilek, 2004). Also, the two oils of *L. stoechas* included in the present study (one from Barcelona and the other one from Sardinia) were chemically similar to the chemotype fenchone/camphor identified in Portuguese *L. pedunculata* oil. In fact, even though both *taxa* are morphologically well distinct, their essential oils consist of the same major components (1,8-cineole, fenchone and camphor) and the same chemical polymorphism. Despite the fact that chemical results may support the classification of *L. pedunculata* as a subspecies of *L. stoechas*, the two *taxa* are morphologically well distinct, justifying the classification that considers two distinct species (Upson and Andrews 2004; Morales, 2010).

L. viridis is a highly aromatic shrub endemic to the South Iberian Peninsula. This species is considered of least concern (LC) in Portugal and Spain but is critically endangered (CR) in Madeira, where recent field observations identified only 20 mature plants in a remote locality near Machico (Upson and Andrews, 2004). This species is also included in the section Stoechas together with *L. luisieri*, *L. pedunculata* and *L. stoechas* but is easily differentiated from the remaining species by its characteristic white flowers and yellowish/green sterile bracts as well as the high concentration of trichomes. Trichome characterization of Portuguese samples showed a very high density of different types of glandular trichomes whereas non-glandular trichomes were very scarce. In *L. viridis* long-stalked capitate trichomes were frequent; these trichomes were not found in the remaining species analysed (see section 2 of this chapter). Essential oil composition showed a very high homogeneity, with the oils from Portuguese samples being very similar to those from Spain (Garcia Vallejo, 1992). Neither subspecies nor varieties are known but hybrids with *L. pedunculata* (*Lavandula* x *Limae*) and *L. luisieri* (*Lavandula alportensis*) have been reported (Upson and Andrews, 2004).

b) Section Lavandula

Section Lavandula comprises 3 species (*L. angustifolia*, *L. lanata* and *L. latifolia*) of small floriferous woody shrubs with narrow entire leaves and no apical bracts. It is the most economically important section since it contains the main cultivated lavender species for essential oil exploitation: *L. angustifolia* (English lavender), *L. latifolia* (spike lavender) and their hybrid (*L.* x *intermedia* (lavandin) (Upson and Andrews, 2004).

Lavandula latifolia is distinguished from the remaining species of the section Lavandula by its felt-like indumentum of short, highly branched hairs and 13-veined calyx. It is widespread and common in France, Spain and Italy. In Portugal, however, only some individuals were found at the south of Coimbra (Rio de Galinhas region). The conservation status of this species is of least concern (LC) but it should be considered critically endangered (CR) in Portugal. In *L. latifolia*, morphological variations in the wild may occur but do not represent taxonomic significance. Trichome analyses of Portuguese samples showed a very high density of non-glandular trichomes and the absence of ramified mixed and bifurcated trichomes (see section 2 of this chapter). Regarding phytochemical studies, different authors (e.g. De Pascual et al., 1983; Ter Heide et al., 1983; Boelens, 1986; Garcia Vallejo, 1992; Muñoz-Bertomeu et al., 2007) have identified 1,8-cineole, linalool, camphor and, in some populations, borneol as the main compounds. In the present study, essential oil analyses were not performed due to limitations in plant quantity.

c) Section Pterostoechas

Section Pterostoechas comprises 16 species. This section is quite distinct due to the single-flowered cymes, arranged in a biseriate or tetragonous spike. All species bear pinatissect to bipinnatisect leaves, being distinguished by differences in indumentum, leaves or bracts (Upson and Andrews, 2004).

Lavandula multifida is the only species of the section *Pterostoechas* native to the European mainland. It occurs commonly in the Mediterranean area (Portugal, Spain, Marroco, Algeria, Tunisia and Lybia). In Portugal it grows spontaneously in the southern region (Sesimbra, Arrábida and Mértola). Confusions registered in these species occur in North America with plants named *L. multifida* being *L. pinnata* (Tucker

and De Baggio, 2000, cited in Upson and Andrews, 2004). However, *L.* multifida has a unique stem indumentum of long white hairs, easily visible and flower spikes with a distinct twist.

In the present study, trichomes characterization reported for the first time, the presence of a particular peltate trichome with a very long stalk and a d istinct arrangement of the eight secretory cells in the head (see section 2 of the present chapter). Moreover, *cis-β*-ocimene was identified for the first time in the essential oil of this species. This lavender was also considered a phenolic-rich lavender due to the high amounts of carvacrol found in all the samples analysed. Gathering all the characteristics referred, *L. multifida* is a species easily distinguished.

In conclusion and taking into account morphological and phytochemical data, the present study considers *L. luisieri, L. pedunculata* and *L. stoechas* as three distinct species. Moreover, it seems quite certain that *L. stoechas* does not occur in Portugal. Recent studies (Morales, 2010) consider *L. stoechas* a ubiquitous species in Portugal. However, taking into account the several field trips performed for plant harvest at different regions of Portugal, the careful and minutious observations of many herbarium specimens and fresh material and the advisement of the taxonomist Jorge Paiva, it seems that the species occurring in Portugal is *L. luisieri* and not *L. stoechas*. Moreover, individual samples were also analysed in order to confirm the presence of necrodane derivatives. The phytochemical studies performed showed the presence of necrodane derivates in all the samples analysed (data not shown). Although the amounts of these compounds may vary, they are only present in *L. luisieri* and have never been reported for *L. stoechas*.

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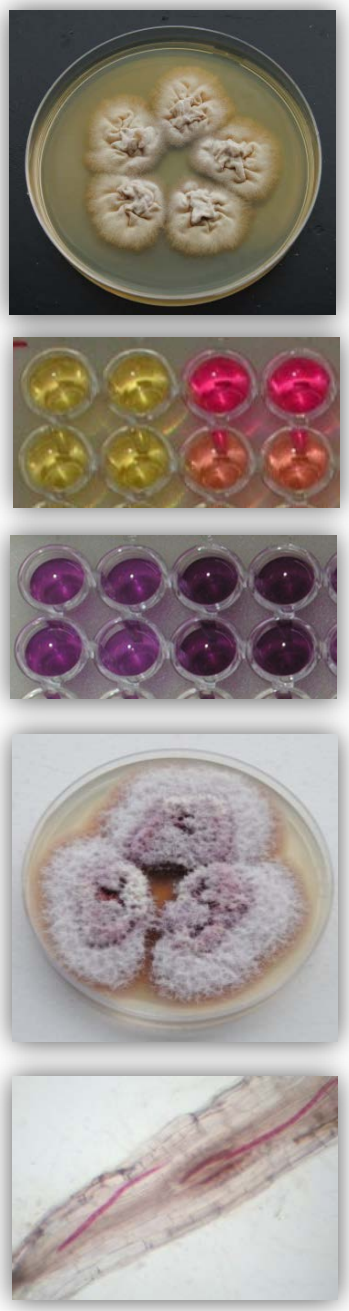

III. Biological Activities and Cytotoxicity of *Lavandula* spp. Essential Oils

1. Antifungal Activity against Human and Animal Pathogens

1. Antifungal Activity against Human and Animal Pathogens

1.1 Introduction

Fungi are responsible for serious pathogenic infections that have increased during the last decades, particularly among high-risk patients (Pfaller et al., 2006; Muñoz et al., 2012; Sipsas, 2012). The range of severity of these infections is related to the host reaction to the metabolic products produced by fungi, the virulence of the infecting strain, the infection site as well as some environmental factors (Weitzman and Summerbell, 1995; Romani, 2007). Fungal infections can be disfiguring, recurrent, and chronic, usually requiring persistent treatments (Shehata et al., 2008). Although conventional antifungals are available, the increased resistance to these compounds and their side-effects, ranging from mild reactions to hepatotoxicity, neurotoxicity, nephrotoxicity and hematologic reactions are frequently responsible for unsuccessful treatments (Andriole, 1994; Del Rosso, 2000; Gupta and Cooper, 2008). Moreover, the effective lifespan of these chemicals is often limited as a consequence of their repeated use as antifungals and immunosuppressive drugs as well as in organ transplantation, lymphomas and HIV secondary infections. Cryptococcosis, candidosis, dermatophytosis and aspergillosis are some of the most threatening fungal infections affecting mainly immunocompromised patients. A summary of their causal agents, symptoms and existing treatments is presented below.

a) Cryptococcosis

Cryptococcosis is an invasive fungal infection, caused mainly by *Cryptococcus neoformans* (Fig. 1) or *Cryptococcus gattii* (Brizendine et al., 2011). The clinical condition of cryptococcosis varies from asymptomatic infections to severe pneumonia and respiratory failure (Brizendine et al., 2011). Management of this disease can vary, according to the characteristics of the affected population (HIV-seropositive, organ transplant recipients or non-transplant/non-HIV), from treatments with amphotericin B, flucytosine or fluconazole (Desalermos et al., 2012). In immunocompromised patients, the disease may spread to the brain and trigger neurological troubles such as meningoencephalitis (Lin and Heitman, 2006), a d isease responsible for more than 600,000 deaths/year worldwide (Desalermos et al., 2012).

Figure 1. *Cryptococcus neoformans* colonies on Sabouraud dextrose agar after 72 h of culture at 35 ºC; bar = 1 cm.

b) Candidosis

This disease results from infections by *Candida* spp. (Fig. 2). These species are commensal microorganisms of the oral cavity, gastrointestinal tract and vagina (Mardh et al., 2002; Mardh et al., 2003). In the last years, *Candida* infections have increased significantly, being *C. albicans*, *C. glabrata* and *C. parapsilopsis* the main agents of nosocomial fungal bloodstream infections (Hajjeh et al., 2004). Vulvovaginal candidosis is one of the most common manifestations of these infections, affecting 70-75 % of women and being recurrent in 40-50 % of the cases (Costa-de-Oliveira, 2008). Vaginal irritation, vulvar burning, pruritus and vaginal discharge are the most common symptoms (Palmeira de Oliveira et al., 2009). Recurrent cases of vulvovaginal candidosis have also increased during the last years, being associated to relapses of persistent yeast strains even after prolonged treatments (Murina et al., 2011). Moreover, pregnancy, diabetes mellitus, oral contraceptive use, antibiotics, tight-fitting clothing, synthetic underwear, dietary excesses or deficiencies, intense sexual activity, and the use of feminine hygiene products have been considered risk factors (Sobel, 2007)

Only a few number of antifungals are effective against *Candida* spp. infections, being amphotericin B, fluconazole and itraconazole the most widely used (Carillo-Munoz et al., 2006). More recently, echinocandins have also been used in systemic infections (Lecha, 2005; Loo, 2006). The pathogenic mechanism behind these infections remains unclear but has been related to germ tube formation. Yeast blastoconidia are involved in asymptomatic colonization of the vagina while germ tubes and hyphae are present in symptomatic cases. Moreover, the structural organization of *Candida* in biofilms seems to be responsible for the resistance to the antifungal agents usually used,

hence reducing the effectiveness of these chemicals (Kuhn et al., 2002). It is known that phenotypic modifications within the fungal cells as well as expression of resistant genes occur during biofilm formation (Chandra et al., 2001; Douglas, 2003). Thus, inhibition of germ tube formation and/or biofilm organization seems to be an excellent target for the management of these infections, avoiding dissemination.

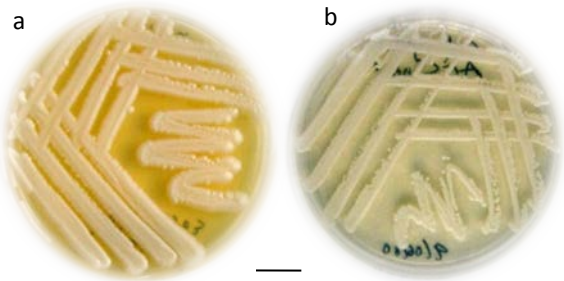

Figure 2. *Candida* spp. colonies on a) Sabouraud dextrose agar and b) potato dextrose agar after 48 h of culture at 35 ºC; bar = 1 cm.

c) Dermatophytosis

Trichophyton, *Microsporum* and *Epidermophyton* species (Fig. 3) are responsible for common infections in skin, hair and nails, generally called dermatophytosis or ringworm infections (Hainer, 2003; Dahdah and Sher, 2008). These fungi colonize keratinized human or animal tissues causing an infection that may vary from mild to very intense. Infections are usually non-invasive, but in immunocomprimised patients they can rapidly progress to life-threatening disseminated infections. Furthermore, some zoophilic and geophilic dermatophytes are responsible for quite severe inflammatory reactions due mainly to delayed hypersensitivity responses to fungi proteases. In addition, in recent years, infections have increased considerably among pediatric and geriatric populations (Mukherjee et al., 2003; Monod, 2008), being also very common among pets and livestock, yet uncommon in wild animals.

Cutaneous dermatophytosis are usually recognized by their scaly patches, with central clearing and sharply demarcated, annular, erythematous, advancing margins, sometimes presenting vesicles, blisters and pustules (Dahdah and Sher, 2008). In some cases, the distinction between dermatophytosis and other clinical conditions that also exhibit similar symptoms such as nummular eczema, subacute cutaneous lupus

erythematosus, pustular psoriasis, subcorneal pustular dermatosis, photoallergic/phototoxic dermatitis, herpes simplex and varicella zoster virus infections is not an easy task (Ziemer et al., 2007).

Treatment of dermatophytosis includes both oral (systemic) and topical formulations mainly from two antifungal drug families: azoles (eg. clotrimazole, miconazole, econazole, oxiconazole, tioconazole) and allylamines (e.g. terbinafine and naftifine) (Gupta and Cooper, 2008). Superficial mycosis such as *tinea pedis*, *t. mannum*, *t. corporis* or *t. cruris* usually respond to topical antifungals (Andrews and Burns, 2008; Dahdah and Sher, 2008; Gupta and Cooper, 2008). Morpholine derivates namely amorolfine and butenafine have been also used (Gupta and Cooper, 2008). For patients displaying wide areas of infection or in cases of severe or persistent infections, oral therapy should be considered, and the same is true for *t. unguium* and *t. capitis* in which terbinafine, itraconazole, fluconazole, griseofulvin, and ketoconazole have been reported as the more adequate. However, oral formulations may be responsible for major side-effects including hepatotoxicity, neurotoxicity, nephrotoxicity, hematologic reactions as well as even uncommon skin problems such as the Stevens-Johnson syndrome (Andrews and Burns, 2008; Gupta and Cooper, 2008). Drug interactions and the consequent reduction of their effectiveness are other causes that must also be evaluated (Andriole, 1994; De Rosso, 2000).

Concerning veterinary, only a reduced number of antifungals are readily available and licensed. The same is true for livestock in which the application of systemic drugs is limited due to the use of these animals and their by-products for human consumption (Chermette et al., 2008).

Figure 3. Morphology of a) *Trichophyton* sp., b) *Microsporum* sp. and c) *Epidermophyton* sp. colonies on Sabouraud dextrose agar after 7 days of culture at 30 ºC; bars = 1 cm.

d) Aspergillosis

Lung diseases caused by *Aspergillus* spp. (Fig. 4) infection of the alveolar tissues are usually known as aspergillosis. These diseases include pulmonary aspergilloma, invasive aspergillosis and allergic bronchopulmonary aspergillosis (Khan and Ahmad, 2011). *A. fumigatus* and *A. niger* are the main strains responsible for aspergillosis, while *A. flavus* and *A. clavatus* seem to be less harmful. Invasive aspergillosis has become a m ajor health concern since it often causes death in immunocompromised patients (Pfaller and Diekema, 2010). The significance of the disease has increased with the growing numbers of patients with impaired immune defense due to cancer, organ transplantation, autoimmune and inflammatory conditions among other conditions, as well as those with chronic obstructive pulmonary disease (Kousha et al., 2011). Moreover, resistance of *Aspergillus* strains to antifungal drugs increased over the last 20 years with the increase use of antifungal drugs (Hadrich et al., 2012). Among the most commonly used antifungals are voriconazole and amphotericin B deoxycholate (Walsh et al., 2008).

Foods, commodities and raw materials are also vulnerable to contamination by *Aspergillus* spp., a major cause of food spoilage in tropical countries, due to the production of powerful mycotoxins (Whitfield, 2003; Hedayati, 2007). These fungi are responsible for food decomposition and the production of allergenic compounds which may occur before any evidence of fungal growth (Pirbalouti et al., 2011).

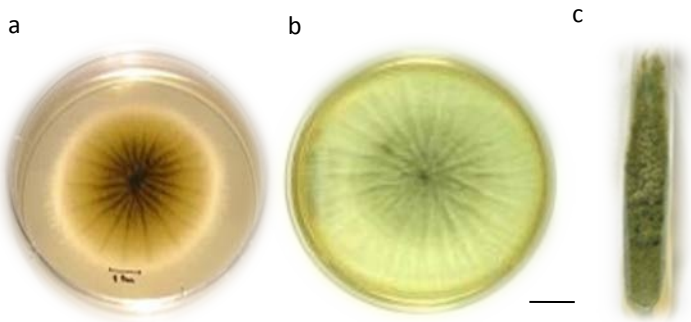

Figure 4. Morphology of a) *Aspergillus niger*, b) *Aspergllus fumigatus* and c) *Aspergillus flavus* colonies on potato and Sabouraud dextrose agar after 48 h of culture at 35 ºC; bars = 1cm.

Taking into account the increasing worldwide incidence of fungal infections, it is urgent to the search for more effective and less toxic antifungals as alternatives to the synthetic ones. For centuries, essential oils have been used as antifungals in traditional remedies (Rios et al., 2005). More recently, several studies have confirmed the huge potential of these natural products as antifungal agents (Pina-Vaz et al., 2004; Pinto et al., 2006; Bakkali et al., 2008; Reichling et al., 2009). Therefore, it is not surprising that essential oils are one of the most promising groups of natural products for the development of broad-spectrum, safer, and cheaper antifungal agents. Although many studies have reported the antifungal activity of essential oils (e.g. Bakkali et al., 2008), the interactions between the oils and microorganisms, which are lately responsible for its activity, remain poorly understood. Furthermore, studies aiming to understand the mechanism of action and the toxicological safety of the oils are missing, hence hampering its potential utilization for industrial purposes.

The genus *Lavandula* provides valuable essential oils for the food (flavoring), perfumery, cosmetic and pharmaceutical industries, being also very popular in aromatherapy. However, many other applications can be envisaged, as has been suggested in several reports on the biological activity of this genus, in particular in what concerns the antifungal properties of some species (e.g. Inouye et al., 2001; D'Auria et al., 2005; Angioni et al., 2006; Cavanagh et al., 2011). In spite of this, studies on the antifungal activity of lavenders growing spontaneously in Portugal are very scarce. As far as it is known, only two studies have been performed with *L. luisieri* oils against *Candida albicans* (Baldovini et al., 2005; Lai et al., 2012).

Considering the lack of information on the biological properties of Portuguese lavenders, the aim of the present study was to evaluate the antifungal activity of the essential oils and their main compounds against several pathogenic fungi responsible for human and animal infections, namely *Candida* spp. *Cryptococcus neoformans*, dermatophytes and *Aspergillus* spp. Moreover, the effect of the oils on the yeast-mycelium transition in *C. albicans,* an important virulence factor, as well as the mechanism of action of selected species was also assessed.

The antifungal activity of the oils against phytopathogenic fungi is reported in section 4 of the present chapter. Although *Aspergillus* strains are important food contaminants, they were included in the present chapter due to their high prevalence in human infections.

1.2 Material and methods

The antifungal activity of the essential oils was evaluated at the Pharmacognosy Laboratory of the Faculty of Pharmacy of the University of Coimbra. Assays on the mechanism of action of the oils were performed in collaboration with team members of the CEQUIMED, Microbiology Service, of the Faculty of Pharmacy of the University of Porto.

1.2.1 Essential oils

The oils selected for the antifungal activity are listed in table 1. For *L. luisieri* two samples (D and L) were selected based on the differences in the amounts of necrodane derivates, sample D being very rich in these compounds, whereas in sample L they were present in very low quantities (see table 3, section 3, chapter II). For *L. pedunculata*, three samples were chosen each one representing a different oil type: sample B3 (rich in 1,8-cineole), sample C6 (with high contents of camphor) and sample D7 (with high amounts of fenchone) (see table 5, section 3, chapter II). For *L. multifida* only sample AI (see table 4, section 3, chapter II) was assessed due to limitations in plant quantity from the remaining sites of collection. Regarding *L. viridis* the samples analyzed showed a similar chemical composition (oils rich in 1,8-cineole, camphor, linalool and α-pinene) being sample A selected as a representative sample (see table 6, section 3, chapter II).

Table 1. Site of collection of *Lavandula* spp. used in the antifungal assays.

Species	Region	Site of Collection	Sample
L. luisieri	Coimbra	Pióbão	D
	Algarve	Cabo de São Vicente	L
L. pedunculata	Bragança	Serra da Nogueira	B3
	Guarda	Celorico da Beira	C6
	Coimbra	Foz de Arouce	D7
L. multifida	Setúbal	Sesimbra	AI
L. viridis	Algarve	Barranco do Velho	A

1.2.2 Pure and reference compounds

Commercial authentic samples of α-pinene (Fluka, 99.0 % purity), camphor (Extrasynthese), carvacrol (Fluka, pure), 1,8-cineole (Merck, 99.5 % purity), *cis-β*-ocimene (SAFC, ≥ 90 % purity), fenchone (Fucka, pure) and linalool (Aldrich, 99.0 % purity) were used. Fluconazole was kindly provided by Pfizer as the pure powder and amphotericin B was from Sigma (80.0 % purity).

1.2.3 Fungal strains

The antifungal activity of the essential oils was evaluated against yeasts and filamentous fungi: four clinical *Candida* strains isolated from recurrent cases of vulvovaginal and oral candidosis (*C. albicans* D5, *C. albicans* M1, *C. krusei* H9 and *C. guillermondii* MAT23); three *Candida* type strains from the American Type Culture Collection (*C. albicans* ATCC 10231, *C. tropicalis* ATCC 13803 and *C. parapsilopsis* ATCC 90018); one *Cryptococcus neoformans* type strain from the Colección Espanõla de Cultivos Tipo (*C. neoformans* CECT 1078); one *Aspergillus* clinical strain isolated from bronchial secretions (*A. flavus* F44) and two *Aspergillus* type strains from the American Type Culture Collection (*A. niger* ATCC 16404 and *A. fumigatus* ATCC 46645); three dermatophyte clinical strains isolated from nails and skin (*Epidermophyton floccosum* FF9, *Trichophyton mentagrophytes* FF7, and *Microsporum canis* FF1), and four dermatophyte type strains from the Colección Espanõla de Cultivos Tipo (*T. mentagrophytes* var. *interdigitale* CECT 2958, *T. rubrum* CECT 2794, *T. verrucosum* CECT 2992, and *M. gypseum* CECT 2908). All strains were stored in Sabouraud dextrose broth with 20 % glycerol at -80 ºC and subcultured in Sabouraud dextrose agar (SDA) or potato dextrose agar (PDA) before each test, to ensure optimal growth conditions and purity.

1.2.4 Antifungal activity

Broth macrodilution methods based on the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) reference protocols M27-A3 (CLSI, 2008a), M27-S3 (CLSI 2008b) and M38-A2 (CLSI, 2008c), for yeasts and filamentous fungi, respectively, were used to determine MICs and MLCs of the essential oils and their major constituents. Briefly, inoculum suspensions were prepared at appropriate

densities in RPMI 1640 broth (with L-glutamine, without bicarbonate, and the pH indicator phenol red) from SDA or PDA cultures and distributed into 12 × 75 mm glass test tubes. Inoculum densities were confirmed by viability counts on SDA. Serial two-fold dilutions of each oil were prepared in DMSO and added to the cell suspensions in order to obtain test concentrations ranging from 0.02 to 20.0 µL/mL (final DMSO concentrations never exceeded 2 % v/v). Oil-free growth controls, as well as sterility and DMSO control tubes, were also included. The test tubes were incubated aerobically at 35 ºC for 48 h/72 h (*Candida* spp. and *Aspergillus*/*Cryptococcus neoformans*) and at 30 ºC for 7 days (dermatophytes). MIC values were determined as the lowest concentration of the oil causing full growth inhibition. Quality control was performed by testing fluconazole and amphotericin B with the reference strains *C. parapsilopsis* ATCC 22019 and *C. krusei* ATCC 6258 and the results were within the predetermined limits. To measure MLCs, 20 µL samples were taken from each negative tube, plus the first tube showing growth (to serve as a growth control) after MIC reading to SDA plates and incubated at 35 ºC for 48 h/72 h (*Candida* spp. and *Aspergillus*/*Cryptococcus neoformans*) or at 30 ºC for 7 days (dermatophytes). MLC values were determined as the lowest concentration of the oil causing fungal death. All experiments were performed in triplicate and repeated whenever the results of each triplicate did not agree. A range of values is presented when different results were obtained.

1.2.5 Mechanism of action

1.2.5.1 Germ tube inhibition assay

Cell suspensions from overnight SDA cultures of *C. albicans* strains ATCC 10231, D5, and M1 were prepared in NYP medium [N-acetylglucosamine (Sigma; 10^{-3} mol/L), Yeast Nitrogen Base (Difco; 3.35 g/L), proline (Fluka; 10^{-3} mol/L), NaCl (4.5 g/L), and pH 6.7 ± 0.1] (Marichal et al., 1986) and adjusted to obtain a density of 1.0 ± 0.2 × 106 CFU/mL. The essential oil was diluted in DMSO and added in 10 µL volumes to 990 µL of the yeast suspensions (final DMSO concentration of 1% v/v), obtaining a series of subinhibitory concentrations (as low as 1/64 of the MIC). The samples were incubated for 3 h at 37 ºC without agitation and 100 cells from each sample were then counted in a haemocytometer. The percentage of germ tubes was determined as the

number of cells showing hyphae at least as long as the diameter of the blastospore. Cells showing a constriction at the point of connection of the hyphae to the mother cell, typical for pseudohyphae, were excluded. The results are presented as averages ± standard deviations (SD) of three separate experiments.

1.2.5.2 Flow cytometry

Yeast suspensions were prepared in phosphate buffered saline (PBS) solution with 2 % D-glucose (w/v) from overnight SDA cultures of *C. albicans* ATCC 10231 at 35 ºC and adjusted, using a haemocytometer, to a final density of $2.0 \pm 0.2 \times 10^6$ CFU mL/L. Serial two-fold dilutions of the essential oil (final concentrations of 0.64 -10.0 µL/mL) and a single solution of amphotericin B (2 µg/mL) in PBS with 2 % D-glucose (w/v) were added to the cell suspensions and the mixtures were incubated at 35 ºC in humid atmosphere without agitation for 30 min or 4 h. D rug-free control tubes were included in each experiment. After this period the cells were washed in PBS and ressuspended in 500 µL of PBS with 2 % D-glucose (w/v) for FUN-1 (Invitrogen, USA) staining and PBS for PI (propidium iodide; Sigma) staining. Five µl of the FUN-1 and PI solutions in DMSO and PBS, respectively, were added to the cell suspensions in order to obtain final concentrations of 0.5 µM of FUN-1 and 1.0 µg/mL of PI. FUN-1-stained cells were incubated for further 20 min at 35 ºC, away from incident light, while PI-stained samples were read after about 10 min at room temperature. Unstained cell suspensions were included as auto-fluorescence controls. The dye FUN-1 freely permeates fungal plasma membranes into the cell and is distributed in the cytoplasm as a bright diffuse green/yellow stain. It is then either metabolized into orange/red cylindrical intravacuolar structures in metabolically active cells or remains in the cytoplasm in a diffuse pattern in cells with impaired metabolism (Millard et al., 1997). On the other hand, the nucleic acid binding fluorescent probe PI, only penetrates dead cells showing severe membrane lesions (Pina-Vaz et al., 2001). Flow cytometry was performed using a FACSCalibur (Becton Dickinson Biosciences, San Jose, CA) flow cytometer with a 488 nm blue argon laser emitting at 15 mW and the results were analyzed using CellQuest Pro Software (Becton Dickinson). Intrinsic parameters (forward and side scatter, for relative cell size and complexity analysis) and fluorescence in the FL2 channel (log yellow/orange fluorescence, bandpass filter 585/42

nm) for FUN-1, and the FL3 channel (log red fluorescence, longpass filter >650 nm) for PI were acquired and recorded for a minimum of 7,500 events/sample using logarithmic scales. Markers (M1) were adjusted to include a maximum of 5 % of the cells in monoparametric histograms of control samples fluorescence intensity and kept unchanged through the analysis of the remaining samples to quantify the percentages of cells showing altered fluorescence in comparison to the drug-free controls. Results are presented as averages ± SD of at least three replicate experiments.

1.3. Results and discussion

1.3.1 *Lavandula luisieri*

1.3.1.1 Antifungal activity

The antifungal activity (MIC and MLC values) of both essential oils (samples D and L) against all the tested strains is summarized in table 2. For dermatophytes, MIC values ranged from 0.16 - 0.32 µL/mL for sample D and 0.32 - 0.64 µL/mL for sample L whereas for *Aspergillus* strains, differences among samples were more significant, with MIC values ranging from 0.32 - 2.5 µL/mL and 1.25 - 10 µL/mL, for sample D and L, respectively. For *Candida* spp. and *Cryptococcus neoformans*, the oils showed very similar activity with MIC values ranging from 0.64 - 2.5 µL/mL, for both samples. Furthermore, MIC and MLC values were very similar for samples D and L, except for *Aspergillus* strains, suggesting a fungicidal activity of the oils.

Table 2. Antifungal activity (MIC and MLC) of the essential oils of *Lavandula luisieri* for *Candida*, *Cryptococcus neoformans*, dermatophyte and *Aspergillus* strains.

Strains	*L. luisieri* D		*L. luisieri* L		Fluconazole		Amphotericin	
	MIC [a]	MLC [a]	MIC [a]	MLC [a]	MIC [b]	MLC [b]	MIC [b]	MLC [b]
Candida albicans ATCC 10231	1.25-2.5	2.5	1.25-2.5	1.25-2.5	1	>128	N.T	N.T
Candida albicans D5	0.64	0.64	0.64	0.64	64	>128	N.T	N.T
Candida albicans M1	1.25	1.25-2.5	1.25	2.5	2	128	N.T	N.T
Candida guillermondii MAT23	1.25	1.25-2.5	1.25	1.25	8	8	N.T	N.T
Candida krusei H9	2.5	2.5	2.5	2.5	64	64-128	N.T	N.T
Candida parapsilopsis ATCC 90018	2.5	2.5-5	2.5	2.5	<1	<1	N.T	N.T
Candida tropicalis ATCC 13803	2.5	2.5-5	2.5	2.5	4	>128	N.T	N.T
Cryptococcus neoformans CECT 1078	0.64	0.64	0.64	0.64-1.25	16	128	N.T	N.T
Epidermophyton floccosum FF9	0.16	0.16	0.32	0.32-0.64	16	16	N.T	N.T
Microsporum canis FF1	0.16-0.32	0.16-0.32	0.64	0.64	128	128	N.T	N.T
Microsporum gypseum CECT 2908	0.32	0.32-0.64	0.64	0.64-1.25	128	>128	N.T	N.T
Trichophyton mentagrophytes FF7	0.32	0.32-0.64	0.64	0.64	16-32	32-64	N.T	N.T
Trichophyton mentagrophytes var. *interdigitale* CECT 2958	0.16-0.32	0.64	0.32-0.64	0.64-1.25	128	≥128	N.T	N.T
Trichophyton rubrum CECT 2794	0.16	0.32	0.32	0.32-0.64	16	64	N.T	N.T
Trichophyton verrucosum CECT 2992	0.32	0.32-0.64	0.32	0.32-0.64	>128	>128	N.T	N.T
Aspergillus flavus F44	1.25-2.5	>20	5-10	≥20	N.T	N.T	2	8
Aspergillus fumigatus ATCC 46645	0.64	10-20	1.25	10	N.T	N.T	2	4
Aspergillus niger ATCC16404	0.32	20	1.25	20	N.T	N.T	1-2	4

[a] MIC and MLC were determined by a macrodilution method and expressed in µL/mL (v/v).
[b] MIC and MLC were determined by a macrodilution method and expressed in µg/mL (w/v).
N.T- not tested.
Results were obtained from three independent experiments performed in duplicate.

1.3.1.2 Mechanism of action

The essential oils were found to inhibit almost completely germ tube formation at concentrations of 0.08 µL/mL (1/16 of the MIC value of 1.25 µL/mL) for *C. albicans* ATCC 10231 and *C. albicans* M1 and 0.04 µL/mL (1/16 of the MIC value of 0.64 µL/mL) for *C. albicans* D5 (Table 3). In general, for concentrations as low as MIC/64 (0.01-0.02 µL/mL) for sample D and MIC/32 (0.02-0.04 µL/mL) for sample L, more than 50 % of germ tube formation was inhibited.

Table 3. Influence of sub-inhibitory concentrations of the essential oils of *Lavandula luisieri* (D and L) on germ tube formation of three *Candida albicans* strains. Results are presented as means ± SD of a minimum of three independent experiments.

		C. albicans ATCC 10231	*C. albicans* D5	*C. albicans* M1
Control [a]		92.0 ± 1.7	75.4 ± 6.7	90.5 ± 1.3
***L. luisieri* D**	MIC/256 (Conc. [b])	78.0 ± 13.0 (0.005)	69.5 ± 24.7 (0.0025)	65.3 ± 14.4 (0.005)
	MIC/128 (Conc.)	74.8 ± 8.5 (0.01)	56.3 ± 22.4 (0.005)	49.0 ± 5.6 (0.01)
	MIC/64 (Conc.)	40.3 ± 13.1 (0.02)	34.7 ± 17.2 (0.01)	37.5 ± 14.5 (0.02)
	MIC/32 (Conc.)	19.7 ± 3.5 (0.04)	19.5 ± 9.4 (0.02)	14.5 ± 6.1 (0.04)
	MIC/16 (Conc.)	**4.0 ± 4.8 (0.08)**	**2.5 ± 2.1 (0.04)**	**2.3 ± 1.7 (0.08)**
	MIC/8 (Conc.)	0.0 ± 0.0 (0.16)	0.0 ± 0.0 (0.08)	0.0 ± 0.0 (0.16)
***L. luisieri* L**	MIC/128 (Conc.)	91.0 ± 7.0 (0.01)	69.0 ± 7.8 (0.005)	78.3 ± 9.3 (0.01)
	MIC/64 (Conc.)	85.0 ± 7.8 (0.02)	63.8 ± 7.7 (0.01)	60.0 ± 7.0 (0.02)
	MIC/32 (Conc.)	66.0 ± 11.5 (0.04)	35.0 ± 8.4 (0.02)	22.7 ± 11.5 (0.04)
	MIC/16 (Conc.)	**9.0 ± 9.1 (0.08)**	**15.2 ± 5.0 (0.04)**	**4.6 ± 4.3 (0.08)**
	MIC/8 (Conc.)	0.2 ± 0.4 (0.16)	1.8 ± 2.5 (0.08)	0.2 ± 0.4 (0.16)

[a] Untreated samples including 1 % (v/v) DMSO.
[b] Absolute concentration in µL/mL.

During the last years, several studies have shown interesting properties of *L. luisieri* essential oils foreseeing several further applications. The biological activities so far reported for these oils include antifeedant effects (González-Coloma et al., 2006; González-Coloma et al., 2011), nematicidal activity (Barbosa et al. 2010), antioxidant

ability (Matos et al. 2009) and antimicrobial activity (Baldovini et al., 2005; Roller et al., 2009; Feijão et al., 2011; Lai et al., 2012; Teixeira et al., 2012). Concerning the antifungal activity of *L. luisieri* oils, the information available is scarce, with only one study reporting interesting antifungal activity against *C. albicans* (Baldovini et al., 2005).

In the present study, several strains of yeast and filamentous fungi, involved in different human and animal infections and also in food contamination, were included. The essential oil with the highest amount of necrodane compounds (D) showed to be the most active against dematophyte and *Aspergillus* strains. Also noteworthy is the inhibition of filamentation in *C. albicans*. Dimorphic transition (yeast to filamentous form) in *C. albicans* has been shown to be essential for virulence (Mitchell, 1998) and its inhibition was described to be sufficient to treat disseminated candidosis (Saville et al., 2006). *L. luisieri* essential oils showed a strong capacity to inhibit this process at concentrations sixteen times lower than MIC which can be very useful for the treatment of candidosis. Therefore, *L. lusieri* essential oils are promising natural products to be used as antifungal agents against foodborne strains (*Aspergillus* spp.) and fungi that are pathogenic to humans and animals (*Candida* spp., *Cryptococcus neoformans* and dermatophytes). Further experiments with necrodane derivatives are being considered to clarify the contribution of these compounds to the antifungal activity of *L. lusieri* oil.

1.3.2 *Lavandula multifida*

1.3.2.1 Antifungal activity

The antifungal activity of the essential oil of *L. multifida* and its major constituents (carvacrol and *cis*-*β*-ocimene) is shown in Table 4. The oil was effective against all the tested strains, showing the highest activity against dermatophyte strains and *Cryptococcus neoformans*, with MIC and MLC values of 0.16 µL/mL and 0.32 µL/mL, respectively. For *Candida* and *Aspergillus* strains MIC values ranged from 0.32 to 0.64 µL/mL and MLC values ranged from 0.32 to 1.25 µL/mL. Carvacrol and *cis*-*β*-ocimene were assayed individually for their antifungal activity. Carvacrol proved to be a very active compound, particularly against dermatophyte strains, with MIC values ranging from 0.04 to 0.16 µL/mL and MLC values ranging from 0.08 to 0.16 µL/mL (Table 4).

Furthermore, *cis*-*β*-ocimene showed MIC and MLC values ranging from 0.16 to 10 µL/mL (Table 4).

Table 4. Antifungal activity (MIC and MLC) of the essential oil of *Lavandula multifida* and its main compounds (carvacrol and *cis*-*β*-ocimene) against *Candida*, *Cryptococcus neoformans*, dermatophyte and *Aspergillus* strains.

	L. multifida		Carvacrol		*cis*-*β*-Ocimene		Fluconazole		Amphotericin B	
Strains	MIC [a]	MLC [a]	MIC [a]	MLC [a]	MIC [a]	MLC [a]	MIC [b]	MLC [b]	MIC [b]	MLC [b]
Candida albicans ATCC 10231	0.32	0.64	0.16	0.16-0.32	0.32-0.64	0.32-0.64	1	>128	N.T	N.T
Candida albicans D5	0.32	0.64	0.16	0.16-0.32	0.32	0.64	64	>128	N.T	N.T
Candida albicans M1	0.32	0.64	0.16	0.32	1.25	1.25-2.5	2	128	N.T	N.T
Candida tropicalis ATCC 13803	0.32	0.64	0.16	0.16-0.32	1.25	1.25	4	>128	N.T	N.T
Candida guillermondii MAT23	0.32	0.32	0.08-0.16	0.16	0.32-0.64	0.32-0.64	8	8	N.T	N.T
Candida krusei H9	0.64	0.64	0.16	016-0.32	0.32-0.64	0.32-0.64	64	64-128	N.T	N.T
Candida parapsilopsis ATCC 90018	0.32	0.64	0.16	0.16-0.32	0.64	0.64	<1	<1	N.T	N.T
Cryptococcus neoformans CECT 1078	0.16	0.32	0.16	0.16	0.16-0.32	0.16-0.32	16	128	N.T	N.T
Epidermophyton floccosum FF9	0.16	0.32	0.08	0.08	0.64	0.64	16	16	N.T	N.T
Microsporum canis FF1	0.16	0.32	0.04	0.08	0.32-0.64	0.32-0.64	128	128	N.T	N.T
Microsporum gypseum CECT 2908	0.16	0.32	0.04	0.08-0.16	0.64	0.64	128	>128	N.T	N.T
Trichophyton mentagrophytes FF7	0.16	0.32	0.04	0.08	0.64	0.64	16-32	32-64	N.T	N.T
Trichophyton mentagrophytes var. *interdigitale* CECT 2958	0.16	0.32	0.08	0.16	0.64	0.64-1.25	128	≥128	N.T	N.T
Trichophyton rubrum CECT 2794	0.16	0.32	0.08	0.08	0.32-0.64	0.32-0.64	16	64	N.T	N.T
Trichophyton verrucosum CECT 2992	0.16	0.32	0.16	0.16	0.64-1.25	1.25	>128	>128	N.T	N.T
Aspergillus flavus F44	0.64	1.25	0.32	0.32	10	10	N.T	N.T	2	8
Aspergillus fumigatus ATCC 46645	0.32	0.64	0.16	0.32	5	5	N.T	N.T	2	4
Aspergillus niger ATCC16404	0.32	1.25	0.16	0.16-0.32	10	10	N.T	N.T	1-2	4

[a] MIC and MLC were determined by a macrodilution method and expressed in µL/mL (v/v).
[b] MIC and MLC were determined by a macrodilution method and expressed in µg/mL (w/v).
N.T- not tested.
Results were obtained from three independent experiments performed in duplicate.

1.3.2.2 Mechanism of action

The essential oil was also found to inhibit almost completely filamentation in the tested *C. albicans* strains at concentrations of 0.08 µL/mL (1/4 of the MIC value of 0.32 µL/mL). Curiously, *cis-β*-ocimene showed a potent effect on inhibition of filamentation, with full inhibition obtained at a concentration of 0.02 µL/mL, corresponding to 1/16 of the MIC (0.32 µL/mL) for *C. albicans* strains ATCC 10231 and D5 and 1/64 of the MIC (1.25 µL/mL) for *C. albicans* M1 (Table 5). Flow cytometry analysis showed a dose-dependent inhibition of cell metabolism after a 30 min. incubation with the oil, with a concentration of 2.5 µL/mL resulting in metabolic arrest in over 80 % of the cells (Fig. 5).

Table 5. Influence of sub-inhibitory concentrations of the essential oil of *L. multifida* and its main compound *cis-β*-ocimene on germ tube formation of three *C. albicans* strains.

		C. albicans ATCC 10231	*C. albicans* D5	*C. albicans* M1
Control [a]		90.9 ± 5.4	87.8 ± 6.0	86.0 ± 6.4
L. multifida	MIC/16 (Conc.[b])	66.3 ± 6.5 (0.02)	39.3 ± 12.6 (0.02)	45.5 ± 12.7 (0.02)
	MIC/8 (Conc.)	33.7 ± 11.7 (0.04)	11.0 ± 6.2 (0.04)	28.8 ± 5.3 (0.04)
	MIC/4 (Conc.)	**5.8 ± 4.4 (0.08)**	**0.9 ± 1.1 (0.08)**	**1.2 ± 1.1 (0.08)**
***cis-β*-Ocimene** [c]	MIC/64 (Conc.)	74.0 ± 15.6 (0.005)	81.5 ± 4.9 (0.005)	46.0 ± 12.7 (0.005)
	MIC/32 (Conc.)	26.0 ± 8.5 (0.01)	35.5 ± 6.4 (0.01)	20.0 ± 14.1 (0.01)
	MIC/16 (Conc.)	**1.5 ± 0.7 (0.02)**	**0.0 ± 0.0 (0.02)**	**0.5 ± 0.7 (0.02)**

[a] Untreated samples including 1 % DMSO.
[b] Absolute concentration in µL/mL.
[c] Test concentrations correspond to MIC/64 (0.02 µL/mL), MIC/128 (0.01 µL/mL), and MIC/256 (0.005 µL/mL) for *C. albicans* M1. Results are means ± S.D. of a minimum of three independent experiments.

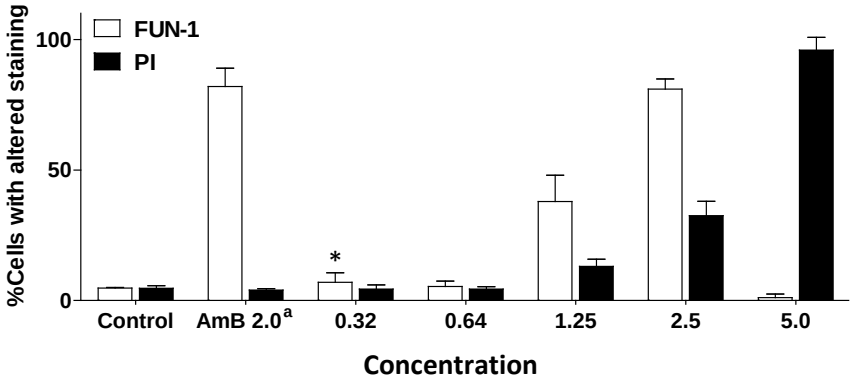

Figure 5. Percentages (and SD) of M1-gated *C. albicans* ATCC 10231 cells, analysed by flow cytometry after treatments with different concentrations of the essential oil of *Lavandula multifida* in comparison to amphotericin B (AmB) and an untreated control. Cells were treated with the compounds for 30 min for staining with FUN-1 and 3 h for staining with PI. * MIC of the essential oil.

L. multifida is widely used in folk medicine in decoctions against rheumatism, colds and also as a d igestive (El-Hilaly et al., 2003; Upson and Andrews, 2004). However, regarding scientific validations, only few studies have been performed, namely on the antioxidant (Messaoud et al., 2012) and antimicrobial (Benbelaid et al., 2012) activities of the essential oils and anti-inflammatory (Sosa et al., 2005) properties of ethanolic and aqueous extracts.

Considering the lack of information on the antifungal activity of *L. multifida*, the present study evaluated the effect of the essential oil on several pathogenic strains and elucidated its mechanism of action on *C. albicans.* The oil was very effective against all the tested strains. Dermatophytes and *Cryptococcus neoformans* were the most sensitive. For *Candida* spp. and *Aspergillus* spp., MIC and MLC values were quite interesting since these strains are not so susceptible to essential oils.

Taking into account the antifungal activity obtained for the main compounds of the oil (carvacrol and *cis*-*β*-ocimene), the results seem to indicate that the antifungal activity of the oil is mainly due to the presence of carvacrol. In fact, several authors have reported the importance of phenols for the biological properties of the oils (eg. Dorman and Deans, 2000; Nostro et al., 2004; Tulio et al., 2007). As referred in section 3 of chapter II, the amounts of carvacrol in this lavender are quite interesting, since

phenolic compounds are mainly related to *Thymus* (Gonçalves et al., 2010) and *Origanum* (Salgueiro et al., 2003) species.

The results also showed that *L. multifida* oil completely inhibited germ tube formation in *C. albicans* at concentrations four times below its MIC. This activity may be explained by its main constituents. In fact, a similar result was obtained with *cis*-*β*-ocimene in absolute concentrations, while representing proportions as low as 1/16 of the MIC (Table 5). Additionally, an important inhibition of filamentation for the oils main constituent, carvacrol, at a concentration of 0.08 µL/mL has also been reported (Vale-Silva et al., 2010). This result is very interesting, since filamentation has long been shown to be essential for virulence in *C. albicans* (Mitchell, 1998) and, in fact, its inhibition alone appears to be sufficient to treat disseminated candidosis (Saville et al., 2006). Regarding the mechanism of action of the oil, the flow cytometry results suggest that the essential oil of *L. multifida* initially causes metabolic arrest and only later leads to cell death. This might exclude direct damage to cell membranes as the mechanism of action, with cell death resulting rather from secondary alterations of a different primary target. This possibility is rather speculative at this point and additional studies need to be carried out to test its validity, namely in terms of electron microscopy analysis and physiological studies.

1.3.3 *Lavandula pedunculata*

1.3.3.1 Antifungal activity

MIC and MLC values of the essential oils (samples B3, C6 and D7) showed various degrees of inhibition against all the fungi tested (Table 6). Dermatophyte strains showed a higher sensibility to the oils in comparison to yeasts and other filamentous fungi. Sample C6 (high contents of camphor, see table 5, section 3, chapter II) proved to be the most active against dermatophyte strains and *Cryptococcus neoformans*, with MIC and MLC values ranging from 0.32 to 0.64 µL/mL. For *Candida* and *Aspergillus* strains, the essential oils showed low activity with MIC and MLC values ranging from 1.25 to >20 µL/mL (Table 6). Even though the MIC and MLC results varied among the tested strains, in most cases the MIC was equivalent to the MLC indicating a fungicidal effect of *L. pedunculata* oils.

The major constituents of the oils (1,8-cineole, fenchone, and camphor) were also assayed individually. Fenchone was the most active constituent, particularly against dermatophyte strains (Table 6). Since the essential oils are complex mixtures of different compounds, it is difficult to attribute their biological activity to a single or a particular constituent. Usually, major compounds are those responsible for the antifungal activity of the essential oils. However, there are studies showing that the whole essential oil has a higher antifungal activity than the combination of the major isolated compounds. Such studies indicate that minor components are critical to the biological activity of the oils (Koroch et al., 2007). In the present study, the higher activity of the oils of *L. pedunculata*, compared to that of the major compounds assayed individually (1,8-cineole, fenchone, and camphor), is presumably due to a synergistic effect among the different compounds present in the oils.

Table 6. Antifungal activity (MIC and MLC) of the essential oil of *Lavandula pedunculata* and its main compounds (1,8-cineole, fenchone and camphor) against *Candida*, *Cryptococcus neoformans*, dermatophyte and *Aspergillus* strains.

	L. pedunculata B3		*L. pedunculata* C6		*L. pedunculata* D7		1,8-Cineole		Fenchone		Camphor		Fluconazole		Anfotericina B	
Strains	MIC[a]	MLC[a]	MIC[a]	MLC[a]	MIC[a]	MLC[a]	MIC[a]	MLC[a]	MIC[a]	MLC[a]	MIC[a]	MLC[a]	MIC[b]	MLC[b]	MIC[b]	MLC[b]
Candida albicans ATCC 10231	2.5	2.5	2.5	2.5	2.5	2.5-5	10	10	5	5	>20	>20	1	>128	N.T	N.T
Candida guillermondii MAT23	1.25	1.25-2.5	1.25	1.25	1.25	1.25	10	10	2.5	2.5	>20	>20	8	8	N.T	N.T
Candida krusei H9	2.5	2.5	1.25	2.5	2.5	2.5-5	10	10	2.5	2.5	>20	>20	64	64-128	N.T	N.T
Candida parapsilosis ATCC 90018	2.5	2.5	2.5	2.5-5	2.5-5	5	10	10	5	5-10	>20	>20	<1	<1	N.T	N.T
Candida tropicalis ATCC 13803	1.25-2.5	1.25-2.5	2.5	2.5	2.5	2.5	20	20	5	5	>20	>20	4	>128	N.T	N.T
Cryptococcus neoformans CECT 1078	1.25	1.25	0.32-0.64	0.32-0.64	1.25	1.25-2.5	5-10	10	2.5	2.5	>20	>20	16	128	N.T	N.T
Epidermophyton floccosum FF9	0.64	0.64	0.32	0.32	0.64	0.64	5	5.0	1.25-2.5	1.25-2.5	>20	>20	16	16	N.T	N.T
Microsporum canis FF1	0.64	0.64-1.25	0.32	0.32-0.64	1.25	1.25	5.0	5.0	2.5-5	2.5-5	>20	>20	128	128	N.T	N.T
Microsporum gypseum CECT 2905	1.25-2.5	0.64	0.64	0.64	1.25	1.25	5-10	5.0	2.5	2.5-5	>20	>20	128	>128	N.T	N.T
Trichophyton mentagrophytes FF7	0.64	0.64	0.64	0.64	1.25	1.25	5.0	5.0	2.5-5	2.5-5	>20	>20	16-32	32-64	N.T	N.T
Trichophyton rubrum CECT 2794	1.25	1.25	0.32	0.32	0.64	0.64	2.5-5.0	5.0	1.25-2.5	1.25-2.5	>20	>20	16	64	N.T	N.T
Aspergillus flavus F44	10	10	5	5-10	10	10-20	>20	>20	20	20	>20	>20	N.T	N.T	2	8
Aspergillus fumigatus ATCC 46645	2.5	10	5	5	5	10	20	20	10	10-20	>20	>20	N.T	N.T	2	4
Aspergillus niger ATCC16404	5.0	20	5	5	5	>20	>20	>20	10	>20	>20	>20	N.T	N.T	1-2	4

[a] MIC and MLC were determined by a macrodilution method and expressed in µL/mL (v/v).

[b] MIC and MLC were determined by a macrodilution method and expressed in µg/mL (w/v).

N.T- not tested.

Results were obtained from three independent experiments performed in duplicate.

Lavandula pedunculata is very popular in Portuguese traditional medicine and for religious ceremonies as well as for melliferous and ornamental purposes. However, studies on its biological activities are very scarce. As far as it is known, only two studies have been performed, one on the acetylcholinesterase inhibition and antioxidant activity of different extracts (Ferreira et al., 2006) and, more recently, a second study evaluating the antibacterial activity of several extracts (Teixeira et al., 2012).

In the present study, the antifungal activity was assessed for the first time in these species. The results showed that the essential oils have an important antifungal activity, particularly the chemotype with high contents of camphor and 1,8-cineole. Also, the activity of the oils seems to be related with a synergistic effect among the compounds, since the activity of the main compounds tested alone was lower than that reported for the essential oil. Therefore, the oil may be useful in the clinical treatment of fungal diseases, particularly dermatophytosis, even though clinical trials are required to evaluate the practical relevance of our *in vitro* research.

In order to guarantee essential oil homogeneity, *in vitro* cultures of the most interesting chemotypes, such as the camphor/1.8-cineole type of *L. pedunculata*, must be considered. In a previous study, a reliable protocol for the rapid and efficient propagation of *L. pedunculata*, through *in vitro* axillary shoot proliferation, has already been developed (Zuzarte et al., 2010). *In vitro* culture techniques applied to *Lavandula* spp. are detailed in chapter IV.

1.3.4 *Lavandula viridis*

1.3.4.1 Antifungal activity

The highest antifungal activity of the oil was observed against dermatophyte strains and *Cryptococcus neoformans,* with MIC and MLC values ranging from 0.32 to 0.64 μL/mL. For *Candida* strains MIC and MLC values ranged from 0.64 to 2.5 μL/mL. The oil was less effective against *Aspergillus* strains (Table 7). For most of the dermatophytes, *C. neoformans* and *Candida* strains, the MIC was equivalent to the MLC, indicating a clear fungicidal effect of *L. viridis* essential oil (Table 7).

The major constituents of the oil (1,8-cineole, camphor, α-pinene and linalool) were also assayed individually for their antifungal activity. 1,8-Cineole and camphor

displayed the lowest antifungal activity against all strains but α-pinene proved to be a very active compound, particularly against dermatophyte strains (Table 7). Therefore, the activity of *L. viridis* essential oil is mainly due to the contribution of α-pinene.

Table 7. Antifungal activity (MIC and MLC) of the essential oil of *Lavandula viridis* ant its main compounds (1,8-cineole, camphor, *α*-pinene, linalool) against *Candida*, dermatophyte and *Aspergillus* strains.

	L. viridis		1,8-Cineol		Camphor		*α*-Pinene		Linalool		Fluconazole		Amphotericin B	
Strains	MIC[a]	MLC[a]	MIC[a]	MLC[a]	MIC[a]	MLC[a]	MIC[a]	MLC[a]	MIC[a]	MLC[a]	MIC[b]	MLC[b]	MIC[b]	MLC[b]
Candida albicans ATCC 10231	2.5	2.5	10	10	>20	>20	0.64-1.25	0.64-1.25	5	5	1	>128	N.T	N.T
Candida albicans D5	1.25	1.25	5-10	5-10	≥20	>20	0.32	0.32	5	5	64	>128	N.T	N.T
Candida albicans M1	2.5	2.5	10	10	>20	>20	0.64-1.25	0.64-1.25	5	5-10	2	128	N.T	N.T
Candida guillermondii MAT23	0.64-1.25	0.64-1.25	10	10	>20	>20	0.64	0.64	5	10	8	8	N.T	N.T
Candida krusei H9	1.25-2.5	1.25-2.5	10	10	>20	>20	0.16-0.32	0.32	10	10	64	64-128	N.T	N.T
Candida parapsilopsis ATCC 90018	1.25	1.25	10	10	>20	>20	0.32	0.32	10	10	<1	<1	N.T	N.T
Candida tropicalis ATCC 13803	1.25-2.5	1.25-2.5	20	20	>20	>20	1.25	1.25-2.5	5	5	4	>128	N.T	N.T
Cryptococcus neoformans CECT 1078	0.64	0.64	5-10	10	>20	>20	0.08	0.32	5	5	16	128	N.T	N.T
Epidermophyton floccosum FF9	0.32	0.32	5	5	>20	>20	0.16	0.16	1.25-2.5	2.5	16	16	N.T	N.T
Microsporum canis FF1	0.32	0.32	5	5	>20	>20	0.16	0.16-0.32	2.5	2.5	128	128	N.T	N.T
Microsporum gypseum CECT 2905	0.64	0.64	5-10	5	>20	>20	0.16	0.16	1.25-2.5	2.5	128	>128	N.T	N.T
Trichophyton mentagrophytes FF7	0.32-0.64	0.64	5	5	>20	>20	0.32	0.32-0.64	1.25	2.5	16-32	32-64	N.T	N.T
Trichophyton mentagrophytes var. *interdigitale* CECT 2958	0.32-0.64	0.64	10-20	10-≥20	>20	>20	0.32	0.32	2.5	2.5-5	128	≥128	N.T	N.T
Trichophyton rubrum CECT 2794	0.32	0.32	2.5-5	5	>20	>20	0.08	0.08	1.25	1.25-2.5	16	64	N.T	N.T
Trichophyton verrucosum CECT 2992	0.32	0.32-0.64	10	10	>20	>20	1.25	1.25	1.25-2.5	1.25-2.5	>128	>128	N.T	N.T
Aspergillus flavus F44	5	10-20	20	20	20	20	1.25	1.25	10	≥20	N.T	N.T	2	8
Aspergillus fumigatus ATCC 46645	2.5	5-10	10	10-20	20	>20	1.25	1.25-2.5	2.5	20	N.T	N.T	2	4
Aspergillus niger ATCC 16404	2.5	20	10	>20	>20	>20	2.5	5	5	≥20	N.T	N.T	1-2	4

[a] MIC and MLC were determined by a macrodilution method and expressed in µL/mL (v/v).
[b] MIC and MLC were determined by a macrodilution method and expressed in µg/mL (w/v).
N.T- not tested.
Results were obtained from three independent experiments performed in duplicate.

1.3.4.2 Germ tube inhibition

The essential oil of *L. viridis* was also found to inhibit filamentation in the tested *C. albicans* strains at concentrations of 0.08 - 0.16 µL/mL, well below the corresponding MICs (Table 8). This marked difference between MICs and filamentation-inhibiting concentrations seems to suggest that different mechanisms may be responsible for these two biological activities. This finding is particularly relevant since inhibition of the dimorphic transition alone has been suggested to be sufficient to treat disseminated candidiasis, thus proving to be a good target mechanism in the development of novel antifungal agents (Saville et al., 2006).

1.3.4.3 Mechanism of action

Flow cytometry analyses after FUN-1 staining have revealed a dose-dependent inhibition of cell metabolism after short incubation periods with the oil at concentrations starting from the respective MIC (Figs. 6 and 7). This change was detected by a reduction of orange fluorescence (FL2 channel) in cells exposed to the essential oil in comparison to untreated controls (Fig. 6 and 7). To observe PI staining of the test cells, on the other hand, 4 h incubation with a concentration of the oil at least two log2 dilutions above the MIC was required (Figs. 6 and 7). The nucleic acid binding fluorescent probe PI penetrates only dead cells showing severe membrane lesions (Pina-Vaz et al., 2001). The observed asymmetry between metabolic inhibition and cell death shows that cells become metabolically inactive in the presence of the essential oil of *L. viridis* clearly before it leads to cell death, thus appearing to exclude a potential mechanism of antifungal action relying on primary leakage of cytoplasmic contents due to direct damage to cell membranes.

Table 8. Percentage of cells showing germ tubes after treatment of three *C. albicans* strains with subinhibitory concentrations of the essential oil of *L. viridis* for three hours in a filamentation-inducing medium at 37 ºC.

	MIC/16 (Conc.[a])	MIC/32 (Conc.)	MIC/64 (Conc.)	Control [b]
C. albicans ATCC 10231	6.7 ± 6.4 (0.16)	54.0 ± 5.6 (0.08)	89.7 ± 2.1 (0.04)	94.0 ± 5.2
C. albicans D5	0.0 ± 0.0 (0.08)	47.5 ± 10.6 (0.04)	66.0 ± 5.7 (0.02)	86.8 ± 9.4
C. albicans M1	0.3 ± 0.6 (0.16)	42.7 ± 10.5 (0.08)	75.0 ± 4.2 (0.04)	87.5 ± 6.6

[a] Concentration in µL/mL (v/v).
[b] Untreated samples including the solvent (1 % DMSO) only.
Results are presented as mean (± SD) values of three independent experiments.

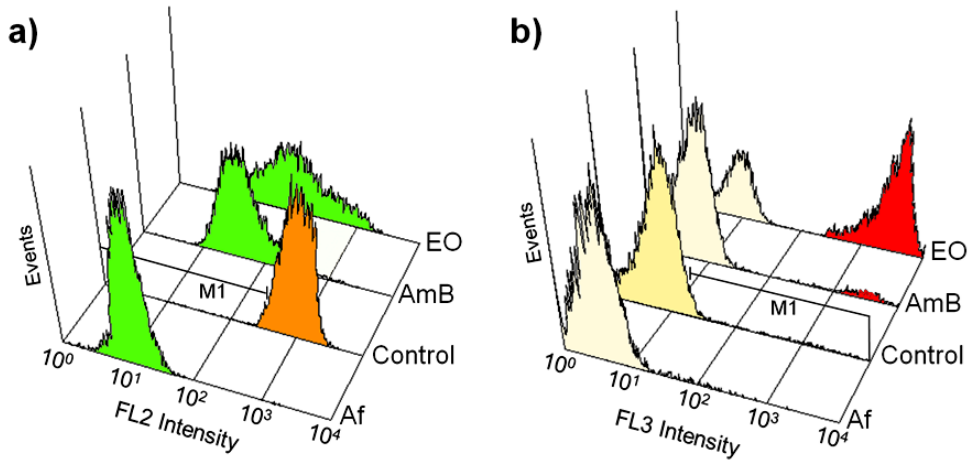

Figure 6. Flow cytometry histograms showing fluorescence intensity *vs* number of events (*C. albicans* ATCC 10231 cells) in relative units. a) Orange fluorescence (FL2 channel) intensity of samples stained with FUN-1. b) Red fluorescence (FL3 channel) intensity of samples stained with PI. Af: autofluorescence of unstained cells; Control: untreated cells; AmB: cells treated with amphotericin B at 2.0 µg/mL; EO: cells treated with the essential oil of *L. viridis* at 10.0 µL/mL.

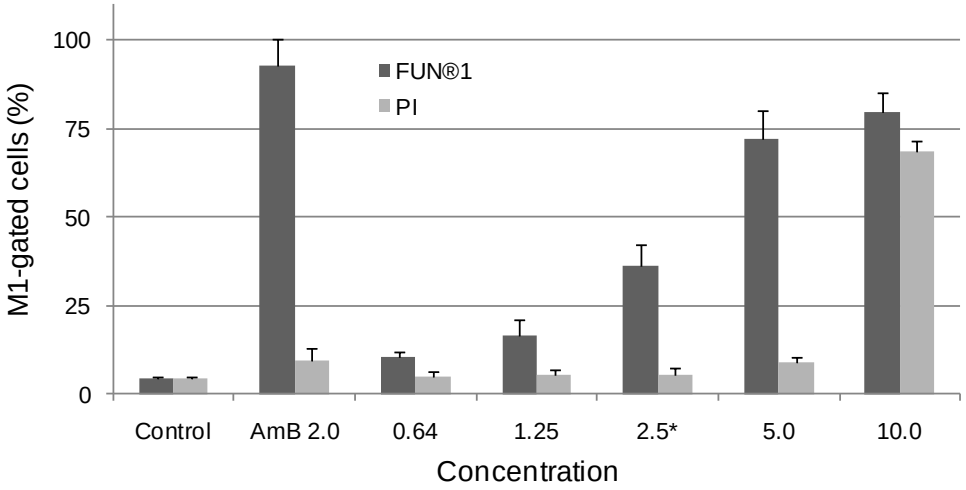

Figure 7. Percentage (and SD) of M1-gated *C. albicans* ATCC 10231 cells, analyzed by flow cytometry, after treatments with different concentrations of the essential oil of *L. viridis* in comparison to amphotericin B (AmB) and an untreated control. Cells were treated with the compounds for 30 min for staining with FUN-1 and 4 hours for staining with PI. * MIC of the essential oil.

The mechanism of action of essential oils remains somewhat controversial. While some studies suggest that the compounds may penetrate the microorganism and react with active sites of enzymes and/or interfere with the cellular metabolism, most evidence supports direct disruption of cellular membranes and concentration-dependent prooxidant cytotoxic effects (Bakkali et al., 2008). Concerning antifungal activity specifically, the mechanism of action of the oils seems to involve penetration through cell walls and direct damage to both cytoplasmic and mitochondrial membranes. This leads to changes in permeability leading to leakage and ultimately resulting in cell death (Bakkali et al., 2008). The present results, for the specific case of *C. albicans* treated with the essential oil of *L. viridis*, are consistent with a mechanism of action starting from damage to mitochondrial membranes, considering the rapid metabolical arrest appearing earlier and in the presence of lower concentrations of the essential oil than those required to cause cell death. Changes in mitochondrial permeability disturb electron flow in the electron transport chain, generating free radicals that proceed to damage essential biomolecules (including lipids, proteins and nucleic acids). Given a high enough concentration and/or exposure time, the oil eventually leads to disruption of cytoplasmic membranes and cell death.

The wide-spectrum antifungal activity and high potency of the oil of *L. viridis* support further validations for its clinical use in the management of superficial and/or mucosal fungal infections.

In conclusion, lavender essential oils can be an excellent option when searching for new antifungals. Being a blend of several compounds, they do not act on specific targets in the fungal cells, and therefore no resistance or adaptation to the oils has been reported (Carson et al., 2002). Moreover, it is likely that several compounds of the essential oils play important roles in cell penetration, lipophilic or hydrophilic attraction, fixation on cell walls and/or membranes, and cellular distribution (Bakkali, 2008). These aspects are particularly relevant since they affect the distribution of the oil in the cells and influence the different types of radical reactions that may occur (Bakkali, 2008).

In order to explore the potential application of lavender oils by the pharmaceutical, cosmetic and food industries, toxicity assays and *in vivo* studies should be considered to evaluate the practical relevance of these *in vitro* assays. The evaluation of the oils toxicity/safety was performed and is presented in section 3 of this chapter.

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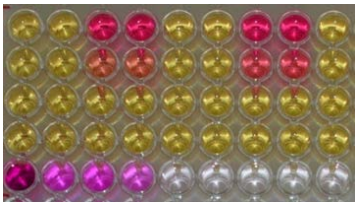


2. Anti-inflammatory activity

2. Anti-inflammatory activity

2.1 Introduction

Inflammation is an adaptive response to restore body homeostasis triggered by several stimuli, such as infections and tissue injury, as well as other adverse conditions responsible for tissue stress and malfunction, not related to host defense or tissue repair (Medzhitov, 2008). During the inflammatory response there is an increase in the permeability of endothelial lining cells, influxes of leukocytes into the interstitium, oxidative burst and release of cytokines, as well as the induction of several enzymes and the metabolization of arachidonic acid into eicosanoids, prostaglandins (PGs) and thromboxane A2. Moreover, the expression of cellular adhesion molecules, namely intercellular adhesion molecules and vascular cell adhesion molecules also takes place (Miguel, 2010).

Activated macrophages play an important role in immune response and are responsible for the production of several pro-inflammatory mediators, including pro-inflammatory cytokines, nitric oxide (NO), PGs and reactive oxygen species (ROS) (Mezhitov, 2008). NO synthase (NOS) is involved in the production of NO from L-arginine and molecular oxygen, whereas cyclooxygenase (COX) catalysis the formation of PGs via arachidonic acid. iNOS and COX-2 are the inducible forms of these enzymes, being activated by several inflammatory stimuli (cytokines, free radicals, infectious pathogens). iNOS and COX-2 pathways interact closely and NO can stimulate COX-2 activity by combining with its heme component (Dudhgaonkar et al., 2004). The high out-puts of NO also contribute to the pathogenesis of septic shock and inflammatory diseases (Zamora et al., 2000; Guzik et al., 2003) whereas PGs amplify the pain mechanism and enhance vascular permeability (González et al., 2003).

The expression of pro-inflammatory mediators is regulated by several transcription factors and signaling pathways. Nuclear transcription factor kappa B (NF-kB) regulates several genes involved in inflammation. NF-κB exists in a latent form in the cytoplasm of cells sequestered by the IκB inhibitor protein (seven members, including IκBα). Briefly, pro-inflammatory stimuli are responsible for the phosphorylation of IκBα allowing free NF-κB to translocate to the nucleus where it binds to κB-binding sites in the promoter regions of target genes. Then, transcription of

pro-inflammatory mediators, like iNOS, COX-2, tumour necrose factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-8 takes place (Scheidereit, 2006; Wong and Tergaonkar, 2009) (Fig. 1).

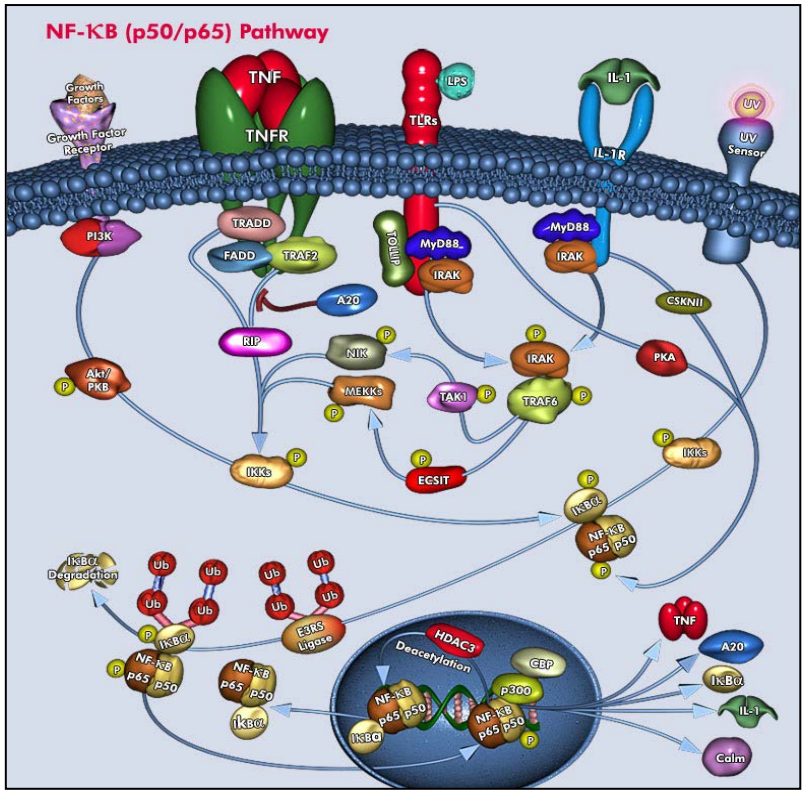


Figure 1. NF-κB pathway. Inflammatory stimuli (LPS, IL-1, TNF) induce phosphorylation of IKBα allowing the translocation of NF-κB to the nucleus and transcription of its target genes.

Mitogen-activated protein kinases (MAPKs) are also involved in the regulation of inflammatory and immune responses. The mammalian MAPK family consists of several isoforms of extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), also known as stress-activated protein kinase (or SAPK) and p38 MAPK (Kim and Choi, 2010). Each MAPK signaling pathway comprises at least three components: MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K), and MAPK. MAP3K phosphorylates MAP2K which in turn phosphorylates and activates MAPKs (Fig. 2). Besides inflammatory response regulation, activated MAPKs regulate several cellular activities, including cell proliferation, differentiation, migration and death (Kim and Choi, 2010).

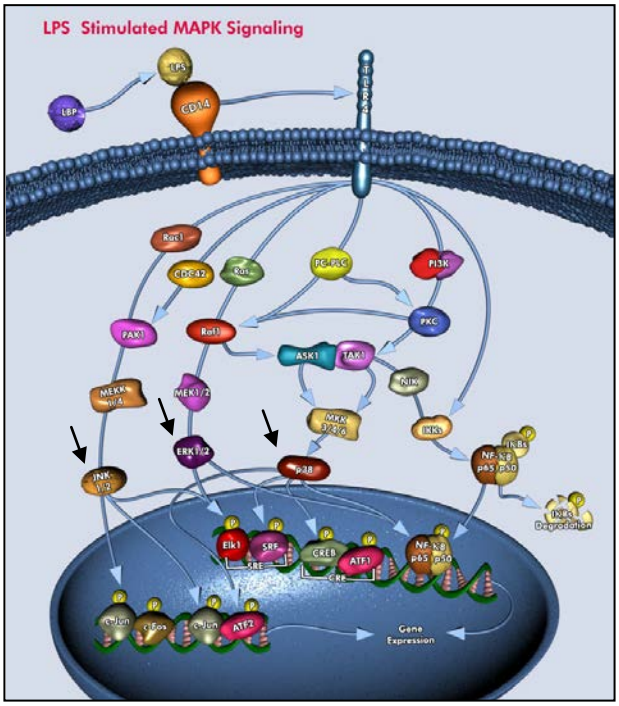


Figure 2. Mitogen-activated protein kinase (MAPK) signaling pathway.

Exacerbated inflammatory responses are involved in several diseases such as cancer (Porta et al., 2009; Khatami, 2012), obesity, diabesity (Schmidt and Duncan, 2003), rheumatoid arthritis, cardiovascular diseases, neurodegenerative diseases (Hunter and Doddi, 2010) and aging. Nowadays, these diseases are a major burden on humanity, despite recent success with biopharmaceuticals. Lack of responsiveness and drug resistance, delivery problems and the cost of biopharmaceuticals justify the search for new anti-inflammatory agents and the development of new approaches to control these diseases. The perfect drug would be able to relieve pain and inflammation and also slow down, stop or, even better, prevent disease progression.

Plants have long been used in traditional medicine to alleviate inflammatory diseases. Also, several lines of research have considered the anti-inflammatory activity of essential oils and/or their constituents showing their ability to scavenge free radicals, to modulate the arachidonic acid metabolism or the production of cytokines. Their roles on the modulation of pro-inflammatory gene expression have also been reported, as summarized in a review by Miguel (2010). The carrageenan-induced mouse paw oedema is frequently used as a model assay to determine the anti-inflammatory activity

of these compounds. However, these experiments provide very little information on the mechanism of action of the oils, a crucial step in the valuation of the potential prophylactic and therapeutic application of essential oils.

Regarding the anti-inflammatory potential of lavender oils only two studies have been reported: *Lavandula angustifolia* (Hajhashemi et al., 2003) and *L. stoechas* (Amira et al., 2012) were able to inhibit carrageneen-induced paw oedema and *L. officinalis* (= *L. angustifolia)* showed strong lipoxygenase inhibitory effect (Wei and Shibamoto, 2010). Additionally, ethanolic and aqueous extracts of *L. multifida* inhibited the Croton oil-induced ear edema in mice, after topical application (Sosa et al., 2005)

Taking into account the lack of information on the anti-inflammatory activity of the essential oils of *Lavandula* species growing spontaneously in Portugal, this work aimed to study the effect of the oils on different pro-inflammatory mediators and signal transduction pathways triggered in an *in vitro* model of inflammation (lipopolysaccharide (LPS)-stimulated macrophages), thus, avoiding animal sacrifices in early stages of drug discovery.

2.2 Material and methods

The anti-inflammatory assays were performed at the Laboratory of Cellular Immunobiology and Oncobiology for the Centre of Neuroscience and Cell Biology of the University of Coimbra.

2.2.1 Essential oils

The oils selected for the anti-inflammatory assays are listed in table 1. The reasons for this selection are referred in the previous section, being sample C6 replaced by sample A0, with higher concentrations of camphor, in order to evaluate the influence of this compound in the anti-inflammatory activity of the oil.

Table 1. Site of collection of *Lavandula* spp. used in the anti-inflammatory assays

Species	Region	Site of Collection	Sample
L. luisieri	Coimbra	Piódão	D
	Algarve	Cabo de São Vicente	L
L. pedunculata	Guarda	Celorico da Beira	A0
	Bragança	Serra da Nogueira	B3
	Coimbra	Foz de Arouce	D7
L. multifida	Setúbal	Sesimbra	AI
L. viridis	Algarve	Barranco do Velho	A

2.2.2 Materials

Lipopolysaccharide (LPS) from *Escherichia coli* (serotype 026:B6) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal calf serum and trypsin were purchased from Invitrogen (Paisley, UK). The protease and phosphatase inhibitor cocktails were obtained from Roche (Mannheim, Germany). The anti-actin antibody was purchased from Millipore (Bedford, MA). Antibodies against phospho-IκB-α, IκB-

α, phospho-p44/p42 MAPK (ERK1/ERK2), phospho-p38 MAPK and phospho-SAPK/JNK were from Cell Signaling Technologies (Danvers, MA, USA). The pan anti-JNK antibody was from R&D Systems (Mineapolis, MN, USA) and pan anti-ERK and p38 MAPK were from Cell Signaling Technologies (Danvers, MA, USA). The alkaline phosphatase-linked secondary antibodies and the enhanced chemifluorescence (ECF) reagent were obtained from GE Healthcare (Chalfont St. Giles, UK), and the polyvinylidene difluoride (PVDF) membranes were from Millipore Corporation (Bedford, MA). Trizol® reagent was purchased from Invitrogen (Barcelona, Spain). iScript kit and SYBR green were obtained from BioRad (Hercules, CA, USA). Primers were from MWG Biotech (Ebersberg, Germany). All other reagents were from Sigma Chemical Co. (St. Louis, Mo, USA) or from Merck (Darmstadt, Germany).

2.2.3 Cell culture

Raw 264.7, a mouse leukaemic macrophage cell line from ATCC (TIB-71), was kindly supplied by Dr. Otília Vieira (Centre for Neuroscience and Cell Biology of the University of Coimbra, Portugal) and cultured on endotoxin-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % (v/v) non inactivated fetal bovine serum, 3.02 g/L sodium bicarbonate, 100 µg/mL streptomycin and 100 U/mL penicillin at 37 ºC, in a humidified atmosphere of 95 % air and 5 % CO_2. The cells were used after reaching 80-90 % confluence. Viable cells were counted on a haemocytometer using trypan blue dye and the morphological appearance of the cells was microscopically monitored during the assays. After 45 passages the cells were discarded.

2.2.4 Cell viability

Assessment of cell respiration, an indicator of cell viability, was performed using a colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), as described by Mosmann (1983). The cells ($6x10^5$ cells/well) were cultured in a 48-well microplates and left to stabilize for 12 h. The cells were then incubated for 24 h with the culture medium alone (control) or with different concentrations of the essential oils. After the treatments, 43 µL of MTT solution (5 mg/mL in phosphate buffered saline) were added to each well and the microplates were

further incubated at 37 ºC for 15 min, in a humidified atmosphere with 5 % CO_2. Supernatants were centrifuged (1000 g during 5 min) to recover viable cells. To dissolve formazan crystals formed in adherent cells, 300 µL of acidified isopropanol (0.04 N HCl in isopropanol) were added to each cell and recovered to the respective microtube containing the pellet formed after centrifugation. Quantification of formazan was performed using an ELISA automatic microplate reader (SLT, Austria) at 570 nm, with a wavelength of 620 nm. All the experiments were performed in triplicate, being the results expressed as mean ± SEM of the indicated number of experiments.

2.2.5 Nitric oxide scavenging activity

The NO scavenging potential of the essential oils was evaluated using a NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP). For these experiments 300 µL of culture medium alone (control) or with different concentrations of the essential oils and SNAP (300 µM) were incubated in 48-well microplates, for 3 h at 37 ºC. After the treatments, 170 µL of the supernatants were mixed with 170 µL of Griess reagent (1 % sulphanilamide and 0.1 % naphthylethylenediamine dihydrochloride in 2.5 % phosphoric acid) and incubated in the dark, for 30 min, at room temperature. The absorbance was then read at 570 nm in a microplate reader. The quantity of nitrites was determined based on a sodium nitrite standard curve. All experiments were performed in triplicate.

2.2.6 Nitric oxide production

The accumulation of nitrites, stable metabolites of NO, was measured in the culture medium by a colorimetric procedure based on Griess reagent. The cells ($3x10^5$cells/well) were cultured in 48-well microplates and left to stabilize for 12 h. The cells were then incubated for 24 h in the culture medium alone (control) or with different concentrations of the essential oils and stimulated with LPS (1 µg/mL). After cells treatments, 170 µL of the supernatants were mixed with 170 µL of Griess reagent and incubated in the dark for 30 min at room temperature. The absorbance was then read at 570 nm in a microplate reader. The quantity of nitrites was determined based on a sodium nitrite standard curve. All experiments were performed in triplicate.

2.2.7 Cell lysate preparation and Western blot analysis

Raw 264.7 cells were plated and allowed to stabilize for 12 h. Then, cells were either maintained in culture medium (control) or pre-incubated with the essential oil for 1 h followed by LPS incubation during the time indicated in the results section. Also incubation of the oil with LPS at the same time was assessed. Total cellular lysates were obtained using lysis buffer (RIPA: 50 mM Tris-HCL, pH 8.0, 1 % Nonidet P-40, 150 mM NaCl, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulphate and 2 mM ethylenediaminetetraacetic acid) freshly supplemented with 1 mM dithiothreitol, protease and phosphatase inhibitor cocktails. The cell lysates were then sonicated (4x, 40 μm peak to peak) in Vibra Cell sonicator (Sonica and Material INC) and centrifuged for 10 min at 4 ºC to remove nuclei and cell debris. Supernatants (total cell lysates) were used to determine protein concentration by the bicinchoninic acid protein assay and cell lysates were denaturated in sample buffer (0.125 mM Tris pH 6.8, 2 % (w/v) sodium dodecyl sulphate, 100 mM dithiothreitol, 10 % glycerol and bromophenol blue). Western blot analysis was performed to evaluate protein levels of iNOS and COX-2, and the activation of MAPKs (ERK1/2, JNK1/2 and p38) and NF-κB signalling pathways. Briefly equivalent amounts of proteins were separated on a 10 % (v/v) sodium dodecylsulphate-polycrylamide gel at 140 v, for 1 h, and transferred to PDVF membranes. Nonspecific IgGs were blocked with 5 % (w/v) milk in Tris-buffered saline (TBS, 50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 2.5 mM KCL) and incubated for 1 h at room temperature. Then, membranes were incubated with specific antibodies against iNOS (1:1000 dilution), COX-2 (1:10,000), phospho-p38 (1:1000), phospho-JNK1/2 (1:1000), phospho-ERK1/2 (1:1000), total IκBα (1:1000), phospho-IκB (1:1000) and actin (1:20000). The membranes were then washed for 25 min with TBS-T, and incubated, for 1 h, at room temperature, with alkaline phosphatase-conjugated secondary antibodies. The immune complexes were detected by membrane exposure to the ECF reagent, during 5 min, followed by scanning for blue excited fluorescence on the Storm 860 (GE Healthcare). The generated signals were analyzed using the ImageQuant TL software. To test whether similar amounts of protein for each sample were loaded, the membranes were stripped and reprobed with antibodies to total ERK1/2, SAPK/JNK, p38 MAPK or with an anti-actin antibody, and blots were

developed with alkaline phosphatase-conjugated secondary antibodies and visualized by enhanced chemifluorescence.

2.2.8 RNA extraction

Cells were plated at $4x10^4$ cells/well in 6-well microplates in a final volume of 2 mL and treated with 1 µg/mL LPS during 6 h. Total RNA was isolated from cells with the Trizol® reagent according to the manufacturer's instructions. Briefly, cells were washed with ice-cold PBS harvested and homogenized in 1 mL of Trizol by pipetting vigorously. After addition of 200 µL of chloroform the samples were vortexed, incubated for 2 min at room temperature and centrifuged at 12,000×g, for 15 min, at 4 ºC. The aqueous phase containing RNA was transferred to a new tube and RNA precipitated with 500 µ L of isopropanol for at least 10 min at room temperature. Following a 10 min centrifugation at 12,000g, the pellet was washed with 1 mL 75 % ethanol and resuspended in 100 µL 60 ºC heated RNase free water. The RNA concentration was determined by OD260 measurement using a Nanodrop spectrophotometer (Wilmington, DE, USA) and quality was inspected for absence of degradation or genomic DNA contamination, using the Experion RNA StdSens Chips in the Experion™ automated microfluidic electrophoresis system (BioRad Hercules, CA, USA). RNA was stored in RNA Storage Solution (Ambion, Foster City, CA, USA) at -80 ºC.

2.2.9 Real-time PCR

One microgram of total RNA was reverse transcribed using the iScript Select cDNA Synthesis Kit. Briefly, 2 µL of random primers and the necessary volume of RNase-free water to complete 15 µL, were added to each RNA sample. The samples were heated at 65 ºC, for 5 min, and snap-chilled on ice for 1 min. After this, 5 µL of a Master Mix containing 1 µL of iScript reverse transcriptase and 4 µL of 5x Reaction Buffer were added to each sample. A protocol for cDNA synthesis was run on all samples (5 min at 25 ºC, 30 min at 42 ºC, 5 min at 85 ºC and then put on hold at 4 ºC). After the cDNA synthesis, the samples were diluted with RNase-free water up to a volume of 100 µL. Real-time PCR was performed in a 20 µL volume containing 5 µL cDNA (50 ng), 10 µL 2x Syber Green Supermix, 2 µl of each primer (250 nM) and 1

µL H_2O PCR grade. Samples were denatured at 95 ºC during 3 min. Subsequently, 40 cycles were run for 10 sec at 95 ºC for denaturation, 30 sec at the appropriate annealing temperature and 30 sec at 72 ºC for elongation. Real-time PCR reactions were run in duplicate for each sample on a Bio-Rad My Cycler iQ5. Primers were designed using Beacon Designer® Software v7.2, from Primier Biosoft International and thoroughly tested. Primer sequences and amplification efficiencies are given in Table 2.

Table 2. Primer sequences for targeted cDNAs

Primer	5'-3' sequence (F: forward; R: reverse)	RefSeq ID
HPRT1	F: GTTGAAGATATAATTGACACTG R: GGCATATCCAACAACAAAC	**NM_013556**
IL-1β	F: ACCTGTCCTGTGTAATGAAAG R: GCTTGTGCTCTGCTTGTG	**NM_008361**
IL-6	F: TTCCATCCAGTTGCCTTC R: TTCTCATTTCCACGATTTCC	**NM_031168**
iNOS	F: GCTGTTAGAGACACTTCTGAG R: CACTTTGGTAGGATTTGACTTTG	**NM_000625.4**

On each real-time PCR plate there was a non-template control present for each pair of primers analyzed. For determination of primer-pair specific efficiencies, a 4 points dilution series of control sample for each pair of primers was run on each experiment (Rasmussen, 2001). Amplification reactions were monitored using a SYBR-Green assay. After amplification, a threshold was set for each gene and Ct-values were calculated for all samples. Gene expression changes were analyzed using the built-in iQ5 Optical system software v2. The software enables analyzing the results with the Pfaffl method (Pfaffl, 2001), a variation of ΔΔCT method corrected for gene-specific efficiencies, and to report gene expression changes as relative fold changes compared to control samples. The results were normalized using a reference gene, HPRT-1, determined with Genex® software (MultiD Analyses AB) as the most stable for the treatment conditions used.

2.2.10 Statistical analyses

Results are expressed as mean ± SEM of the indicated number of experiments. Statistical analysis comparing a treatment condition to control was performed using two-sided unpaired t-test. When comparing the effect of different treatments to LPS-stimulated cells, a multiple group comparison was performed and one-way ANOVA followed by Dunnett's multiple comparison test or Bonferroni's test with significance levels *p<0.05, **p <0.01, ***p<0.001. These tests were applied using GraphPadPrism version 5.02 (GraphPad Software, San Diego, CA, USA).

Since the real-time PCR results are presented as ratios of treated samples over untreated (control) or LPS treated cells, the distribution of the data does not follow a normal distribution. A two-base logarithmic transformation was therefore used to make observations symmetric and closer to a normal distribution. If x represent the fold change of a gene in one sample, then the two-base logarithmic transformation is: $\log2(x) = \ln(x1)/\ln(2)$. This way, fold changes of 2 and 0.5 correspond to mean log2 values of 1 and −1, respectively.

2.3. Results and discussion

NO is a pro-inflammatory mediator produced in high quantities during inflammatory related disorders. Incubation of macrophages with LPS, during 24 h, induces an increase in NO production that mimics an inflammatory condition in the body, and therefore may be used to screen *in vitro* anti-inflammatory potential of drugs.

In the present study the essential oils of *L. luisieri* (2 samples), *L. pedunculata* (3 samples), *L. multifida* and *L. viridis* were assessed for their capacity to inhibit NO production in LPS-stimulated macrophages. Moreover, the NO scavenging potential of the oils that showed inhibition of NO production by cells was also evaluated, in order to clarify whether the inhibition was due to a scavenging activity of the oil or to an inhibition of the inducible NOS expression. A summary of the results including the effect of the oils on macrophages viability is presented below for each sample analysed.

2.3.1 *Lavandula luisieri*

2.3.1.1 Inhibition of NO production

L. luisieri essential oil with low amounts of necrodane derivatives (sample L, Fig. 3) induced a significant inhibitory effect on the LPS-induced NO production in a dose-dependent manner (0.08-0.32 μL/mL; Fig. 3a), without affecting cell viability (Fig. 3b). On the other hand, the sample with high amounts of necrodane derivatives (sample D; Fig. 4) did not inhibit NO production (Fig. 4a) at concentrations without cytotoxicity to macrophages (Fig. 4b)

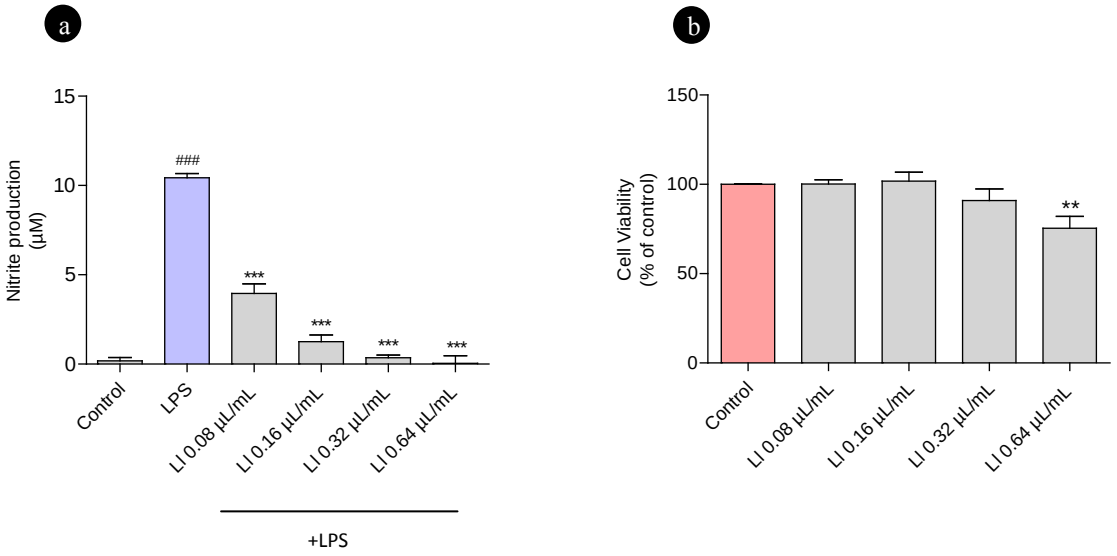


Figure 3. Effect of *Lavandula luisieiri* (Ll) essential oil with low amounts of necrodane derivatives on: a) NO production triggered by LPS in macrophages. The cells ($3x10^5$ cells/well) were maintained in culture medium (control), or incubated with 1 µg/mL LPS, or with LPS in the presence of different concentrations of the oil (0.08-0.64 µL/mL), for 24 h. Nitrite concentration was determined from a sodium nitrite standard curve and the results are expressed as concentration (µM) of nitrite in culture medium; b) Macrophages viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate (###p<0.001, compared to control; **p<0.01 and ***p<0.001, compared to LPS).

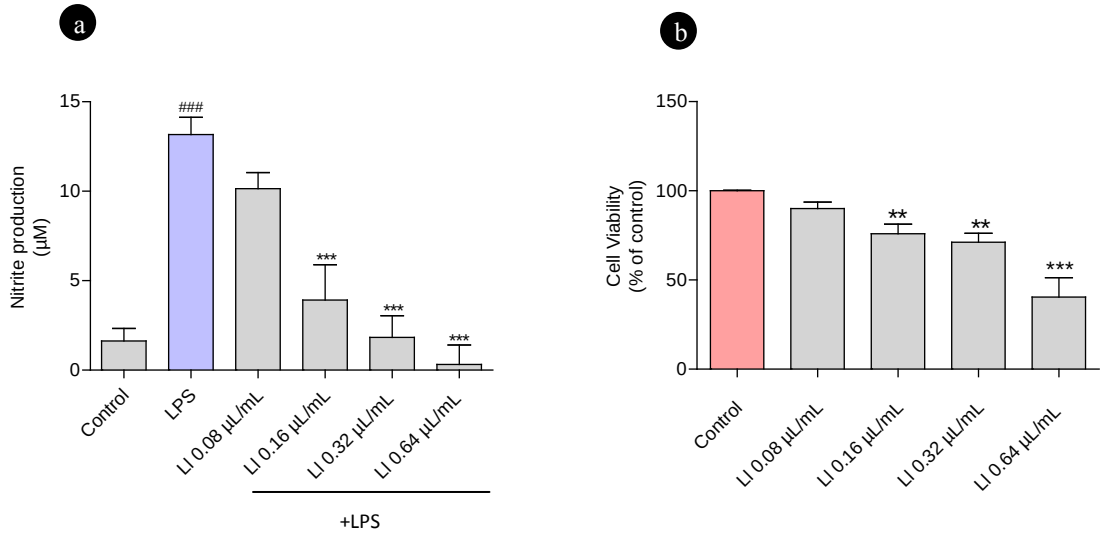


Figure 4. Effect of *Lavandula luisieiri* (Ll) essential oil with high amounts of necrodane derivatives on: a) NO production triggered by LPS in macrophages. The cells ($3x10^5$ cells/well) were maintained in culture medium (control), or incubated with 1 µg/mL LPS, or with LPS in the presence of different concentrations of the oil (0.08-0.64 µL/mL), for 24 h. Nitrite concentration was determined from a sodium nitrite standard curve and the results are expressed as concentration (µM) of nitrite in culture medium. b) Macrophages viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate (###p<0.001, compared to control; **p<0.01 and ***p<0.001, compared to LPS).

2.3.1.2 NO scavenging

Since sample L was able to inhibit NO production, the scavenging activity of the oil was also assessed (Fig. 5). In all the concentrations tested the values of nitrites in the culture medium remained very similar in comparison to the positive control (SNAP alone). Therefore, the capacity of the oil to inhibit NO production is not related to a scavenging ability of the oil and may be due to its effect directly on the iNOS pathway.

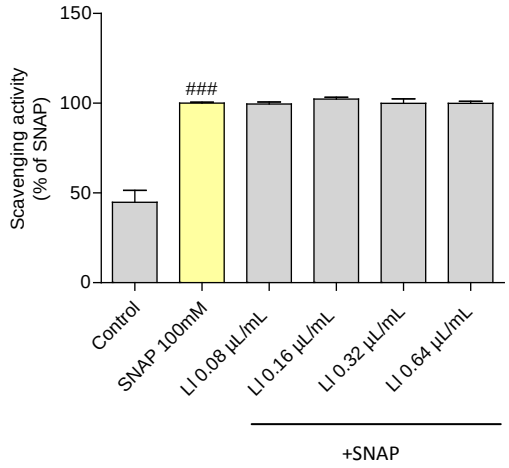


Figure 5. Effect of *Lavandula luisieiri* (Ll) essential oil with low amounts of necrodane derivatives on the nitric oxide scavenging activity. Different concentrations of essential oil (0.08-0.64 µL/mL) were incubated with 0.9 µL/mL of the NO donor, SNAP (100 mM), in culture medium for 3 h. Results are expressed as percentage of NO release triggered by SNAP. Each value represents the mean ± SEM from three experiments, performed in duplicate (### $p<0.001$, compared to control).

2.3.2 *Lavandula multifida*

2.3.2.1 Inhibition of NO production

L. multifida oil did not inhibit NO production (Fig. 6a) at concentrations without affecting macrophages viability (Fig. 6b). Therefore this species did not show anti-inflammatory potential and was not considered in further experiments.

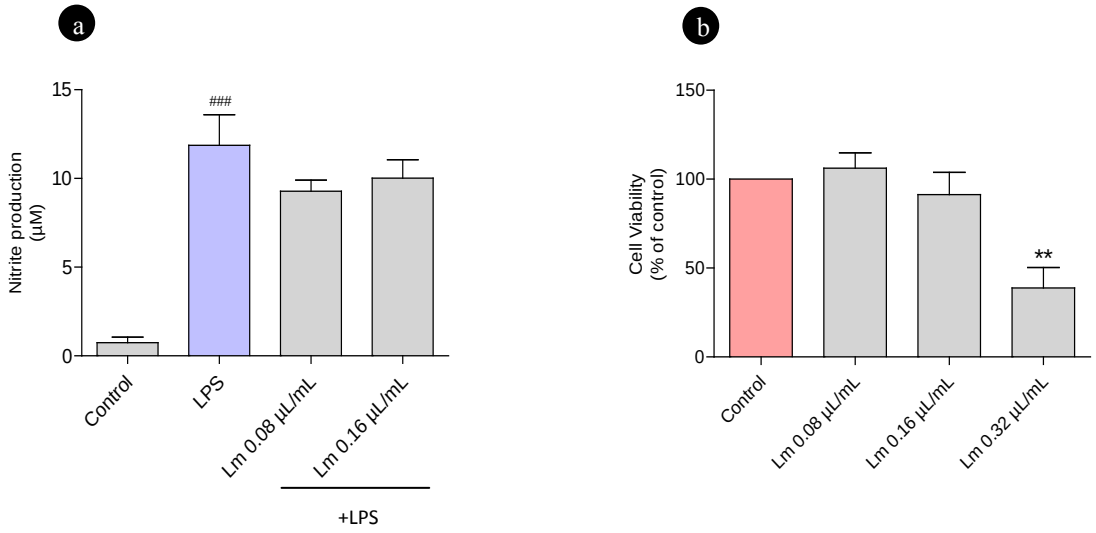


Figure 6. Effect of *Lavandula multifida* (Lm) essential oil on: a) NO production triggered by LPS in macrophages. The cells ($3x10^5$ cells/well) were maintained in culture medium (control), or incubated with 1 µg/mL LPS, or with LPS in the presence of different concentrations of the oil (0.08-0.16 µL/mL), for 24 h. Nitrite concentration was determined from a sodium nitrite standard curve and the results are expressed as concentration (µM) of nitrite in culture medium. b) Macrophages viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate (###$p<0.001$, compared to control; **$p<0.01$, compared to LPS).

2.3.3 *Lavandula pedunculata*

2.3.3.1 Inhibition of NO production

Three samples of *L. pedunculata* with distinct chemical compositions: sample A0 (high amounts of camphor; Fig. 7), sample B3 (high amounts of 1,8-cineole; Fig. 8) and sample D7 (high amounts of fenchone; Fig. 9) were assessed.

The samples with high amounts of camphor (A0; Fig 7a) and high amounts of 1,8-cineole (B3; Fig. 8a) were the most effective on NO inhibition, being the later slightly more active. The sample with high amounts of fenchone was also able to significantly inhibit NO production, but was less effective in comparison to samples A0 and B3 (Fig. 9a). For all the samples analysed, no cytotoxicity on macrophages was recorded from 0.08-0.64 µL/mL (Fig. 7b, 8b and 9b).

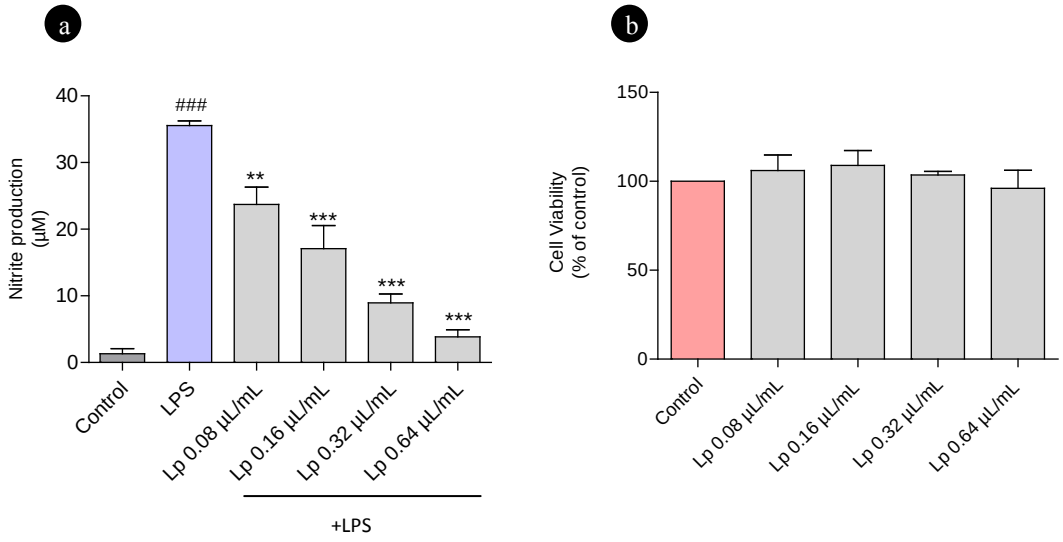


Figure 7. Effect of *Lavandula pedunculata* (Lp) essential oil with high amounts of camphor on: a) NO production triggered by LPS in macrophages. The cells ($3x10^5$ cells/well) were maintained in culture medium (control), or incubated with 1 µg/mL LPS, or with LPS in the presence of different concentrations of the oil (0.08-0.64 µL/mL), for 24 h. Nitrite concentration was determined from a sodium nitrite standard curve and the results are expressed as concentration (µM) of nitrite in culture medium. b) Macrophages viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate (###$p<0.001$, compared to control; **$p<0.01$ and ***$p<0.001$, compared to LPS).

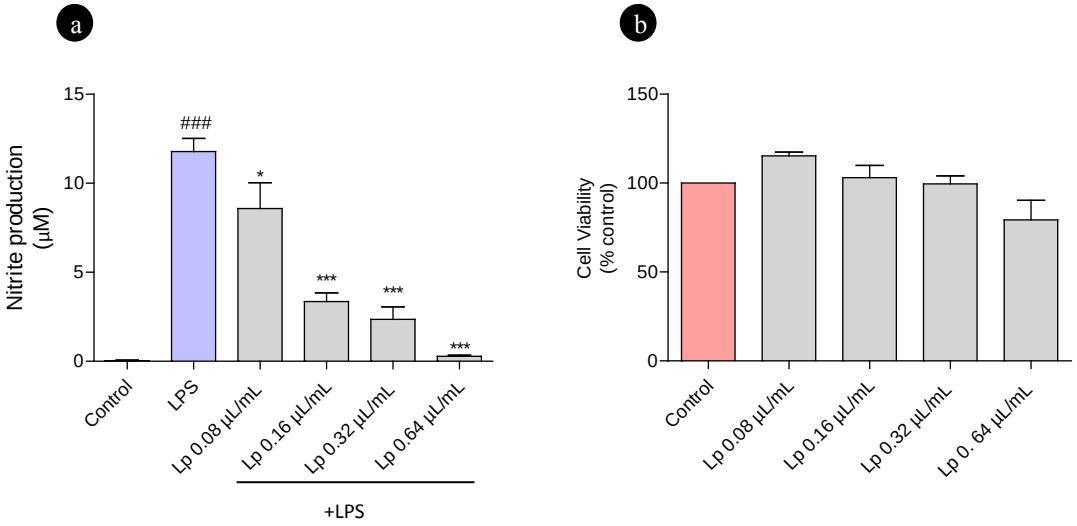


Figure 8. Effect of *Lavandula pedunculata* (Lp) essential oil with high amounts of 1,8-cineole on: a) NO production triggered by LPS in macrophages. The cells ($3x10^5$ cells/well) were maintained in culture medium (control), or incubated with 1µg/mL LPS, or with LPS in the presence of different concentrations of the oil (0.08-0.64 µL/mL), for 24 h. Nitrite concentration was determined from a sodium nitrite standard curve and the results are expressed as concentration (µM) of nitrite in culture medium. b) Macrophages viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate (###p<0.001, compared to control; *p<0.05 and ***p<0.001, compared to LPS).

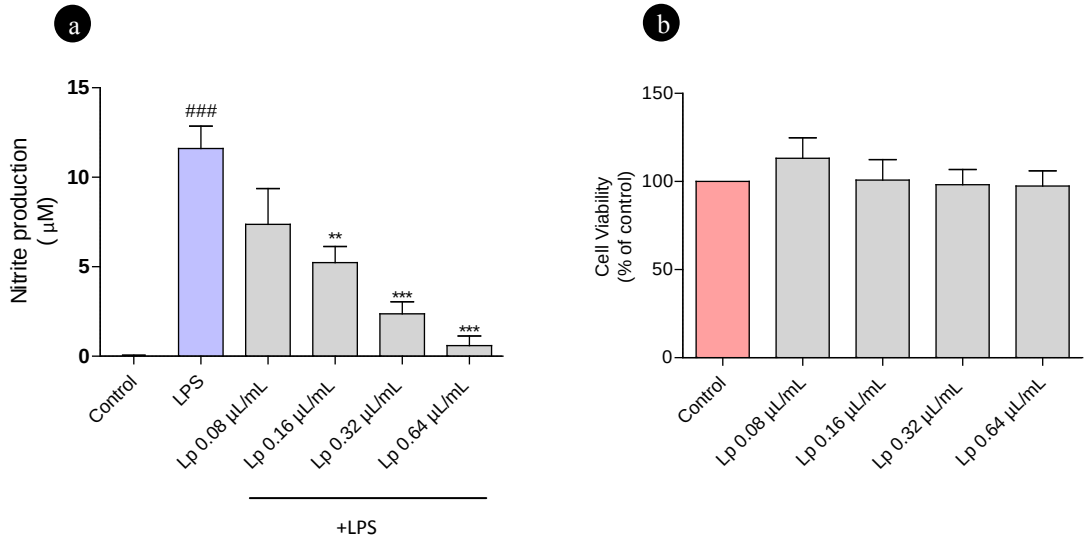
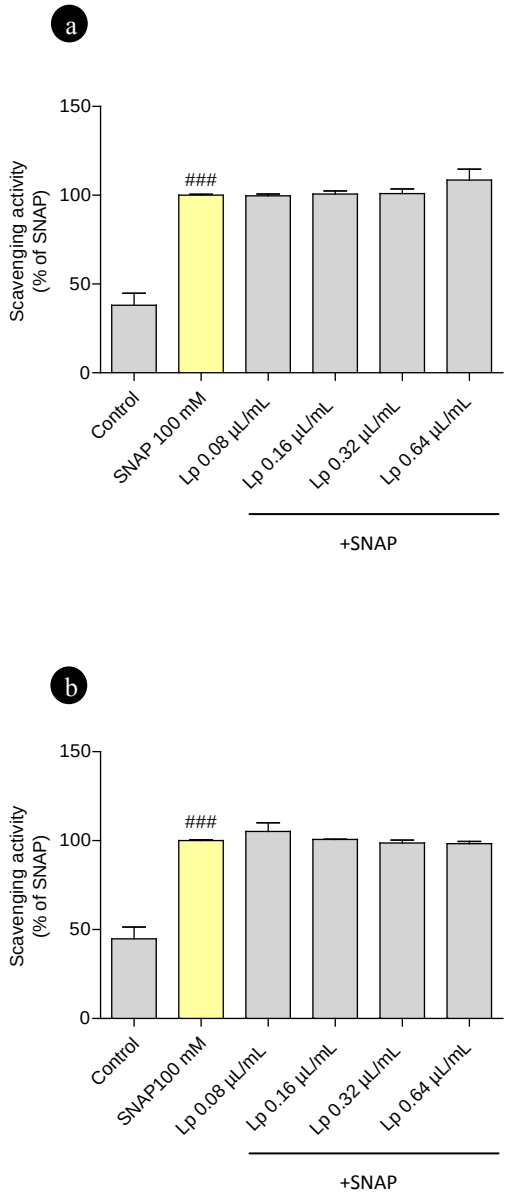


Figure 9. Effect of *Lavandula pedunculata* (Lp) essential oil with high amounts of fenchone on: a) NO production triggered by LPS in macrophages. The cells ($3x10^5$ cells/well) were maintained in culture medium (control), or incubated with 1µg/mL LPS, or with LPS in the presence of different concentrations of the oil (0.08-0.64 µL/mL), for 24 h. Nitrite concentration was determined from a sodium nitrite standard curve and the results are expressed as concentration (µM) of nitrite in culture medium. b) Macrophages viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate (###p<0.001, compared to control; **p<0.01 and ***p<0.001, compared to LPS).

2.3.3.2 NO scavenging

For all the oils of *L. pedunculata*, the results were very similar, with no NO scavenging ability found (Fig. 10a-10c). Therefore, the inhibition of NO is not related to a potential scavenging activity and may be related to the inhibition of iNOS expression.



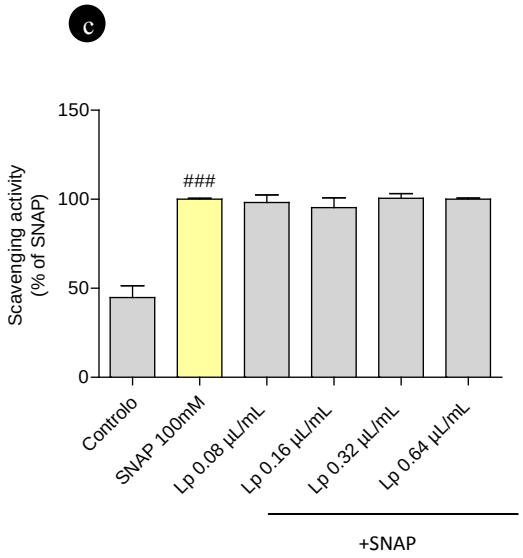


Figure 10. Effect of *Lavandula pedunculata* (Lp) essential oil with high amounts of camphor (a), 1,8-cineole (b) and fenchone (c) on the nitric oxide scavenging activity. Different concentrations of essential oil (0.08-0.64µL/mL) were incubated with 0.9 µL/mL of the NO donor, SNAP (100mM), in culture medium for 3 h. Results are expressed as percentage of NO release triggered by SNAP. Each value represents the mean ± SEM from three experiments, performed in duplicate (### $p<0.001$, compared to control).

2.3.4 *Lavandula viridis*

2.3.4.1 Inhibition of NO production

In LPS-stimulated macrophages pre-incubated with *L. viridis* essential oils the production of NO was significantly inhibited at all the tested concentrations (Fig. 11a) without affecting cell viability (Fig. 11b).

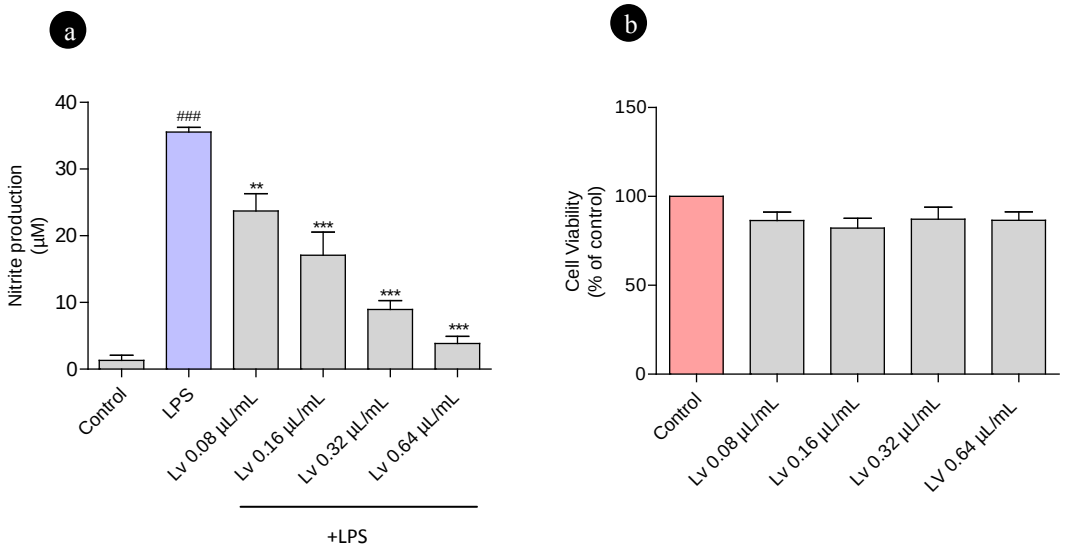


Figure 11. Effect of *Lavandula viridis* (Lv) essential oil on NO production triggered by LPS in macrophages. The cells ($3x10^5$ cells/well) were maintained in culture medium (control), or incubated with 1 µg/mL LPS, or with LPS in the presence of different concentrations of the oil (0.08-0.64 µL/mL), for 24 h. Nitrite concentration was determined from a sodium nitrite standard curve and the results are expressed as concentration (µM) of nitrite in culture medium. b) Macrophages viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three experiments, performed in duplicate (###$p<0.001$, compared to control; **$p<0.01$ and ***$p<0.001$, compared to LPS).

2.3.4.2 NO scavenging

As in all the lavenders reported previously, no N O scavenging activity was found for *L. viridis* (Fig. 12). Once again, the inhibition of NO production is not related to this ability, justifying further investigations in intracellular signalling pathways activated during inflammation and involved in iNOS expression.

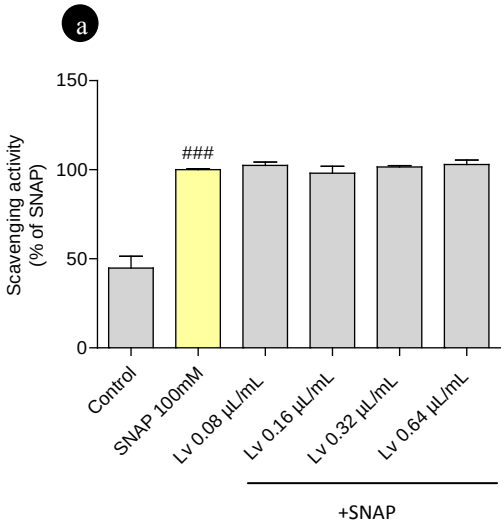


Figure 12. Effect of *Lavandula viridis* (Lv) essential oil on the nitric oxide scavenging activity. Different concentrations of the essential oil (0.08-0.64 µL/mL) were incubated with 0.9 µL/mL of the NO donor, SNAP (100 mM), in culture medium for 3 h. Results are expressed as percentage of NO release triggered by SNAP. Each value represents the mean ± SEM from three experiments, performed in duplicate (### $p<0.001$, compared to control).

Gathering the information of the screening assays, 4 samples, namely the oils from *L. luisieri* with low amounts of necrodanes (L), *L. pedunculata* rich in camphor (A0), *L. pedunculata* with high amounts of 1,8-cineole (B3) and *L. viridis* showed a significant inhibitory effect on NO production and therefore can be pointed out as potential anti-inflammatory agents, justifying further experiments.

In the present work a more detailed study was performed with *L. viridis* and included: the effect of the oil on the protein levels of iNOS and COX-2; activation of the major regulator of inflammation, NF-κB; quantification of mRNA levels of iNOS and the pro-inflammatory cytokines (IL-1β and IL-6). This species was selected due to the scarce information on its biological properties and its high essential oil yield (see section 3, chapter II), strongly relevant for a future commercial exploitation. Moreover, the main compound of the oil (1,8-cineole) was also tested to evaluate its effect on NO production.

For the remaining species (*L. luisieri* and *L. pedunculata*) detailed studies are currently being conducted and preliminary results concerning the effect of the oils on iNOS and COX-2 protein levels have shown very promising results, justifying further experiments. Both oils were able to inhibit iNOS protein levels by more than 50 %, although *L. pedunculata* was slightly more active than *L. luisieri* (data not shown).

2.3.4.3 iNOS and COX-2 protein levels inhibition

The effect of *L. viridis* oil on iNOS (Fig. 13) and COX-2 (Fig. 14) protein levels triggered by LPS was evaluated. In untreated cells (control) and in cells treated with essential oil, without LPS stimulation, no iNOS (Fig. 13) and COX-2 (Fig. 14) proteins were detected. However, after macrophages treatment with LPS, for 24 h , the expression of both enzymes was strongly increased (Fig. 13 and 14). Pre-treatment of cells with *L. viridis* oil significantly reduced the LPS induced iNOS expression in macrophages by 97.40 % (Fig. 13). Regarding COX-2 expression, an inhibition of 53.9 % was achieved (Fig. 14). These results are quite interesting since COX-2 protein levels inhibition by natural products is not frequent.

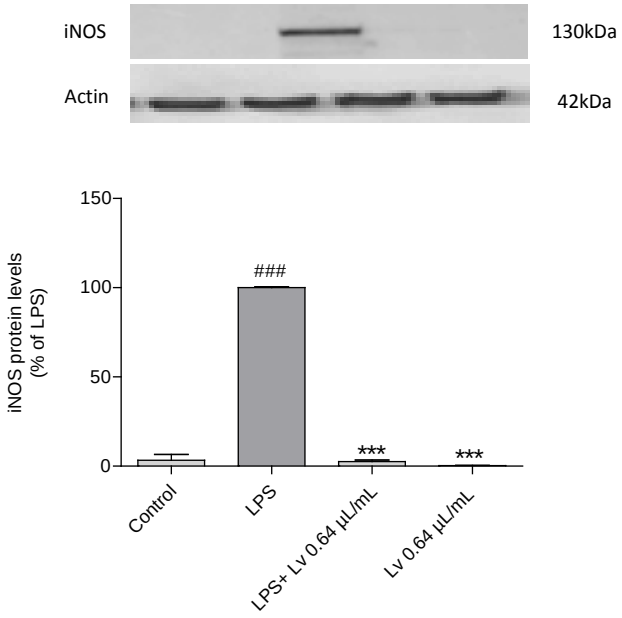


Figure 13. Inhibitory effect of *Lavandula viridis* (Lv) essential oil on LPS-induced iNOS protein expression in macrophages. Raw 264.7 cells ($1.2x10^6$ cells/well) were maintained in culture medium (control), or incubated with 1 µg/mL LPS, or incubated with essential oil (0.64 µL/mL) alone or simultaneously with 1 µg/mL LPS for 24 h. Results are expressed as percentage of iNOS protein levels relatively to LPS. Each value represents the mean ± SEM from at least 3 experiments (### $p<0.001$, compared to control; *** $p<0.001$, compared to LPS).

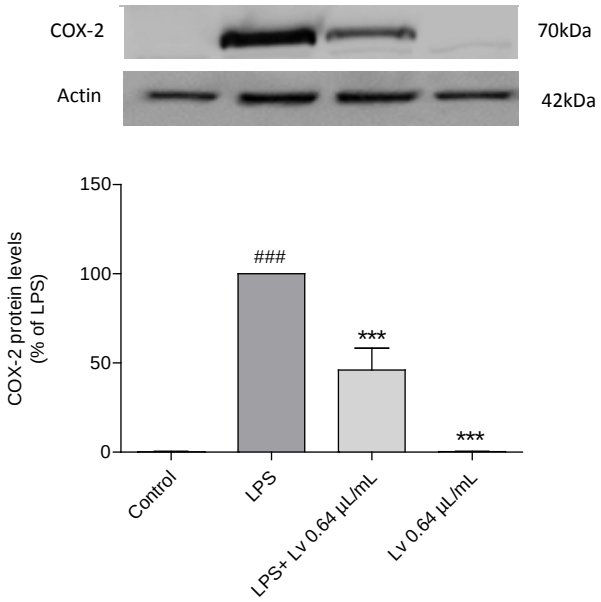


Figure 14. Inhibitory effect of *Lavandula viridis* (Lv) essential oil on LPS-induced COX-2 protein expression in macrophages. Raw 264.7 cells ($1.2x10^6$ cells/well) were maintained in culture medium (control), or incubated with 1 µg/mL LPS, or incubated with essential oil (0.64 µL/mL) alone or simultaneously with 1µg/mL LPS for 24 h. Results are expressed as percentage of iNOS protein levels relatively to LPS. Each value represents the mean ± SEM from at least 3 experiments (### $p<0.001$, compared to control; *** $p<0.001$, compared to LPS).

2.3.4.4 MAPKs activation

The effect of *L. viridis* oil on the activation of MAPKs was also assessed. Macrophages were incubated for 10, 15 and 30 min with LPS (Fig. 15a, 15b and 15c, respectively, and Fig. 15d). Pre-incubation with *L. viridis* essential oil for 1 h prior to LPS treatment inhibited ERK1/2 phosphorylation (phospho-ERK1/2), with a significant decrease after 10 min of LPS incubation (Fig. 15a). On the other hand, no effects were observed for phospho-JNK1/2 and phospho-p38 (data not shown).

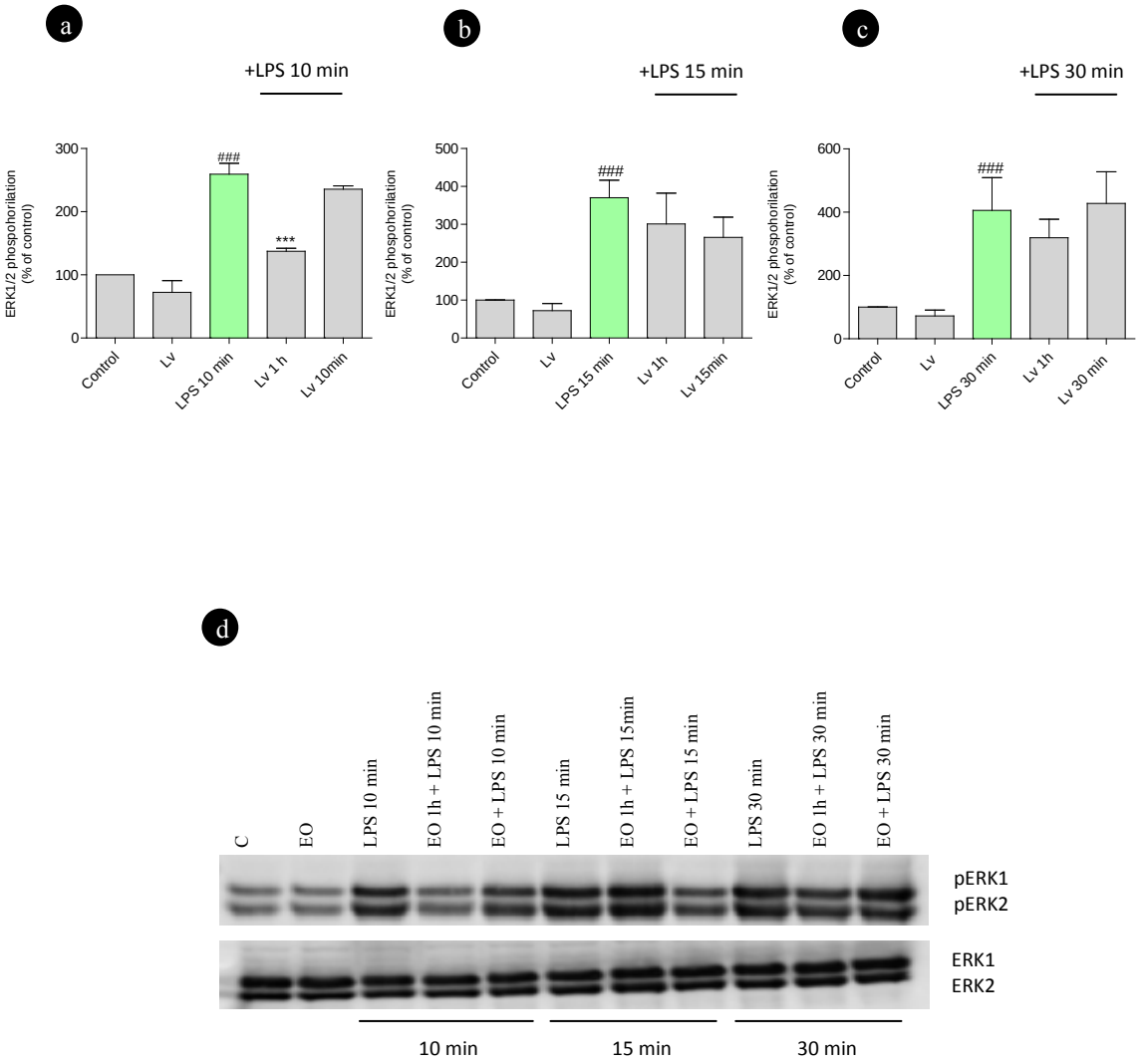


Figure 15. Inhibitory effect of *Lavandula viridis* (Lv) essential oil on ERK1/2 activation triggered by LPS. Raw 264.7 cells ($1.2x10^6$ cells/well) were maintained in culture medium (control), or pre-incubated for 1h with 0.64 µL/mL of *L. viridis* oil and then treated with 1µg/mL LPS for 10 (a), 15 (b) and 30 (c) min; the simultaneously incubation of cells with LPS plus essential oil was also tested. Total cell lysates were analysed by western blot using antibodies against phospho-ERK 1/2 and total ERK 1/2. The blot (d) shown is representative of three blots yielding similar results (### $p<0.001$, compared to control; *** $p<0.001$, compared to LPS).

2.3.4.5 NF-κB activation

The effect of the oil on the activation of NF-κB was evaluated by measuring the proteolytic degradation of its inhibitor, IκBα (Fig. 16). Incubation of the cells with the oil significantly inhibited IκBα phosphorylation triggered by LPS, at 10 and 30 min time points. The inhibition was more pronounced when the oil was pre-incubated 1 h before LPS stimulation for 30 min (Fig. 16c), but curiously, for 10 min stimulation, inhibition only occurred if the oil was added simultaneously with LPS (Fig. 16a). Through inhibition of IκBα phosphorylation and degradation, NF-κB is not activated and therefore, its target genes encoding pro-inflammatory proteins are not transcribed.

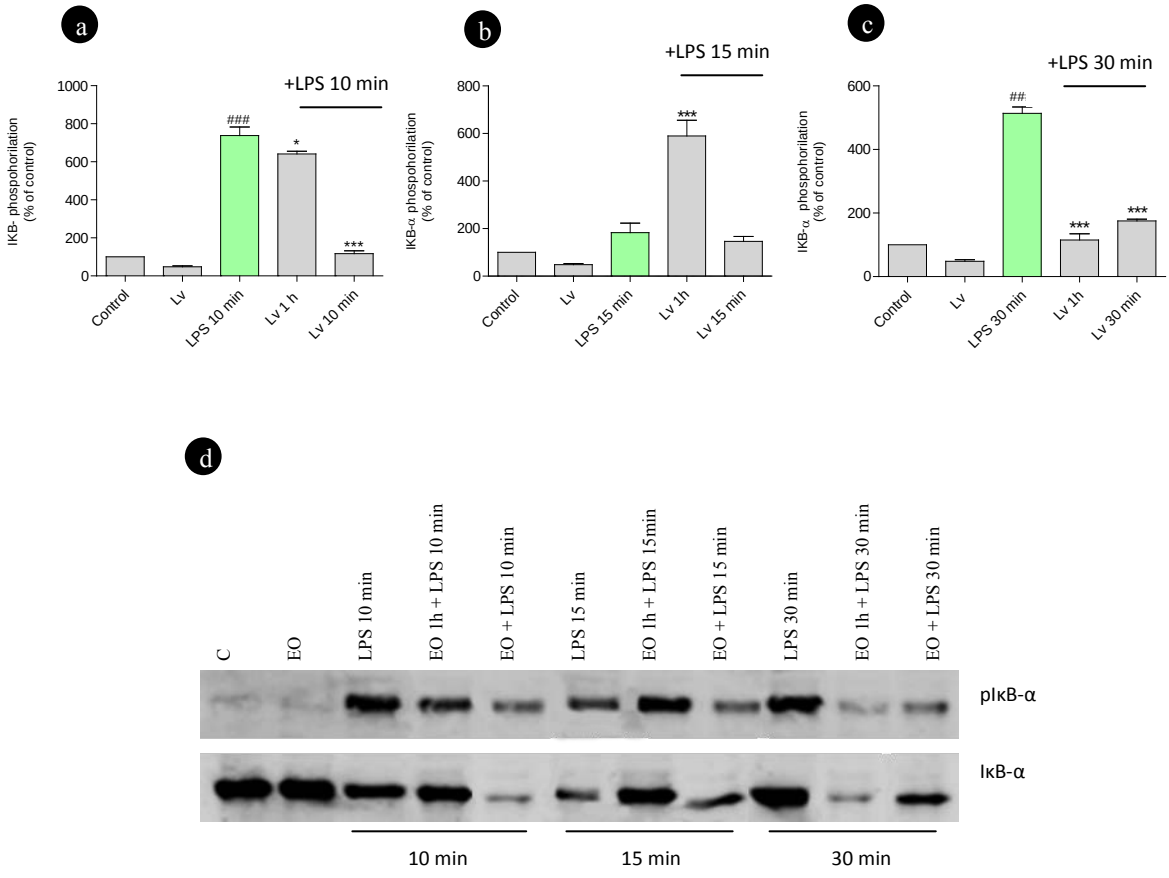
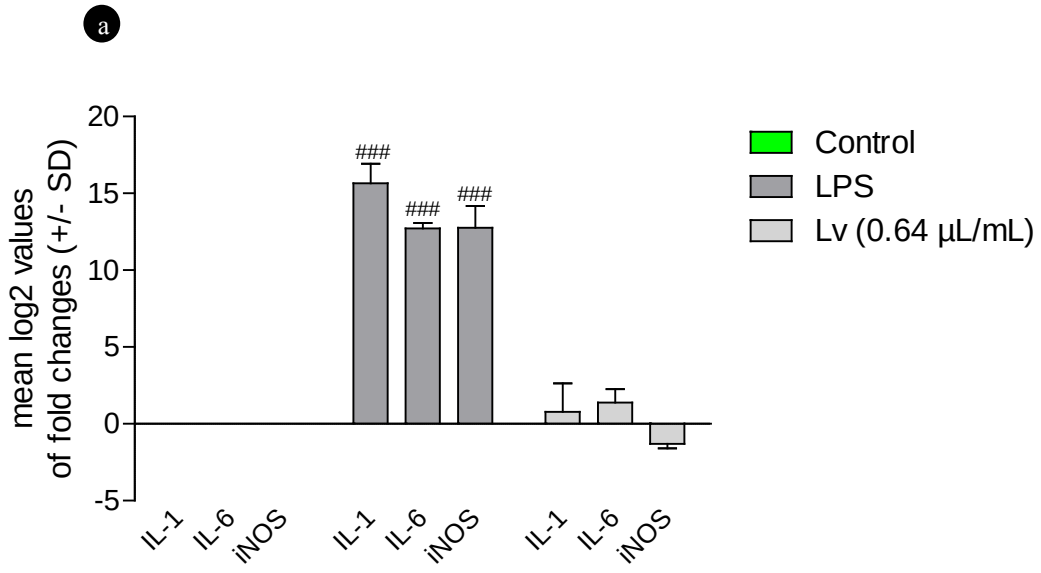


Figure 16. Inhibitory effect of *Lavandula viridis* (Lv) essential oil on NF-kB activation triggered by LPS. Raw 264.7 cells ($1.2x10^6$ cells/well) were maintained in culture medium (control), or pre-incubated for 1 h with 0.64 µL/mL of *Lavandula viridis* oil and then treated with 1µg/mL LPS for 10 (a), 15 (b) and 30 (c) min; the simultaneously incubation of cells with LPS plus essential oil was also tested. Total cell lysates were analysed by western blot using antibodies against phospho-IκB-α and IκB- α. The blot shown (d) is representative of three blots yielding similar results. (### $p<0.001$, compared to control; * $p<0.05$ and *** $p<0.001$, compared to LPS).

2.3.4.6 mRNA levels for iNOS, IL-1β, IL-6

LPS increased both cytokines (IL-1β and IL-6) and iNOS mRNA levels (dark grey bars; Fig. 17a) in macrophages, whereas the essential oil tested alone had very low detrimental effects, showing that the oil has no pro-inflammatory effect and was free of eventual endotoxin contamination (light grew bars; Fig. 17 a). LPS-induced expression of IL-1β, IL-6 and iNOS was significantly decreased in the presence of the essential oil (Fig. 17b). A most significant inhibition was achieved for IL-1β and iNOS when the oil was pre-incubated for 1 h before LPS stimulation (blue bars, Fig. 17b). Therefore, the essential oil from *L. viridis* was able to revert the pro-inflammatory profile elicited by LPS in both a prophylactic (before the inflammatory process; green bars, Fig. 17b) and therapeutic (during the inflammatory process; blue bars, Fig. 17b) situation.



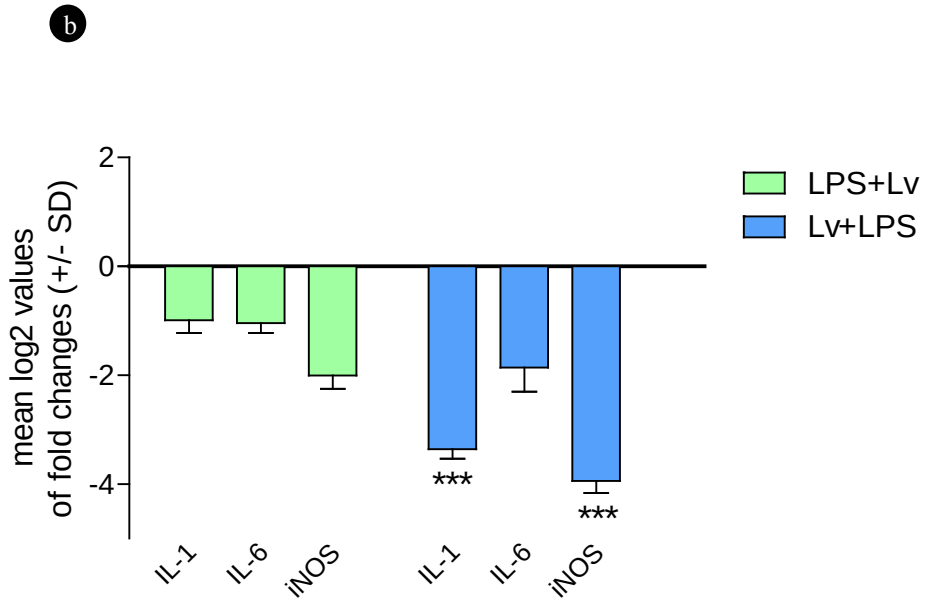


Figure 17. a) Transcription of pro-inflammatory cytokines and iNOS modulated by LPS and *L. viridis* essential. b) Effect of *L. viridis* essential oil on LPS-induced expression of IL-1β, IL-6 and iNOS. Cells were plated at 4 x 10^4 cells/well in 6-well microplates in a final volume of 2 ml of medium and treated with 1 µg/mL LPS during 6 h. Total RNA was isolated and retrotranscribed as indicated in experimental procedures. The mRNA levels were assessed by quantitative Real-Time RT-PCR. Gene expression is indicated as mean log2 values of fold changes relatively to control. Each value represents the mean±S.D.from three independent experiments (###$p < 0.001$, compared to control; *** $p < 0.001$, compared to the addition of essential oil simultaneously with LPS).

2.3.4.7 NO inhibition of 1,8-cineole

The major compound of *L. viridis* essential oil was used to evaluate its capacity to inhibit NO production in order to disclose whether this compound is responsible for the anti-inflammatory profile of the oil. The results showed that a higher concentration of 1,8-cineole is required to significantly reduce NO production (Fig. 18), in comparison to the oil, suggesting that other compounds are responsible for the activity of the oil. In this way, further experiments are being considered, including evaluation of the anti-inflammatory activity of the remaining main compounds (camphor, α-pinene and linalool) and/or oil fractions, in order to identify the most promising fraction and furthermore, a potential bioactive compound.

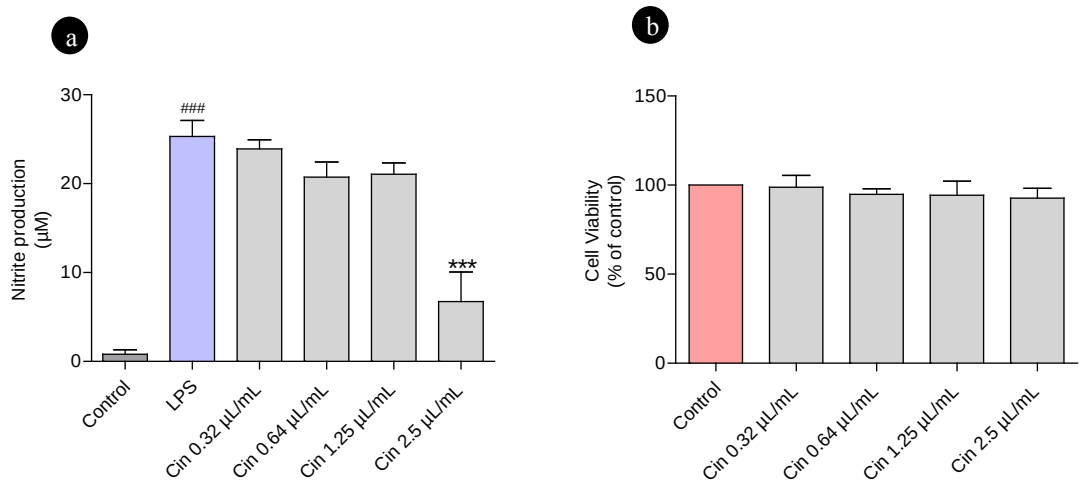


Figure 18. Effect of 1,8-cineole on: a) NO production triggered by LPS in macrophages. The cells ($3x10^5$ cells/well) were maintained in culture medium (control), or incubated with 1µg/mL LPS, or with LPS in the presence of different concentrations of the compound (0.08-0.64 µL/mL), for 24 h. Nitrite concentration was determined from a sodium nitrite standard curve and the results are expressed as concentration (µM) of nitrite in culture medium. b) Macrophages viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate (###$p<0.001$, compared to control; ***$p<0.001$, compared to LPS).

The present study clearly demonstrated the anti-inflammatory potential of *L. viridis* essential oil, which was due to the selective inhibition of NO production, through modulation of the pro-inflammatory signaling cascades ERK 1/2 and NF-κB. Moreover, inhibition at transcriptional and translational levels was also suggested as evidenced by reduction in mRNA levels of IL-1β, IL-6 and iNOS, as well as inhibition of iNOS and COX-2 protein levels.

NF-κB plays a critical role in the regulation of cell survival genes and coordinates the expression of several pro-inflammatory enzymes and cytokines like iNOS, COX-2, TNF-α and IL-6 (Edwards et al., 2009; Wong and Tergaonkar, 2009). Hence, it seems reasonable to consider that the inhibitory effects of *L. viridis* oil on the production of NO, IL-1β, IL-6 as well as on the expression of iNOS and COX-2, occurs via NF-κB signalling pathway modulation. However, it must be stated that the inhibition of other transcription factors can also occur, thus contributing for the anti-inflammatory profile of *L. viridis* oil.

On the other hand, MAPKs play an important role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stressors. Additionally, these kinases are involved in LPS-induced signalling pathways, through which iNOS is expressed (Malemud and Miller, 2008). In the present study, treatment with *L. viridis* oil was found to selectively inhibit ERK 1/2, suggesting that this kinase is also involved in the inhibition of LPS-stimulated NF-κB in Raw 264.7. This aspect deserves further investigation since previous studies have suggested that only JNK 1/2 and p38 MAPK but not ERK 1/2 modulate iNOS expression and NO production in LPS-stimulated macrophages Raw264.7 (Zhou et al., 2008; Chen and Wang, 1999; Francisco et al., 2011).

Although several essential oils have been assessed for its anti-inflammatory potential, as pointed out in a recent review describing the antioxidant and anti-inflammatory activities of these compounds (Miguel, 2010), studies on *Lavandula* spp. oils are very scarce. For example, *L. officinalis* oil showed a strong lipoxygenase inhibitory effect related to the presence of 1,8-cineole (Wei and Shibamoto, 2010) that also inhibited the production of the pro-inflammatory mediators prostaglandins (Juergens, 1998) and showed inhibitory effects on paw oedema induced by carrageenan and cotton pellet-induced granuloma (Santos and Rao, 200). *L. viridis* essential oil is

also characterized by high amounts of 1,8-cineole (see table 6, section 3, chapter II), suggesting that this compound must be partially involved in the anti-inflammatory activity of the oil. Indeed, our results demonstrated that the isolated compound inhibited NO production but at a higher concentration than that used for the essential oil, suggesting that other compounds are also responsible for the anti-inflammatory profile of the oil.

The non-steroidal anti-inflammatory drugs (NSAIDS) have long been used to combat inflammation. Recently, cyclooxygenase 2 inhibitors, that block the production of prostaglandins, have proved to be some of the most efficacious compounds. However, the application of these agents is limited because of the well-documented ulcerogenic effects and, more recently, the increased risk of heart disease, stroke and adverse renal effects (Süleyman et al., 2007; Harirforoosh and Jamali, 2009; Ritter et al., 2009). Since *L. viridis* oils were able to inhibit COX-2, it could be used as a natural alternative for NSAIDs. Further investigation regarding the effects of the oils on COX-1 enzyme is being conducted in order to identify oils that selectivity target the inducible form of the enzyme.

Furthermore, two different types of conventional anti-inflammatory drugs were also assessed at our laboratory: dexamathasone (7,85 ug/mL), a glucocorticoid class of steroid drugs, inhibited NO production by 70.56 % (Francisco, 2011), whereas indomethacine (5.36 µg/mL), a non-steroidal anti-inflammatory drug, induced an inhibition around 50 % in NO production and decreased iNOS expression about 43 % (Tavares, 2012). Although in this work we used higher concentrations of *L. viridis* oil, a stronger inhibition in both NO production (above 95 %) and iNOS expression (near 100 %) were detected, without affecting cell viability. These results are quite interesting since dexamathasone and indomethacine were toxic to macrophages at higher concentrations.

Taken together these results suggest that the *L. viridis* essential oils may be a potential natural source of new anti-inflammatory drugs. Further experiments are justified to identify which compound or compounds(s) are responsible for the anti-inflammatory properties of the oil.

2.4 References:

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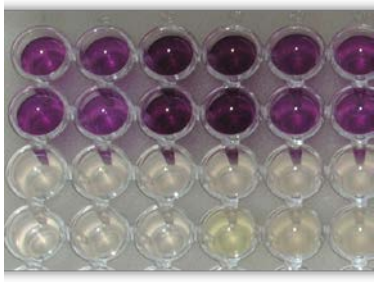

3. Cytotoxicity (Cell Viability)

3. Cytotoxicity (Cell Viability)

3.1 Introduction

The use of natural products has become increasingly popular as a source of bioactive compounds for food and health purposes, besides their recognized use in the cosmetic and perfumery industries (Lalhou, 2004). Nowadays, aromatic plants and their essential oils are widely used in aromatherapy to improve mood, anxiety and depression (Edris, 2007). The essential oils are usually applied by inhalation, dermal application and oral administration (Reichling, 2009). The small lipophilic molecules are able to reach the blood, cross the blood-brain barrier and enter the central nervous system, triggering several effects. Also, the essential oils and their terpenes have been used as natural alternatives to synthetic skin penetration enhancers (Narishetty and Panchagnula, 2004). The evaluation of the sensitization, carcinogenicity and toxicity of these oils is continuously carried out by organizations, such as the Research Institute for Fragrance Materials (RIFM), International Flavour and Fragrance Association (IFRA), Flavour Essence Manufacturers Association (FEMA) and the National Toxicology Program (NTP) with several international public health groups evaluating the toxicity of some oil components and establishing their NO Adverse Effect Level (NOAEL) (Vigan, 2010).

Despite the long traditional use of medicinal and aromatic plants, many uses are supported on a necdotal evidence rather than scientific research hence justifying the implementation of judicious and well-designed studies to avoid misleading reports, and clarify many questions regarding efficacy and safety of such compounds. Besides animal and clinical validations, safety assays must also be considered. Cytotoxic activities of the essential oils and their major compounds have been demonstrated *in vitro* in mammalian cells using short-term viability assays based on specific cell staining or florescent dyes. The main tests performed include neutral red uptake (NRU) (Söderberg et al., 1996; Stammati et al., 1999; Dijoux et al., 2006)., 3-(4,5-dimethylthiazol-2-yl)-2,5-diohenyl-tetrazolium bromide (MTT) test (Sun et al., 2005; Yoo et al., 2005; Chung et al., 2007), almar blue (resazurin) test (O'Brien et al., 2000), trypan blue exclusion test (Horvathova et al., 2006; Slamenova et al., 2007), propidium

iodide test (Fabian et al., 2006) and more recently, unscheduled DNA synthesis (to detect the presence and removal of adducts of DNA and repair DNA synthesis) (Burkey et al., 2000).

A number of investigations in cell culture systems have been carried out in order to predict the toxicity of essential oils to mammalian cells. Cell lines are more widely used for general toxicity studies than primary cell cultures because they are well characterized and more easily cultured. Most of the *in vitro* tests are performed on human fibroblasts or skin epithelial cells, although tumor cell lines are also used (Reichling et al., 2009). Concerning a summary reported by this author, the essential oils have shown cytotoxic effects *in vitro* at cytotoxic concentration (CC_{50}) values from 5.0-1950 µg/mL depending on incubation time (1-96 h). However, *in vitro* cytotoxicity data may overestimate the toxicity of a substance *in vivo*, since in cell cultures neither tissue structures, nor biotransformation or transport processes are simulated. In fact, most cell culture models use a monolayer of cells, being the essential oils directly applied to the culture medium. Therefore, the worst scenario, very unlikely to occur *in vivo*, is assessed (Reichling et al., 2009). For this reason, correlations between *in vitro* and *in vivo* toxicity data have been carried out in order to develop models that allow the prediction of systemic toxicity *in vivo* from cell culture data (Forsby et al., 2009).

The toxicity of essential oils varies according to oil composition, rout of administration and also the health of the person exposed to the oil (Vigan, 2010). Some essential oils have been reported to be toxic (e.g. Hayes and Markovic, 2003; Prashar et al., 2004), while others cause adverse reactions, including local irritation on skin and mucous membranes and allergic reactions (e.g. Schnuch et al., 2004; Veien et al., 2004; Willms et al., 2005). The cytotoxicity of the oils has been related to the induction of apoptosis and necrosis (Bakkali et al., 2008). Also, oil ingestion has been responsible for intoxication, including vomiting, respirational failure, and unconsciousness (Woolf, 1999). Moreover, drug interactions with conventional medicines may also occur (Reichling et al., 2009)

Regarding *Lavandula* spp. few toxicity assays have been performed. The cytotoxicity of *L. stoechas* oils was evaluated on several cell lines: epidermoid carcinoma (KB), drug-resistant KB (KB-V), mamma carcinoma (BC-1), lung carcinoma (Lu-1), colon carcinoma (Col-2), prostate carcinoma (LNCaP), murine leukemia (P388)

and fibroblasts (3T6); with the cytotoxic concentration (CC_{50}) varying from 5 to 286.8 (Gören et al., 2002). For *L. angustifolia*, the oils toxicity was evaluated on microvascular endothelial cells (HMEC-1), dermal fibroblasts (HNDF) and fibroblasts (153BR) with CC_{50} varying from 1690 to 1950 µg/mL (Prashar et al., 2004).

The essential oils from lavenders growing wild in Portugal showed very interesting antifungal and anti-inflammatory properties (see section 1 a nd 2 of the present chapter), justifying further experiments to evaluate the practical relevance of the *in vitro* results. Therefore, the potential toxicity of *L. luisieri*, *L. multifida*, *L. pedunculata* and *L. viridis* essential oils were assessed in order to explore their future utilization by the pharmaceutical and cosmetic industries. For oils with higher chemical polymorphism, namely *L. pedunculata* and *L. luisieri*, different oil samples were considered. Toxicological evaluation was performed on c ell line cultures, avoiding animal sacrifices at this early stage of validation. Three distinct cell lines were selected: keratinocytes (HaCat, Fig. 1a), alveolar epithelial cells (A549, Fig. 1b) and macrophages (Raw 264.7, Fig. 1c), in order to predict the effect of the oils based on its mode of administration, topical, through inhalation and oral, respectively. Although the effect of the oils on macrophages was already shown in the previous section, results are repeated in this section in order to simplify comparisons with the other cell lines.

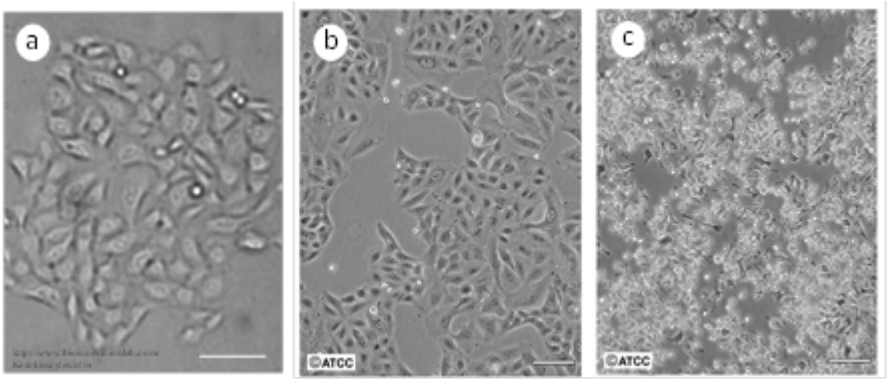

Figure 1. Morphological appearance of the cell lines used to evaluate the cytotoxicity of *Lavandula* spp. essential oils: a) HaCat (DKFC). b) A-549 (ATCC number CCL-185); c) Raw 264.7 (ATCC number TIB-71). Bars = 100 µm.

3.2 Material and methods

The cytotoxicity of the essential oils was evaluated at the Laboratory of Cellular Immunology and Oncobiology of the Centre of Neuroscience and Cell Biology of the University of Coimbra.

3.2.1 Essential oils

The oils selected for cytotoxicity evaluation are listed in table 1. The reasons for this selection are referred in previous sections.

Table 1. Site of collection of *Lavandula* spp. for toxicological evaluation.

Species	Region	Site of Collection	Sample
L. luisieri	Coimbra	Pióbão	D
	Algarve	Cabo de São Vicente	L
L. pedunculata	Guarda	Celorico da Beira	A0
	Bragança	Serra da Nogueira	B3
	Coimbra	Foz de Arouce	D7
L. multifida	Setúbal	Sesimbra	AI
L. viridis	Algarve	Barranco do Velho	A

3.2.2 Cell culture and materials

The fetal calf serum was from Biochrom KG (Berlin, Germany) and trypsin from Gibco (Paisley, UK). MTT and all the other reagents were from Sigma Chemical Co. (Saint Louis, MO).

The human keratinocyte cell line, HaCat, obtained from DKFZ (Heidelberg), was kindly supplied by Dr. Eugénia Carvalho (Centre for Neuroscience and Cell Biology of the University of Coimbra, Portugal). The human alveolar epithelial cell line A549 was purchased at ATCC (number CCL-185).

Keratinocytes and epithelial lung cells were cultured in DMEM medium supplemented with 10 % (v/v) inactivated fetal bovine serum, 3.02 g/L sodium bicarbonate, 100 µg/mL streptomycin and 100 U/mL penicillin at 37 ºC in a humidified atmosphere of 95 % air and 5 % CO_2.

Raw 264.7, a mouse leukaemic monocyte macrophage cell line from ATCC (number TIB-71), was kindly supplied by Dr. Otília Vieira (Centre of neuroscience and Cell Biology of the University of Coimbra, Portugal), and cultured on D MEM supplemented with 10 % (v/v) non i nactivated fetal bovine serum, 3.02 g/L sodium bicarbonate, 100 µg/mL streptomycin and 100 U/mL penicillin at 37 ºC in a humidified atmosphere of 95 % air and 5 % CO_2.

Along the experiments, HaCat, A549 and Raw cells were monitored by microscope observation in order to detect any morphological change.

3.2.3 Cytotoxicity of the oils

Assessment of metabolically active cells was performed using the MTT reduction colorimetric assay as previously reported (Mosmann, 1983). Macrophages (3×10^5cells/well), keratinocytes ($2x10^5$ cells/well) and lung cells (5×10^4 cells/well) were plated in 48-well microplates and allowed to stabilize for 12 h. Then, cells were either maintained in culture medium or incubated with different concentrations of the essential oils for 24 h. After cells treatment, the MTT solution (5 mg/mL in phosphate buffered saline) was added and cells incubated at 37 ºC for 15 min (Raw 264.7), 30 min (HaCat), or 2.5 h (A549) in a humidified atmosphere of 95 % air and 5 % CO_2. Supernatants were then removed and blue formazan crystals solubilized with 300 µL acidic isopropanol (0.04 N HCl in isopropanol). Quantification of formazan was performed using an ELISA microplate reader (SLT, Austria) at 570 nm , with a reference wavelength of 620 nm.

A cell-free control was performed in order to exclude non-specific effects of the oils on MTT (data not shown). All the experiments were performed in duplicate, being the results expressed as mean ± SEM of three independent experiments. The means were statistically compared using one-way ANOVA, with a Dunnett's multiple

comparison test. The statistical tests were applied using GraphPad Prism, version 5.02 (GraphPad Software, San Diego, CA, USA).

3.3 Results and discussion

The essential oils of *Lavandula luisieri* (2 samples), *L. pedunculata* (3 samples), *L. multifida* and *L. viridis* were tested on different cell lines (HaCat, A549 and Raw 264.7), in order to evaluate their potential toxicity regarding the three main ways of essential oils administration. As far as it is known this is the first report on the cytotoxicity evaluation of these lavenders.

3.3.1 *Lavandula luisieri*

To evaluate *L. luisieri* essential oils toxicity sample L with low amounts of irregular monoterpenes (necrodane derivatives) and sample D with high amounts of those compounds were assessed.

In general, keratinocytes were more sensible to the oils than alveolar epithelial cells and macrophages. Both oil samples (L and D) showed similar toxicity to keratinocytes (toxicity at 0.32 µL/mL; Fig. 2a and 2b) and were less toxic to alveolar epithelial cells (toxicity at 0.64 µL/mL; Fig. 3a and 3b). Regarding macrophages, the samples showed different toxicities, being the oil rich in necrodane derivatives (D) more toxic (at 0.16 µL/mL; Fig. 4b) than the oil with lower amounts of these compounds (L) that was only toxic at 0.64 µL/mL (Fig. 4a).

Cytotoxicity on HaCat

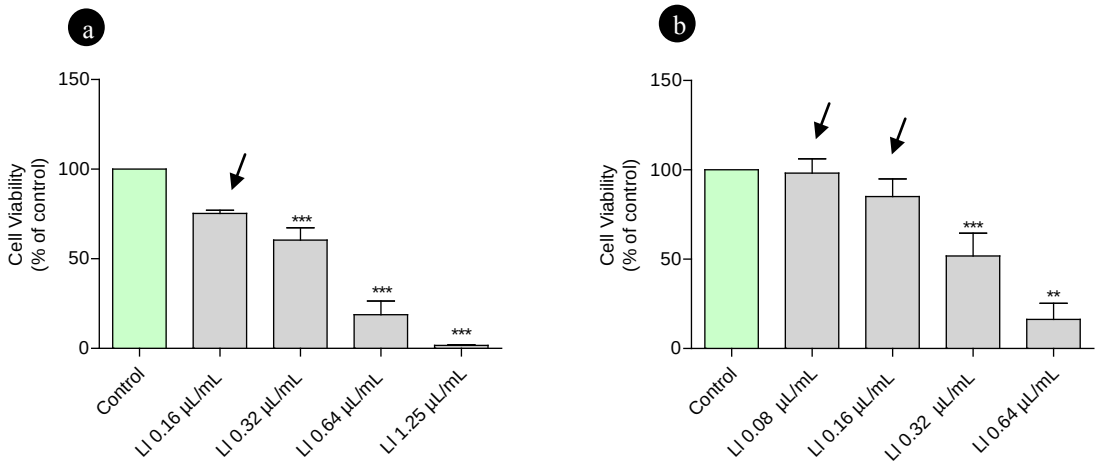

Figure 2. Effect of *Lavandula luisieiri* (Ll) essential oils with low amounts (a) and high amounts (b) of necrodane derivates on keratinocytes viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate (**p<0.01 and ***p<0.001, compared to control). Arrows indicate non-toxic concentrations

Cytotoxicity on A549

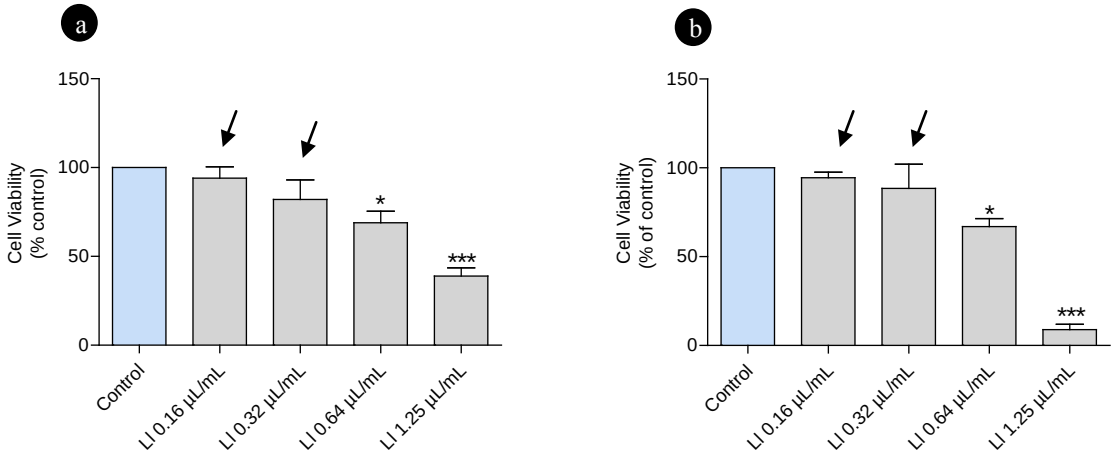

Figure 3. Effect of *Lavandula luisieiri* (Ll) essential oils with low amounts (a) and high amounts (b) of necrodane derivates on alveolar epithelial cells viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate (*p<0.05 and ***p<0.001, compared to control). Arrows indicate non-toxic concentrations.

Cytotoxicity on Raw 264.7

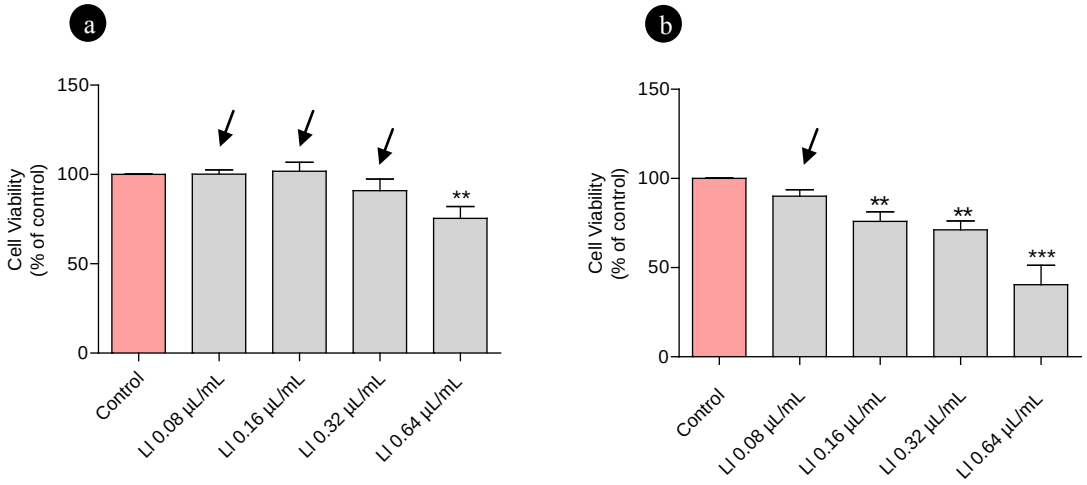

Figure 4. Effect of *Lavandula luisieiri* (Ll) essential oils with low amounts (a) and high amounts (b) of necrodane derivates on macrophages viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate (**p<0.01 and ***p<0.001, compared to control). Arrows indicate non-toxic concentrations.

3.3.2 *Lavandula multifida*

To evaluate *L. multifida* essential oils toxicity, the oil from Sesimbra region (sample AI) was tested. The oil was characterized by high amounts of carvacrol (see table 4, section 3, chapter II).

L. multifida oil showed a higher toxicity for keratinocytes (toxicity above 0.08 µL/mL; Fig 5) whereas for alveolar epithelial cells and macrophages a safer profile was determined, with toxicity only occurring at 0.64 µL/mL, for alveolar epithelial cells (Fig. 6) and 0.32 µL/mL, for macropaghes (Fig. 7). These results are quite interesting since the oils major compound, carvacrol, is known as a very toxic compound. The toxicity of carvacrol was assessed on m acrophages (graphic not shown), being the compound toxic above 0.02 µL/mL. Accordingly, several authors have reported the high toxicity of this compound (Monzote et al., 2009; Mehdi et al., 2011). The present results show that the phenolic rich *L. multifida* oil may be used in therapy in higher concentrations than carvacrol, without affecting cells viability.

Cytotoxicity on HaCat

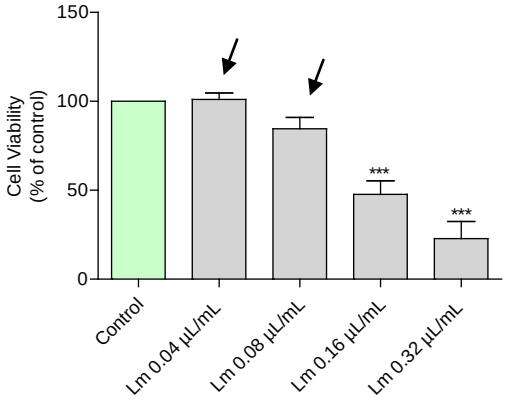

Figure 5. Effect of *Lavandula multifida* (Lm) essential oils on keratinocytes viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate (***p<0.001, compared to control). Arrows indicate non-toxic concentrations.

Cytotoxicity on A549

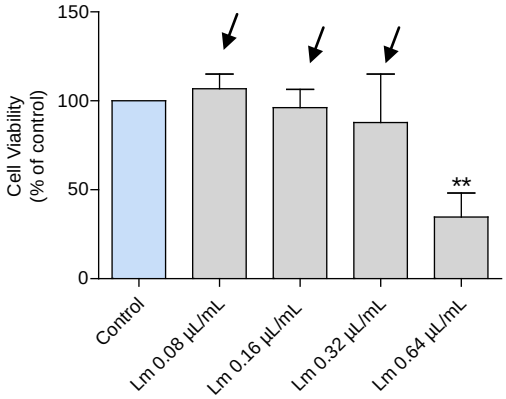

Figure 6. Effect of *Lavandula multifida* (Lm) essential oils on alveolar epithelial cells viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate (**p<0.01, compared to control). Arrows indicate non-toxic concentrations.

Cytotoxicity on Raw 264.7

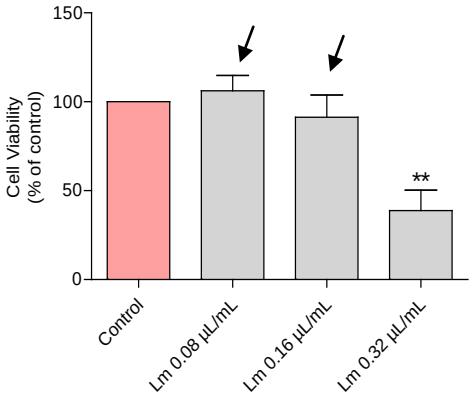

Figure 7. Effect of *Lavandula multifida* (Lm) essential oils on macrophages viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate (**p<0.01, compared to control). Arrows indicate non-toxic concentrations.

3.3.3 *Lavandula pedunculata*

Taking into account the high polymorphism observed in *L. pedunculata* oils, three distinct oil samples were selected to evaluate their toxicity: sample B3 (high amounts of 1,8-cineol), sample D7 (high amounts of fenchone) and samples A0 (high amounts of camphor) (see table 5, section 3, chapter II).

Keratinocytes were one again, the most sensitive cells to all samples (Fig. 8a-8c), being chemotype camphor (A0) the most toxic, affecting cell viability at 0.16 µL/mL (Fig. 8c) whereas chemotype fenchone (D7) only showed toxic effects at 0.64 µL/mL (Fig. 8b).

For alveolar epithelial cells, the chemotype 1,8-cineole (B3) affected cell viability at 0.64 µL/mL (Fig. 9a) whereas the other two samples (A0 and D7) showed toxic effects at a higher concentration (1.25 µL/mL; Fig 9b and 9c, respectively).

Regarding macrophages, no di fferences were found among the sample tested, being all chemotypes safe at the tested concentrations (Fig. 10a, 10b and 10c).

Cytotoxicity on Hacat

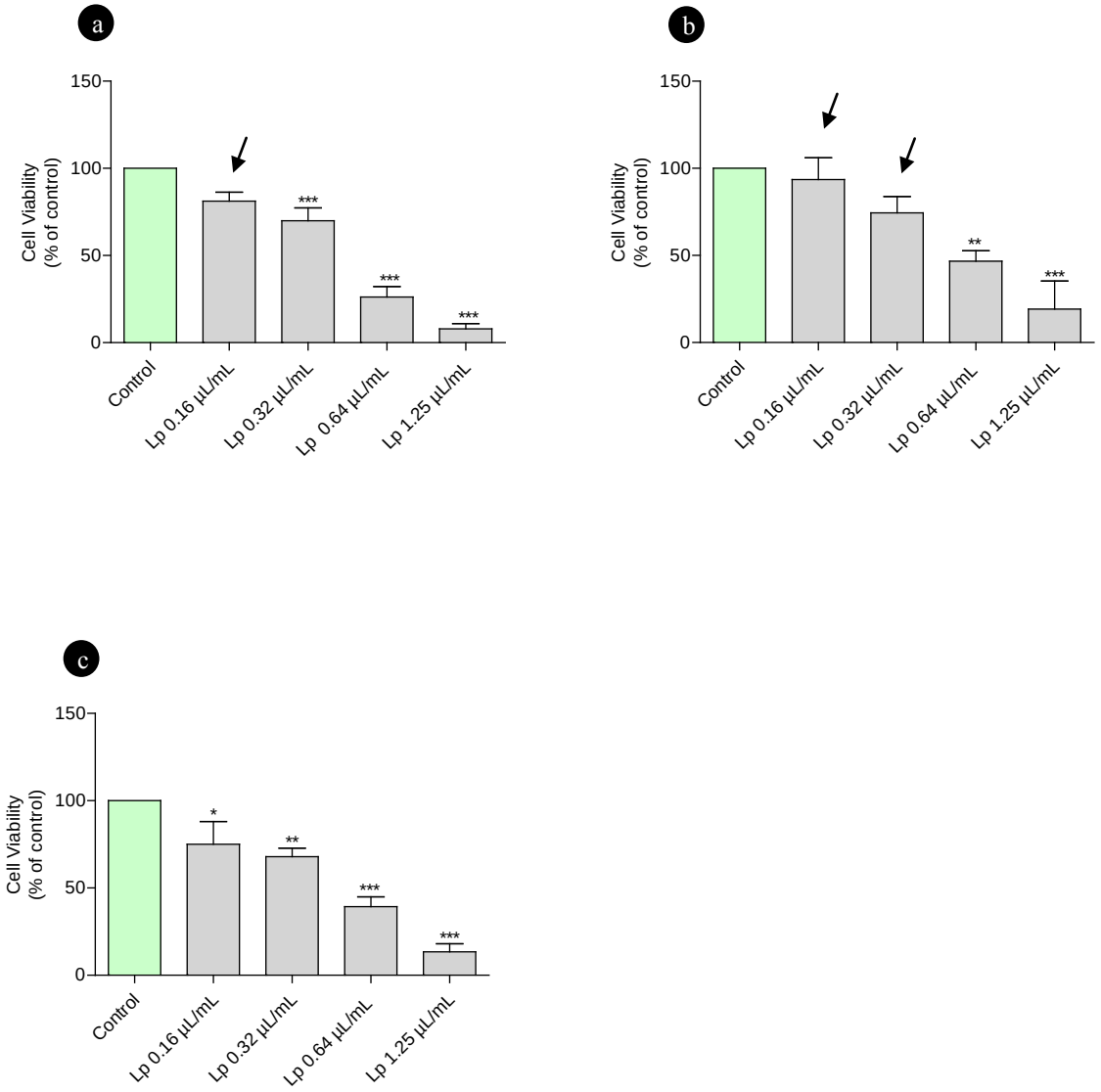

Figure 8. Effect of *Lavandula pedunculata* (Lp) essential oils with high amounts of 1,8-cineole (a), fenchone (b) and camphor (c) on keratinocytes viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate (*p<0.05, **p<0.01 and ***p<0.001, compared to control). Arrows indicate non-toxic concentrations.

Cytotoxicity on A549

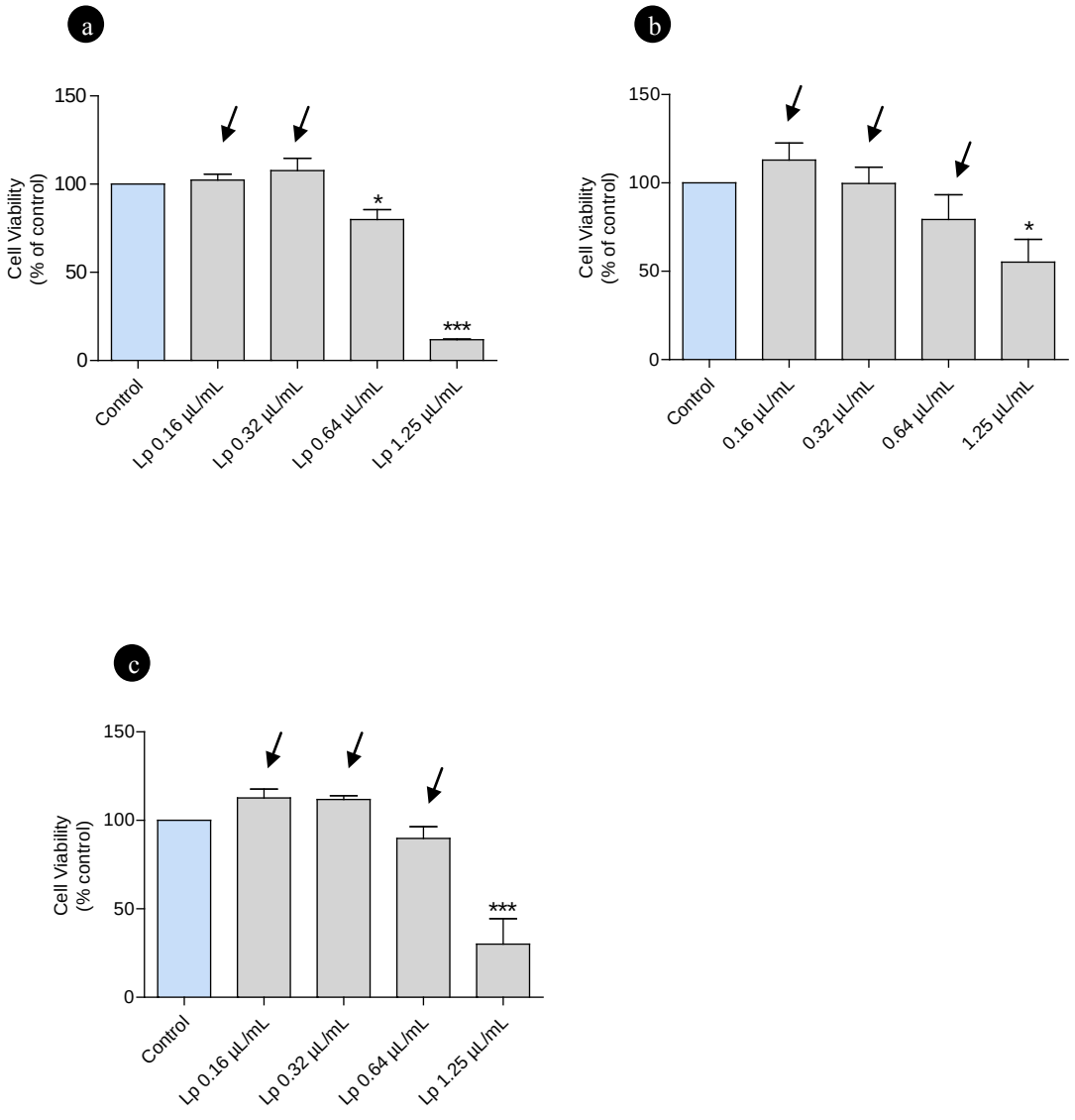

Figure 9. Effect of *Lavandula pedunculata* (Lp) essential oils with high amounst of 1,8-cineole (a), fenchone (b) and camphor (c) on alveolar epithelial cells viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate (*$p<0.05$ and ***$p<0.001$, compared to control). Arrows indicate non-toxic concentrations.

Cytotoxicity on Raw 264.7

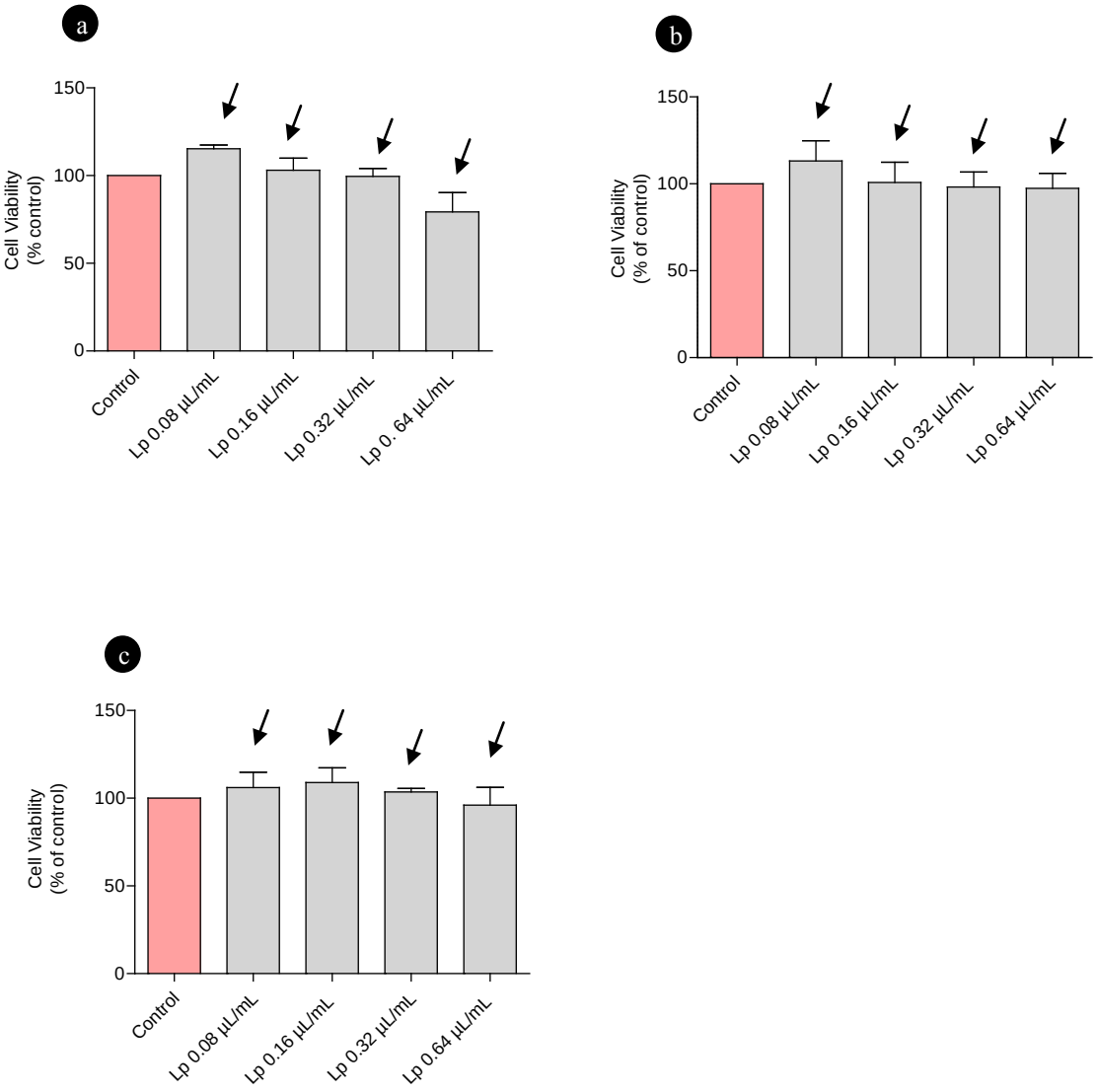

Figure 10. Effect of *Lavandula pedunculata* (Lp) essential oils with high amounts of 1,8-cineole (a), fenchone (b) and camphor (c) on macrophages viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate. Arrows indicate non-toxic concentrations. Arrows indicate non-toxic concentrations.

3.3.4 *Lavandula viridis*

L. viridis oils are very homogenous and therefore only one oil sample, collected at Barranco do Velho region, was selected to evaluate its toxicity.

The results showed that the oil was less toxic to keratinocytes than the other species (toxic at concentrations ≥ 0.64 µL/mL; Fig. 11). For alveolar epithelial cells, toxicity was detected at 1.25 µL/mL (Fig. 12) and for macrophages the oil did not affect cell viability at the tested concentrations (Fig 13). These results showed that *L. viridis* essential oils does not affect cell viability at concentrations with biological activity.

Cytotoxicity on HaCat

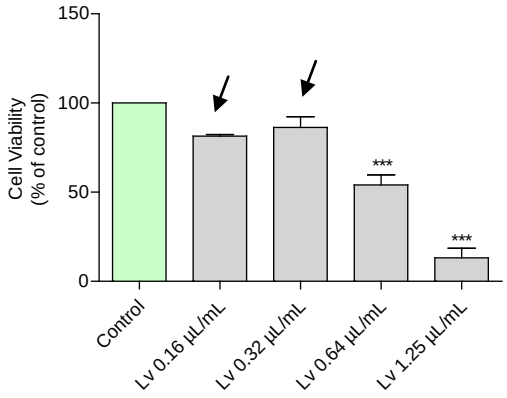

Figure 11. Effect of *Lavandula viridis* (Lv) essential oils on keratinocytes viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate (***p<0.001, compared to control). Arrows indicate non-toxic concentrations.

Cytotoxicity on A549

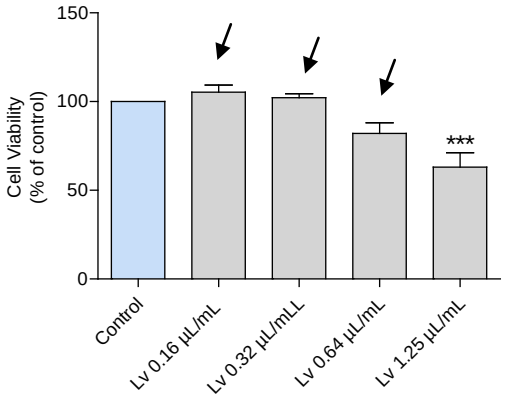

Figure 12. Effect of *Lavandula viridis* (Lv) essential oils on alveolar epithelial cells viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate (***p<0.001, compared to control). Arrows indicate non-toxic concentrations.

Cytotoxicity on Raw 264.7

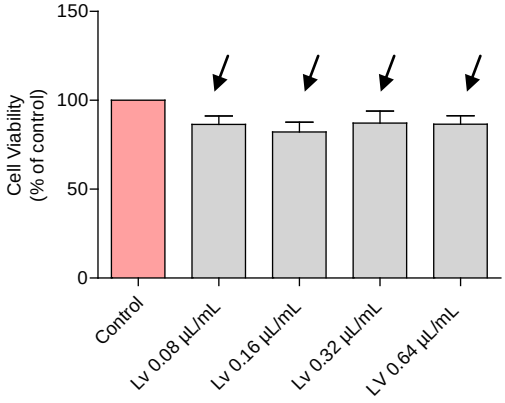

Figure 13. Effect of *Lavandula viridis* essential oils on macrophages viability. Results are expressed as percentage of MTT reduction by control cells maintained in culture medium. Each value represents the mean ± SEM from three independent experiments, performed in duplicate. Arrows indicate non-toxic concentrations

The evaluation of the oils cytotoxicity is a crucial step for developing an antifungal or anti-inflammatory agent. In the last few years, several *in vitro* toxicity assays have been developed and validated in order to replace animal testing methods (Bhanushali et al., 2010).

Regarding *Lavandula* essential oils toxicity, few studies have been performed namely with *L. stoechas* (Gören et al., 2002) and *L. angustifolia* (Prashar et al., 2004) oils. Since different cell lines were used in these sudies, comparisons cannot be made. *L. stoechas* oil exhibited a very high toxicity on several carcinoma lines, with CC_{50} values ranging from 5.0 to 20 µg/mL. The oil was much less toxic on fibroblasts with CC_{50} of 286.8 µg/mL. Regarding *L. angustifolia* oil, its CC_{50} values ranged from 1950-1690 µg/mL in fibroblast and endothelial cells, respectively; the authors suggested the use of the oil with caution and in highly diluted forms for skin application (Prashar et al., 2004).

In the present study, the oils from *Lavandula* spp. growing wild in Portugal showed different degrees of toxicity, being in general more toxic to keratinocytes. Moreover, *L. viridis* and *L. pedunculata* (chemotype fenchone) showed the higher safety profile and did not affect cell viability at concentrations up to 0.32 µL/mL (HaCat) and 0.64 µL/mL (A549 and Raw 264.7); *L. multifida* was significantly less toxic than its main compound carvacrol, known as a very toxic phenol; *L. luisieri* rich in necrodane derivates was very toxic to macrophages, affecting cell viability at concentrations > 0.08 µL/mL. In fact, necrodane derivatives have previously been reported for their toxicity (antifeedant effects) against several insects (González-Coloma, 2011).

In this way, *Lavandula* spp. oils seem to be promising natural products to be further explored as antifungal and anti-inflammatory agents. Moreover, suitable doses of the oils with biological activity and without, or with very low detrimental effect on cells, were identified. Further *in vivo* assays are being considered to validate these *in vitro* results.

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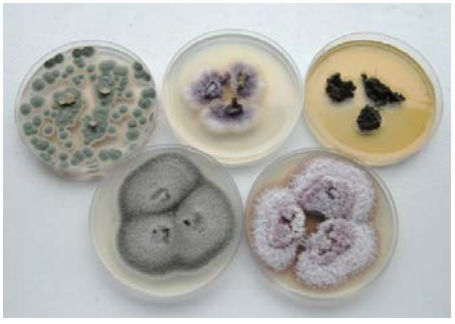

4. Antifungal Activity against Phytopathogenic Fungi

4. Antifungal Activity against Phytopathogenic Fungi

4.1. Introduction

Crops, foods, commodities and raw materials are vulnerable to contamination by many fungi. Besides reduction in crop yield and losses of quality, these fungi are a source of some very toxic compounds generally known as mycotoxins. Fungal contaminations are a severe problem that depends on production and storage practices and varies with the type of food. Moreover, mycotoxin production may occur before any detectable evidence of fungal growth (Pirbalouti et al., 2011) causing severe health concerns (e.g. hemorrhages, hepatitis, esophageal cancer and kidney failure). Fungal toxins of most concern are produced by species of the genera *Aspergillus*, *Fusarium* and *Penicillium* and frequently occur in major field crops and continue to contaminate them during storage (Reddy et al., 2010). In fact, several outbreaks of mycotoxicoses in humans and animals have been reported after the consumption of food and feed contamination (Reddy and Raghavender, 2007), being the mycotoxins aflotoxin, fumonisin and ochratoxin A the most toxic to humans (Donmez-Altuntas et al., 2003; Reddy et al., 2010).

Humans have been preserving food to protect it from spoilage for thousands of years. Different techniques including curing with salt, pickling, dehydrating, pasteurizing, and freezing have been applied to maintain the rich nutrition of foodstuffs. Currently, synthetic fungicides have also been used to control these contaminations. However, the continued applications of fungicides may disrupt equilibrium of ecosystems, leading to dramatic disease outbreaks, development of fungicide-resistant strains, toxicity to non-target organisms and environmental concerns (Lee et al., 2009). Furthermore, growing concerns on the presence of chemical residues in the food chain (Lee et al., 2008) together with revocations in registration of some of the most effective fungicides have highlighted the need to develop alternative control strategies or innovative crop protection and postharvest methods with reduced use of conventional fungicides or without synthetic chemicals at all (Kim et al., 2003).

Research on plant-derived fungicides is gaining interest, as it becomes evident that these substances have enormous potential to improve future agrochemical

technology. Since secondary plant metabolites are biodegradable to nontoxic products, they are potentially useful in integrated pest management programs and could allow the development of a new class of safer disease control substances.

Several studies have pointed out the use of essential oils to control postharvest fruit and vegetable diseases (Bhaskara-Reddy, 1998; Chebli et al, 2004; Tzortzakis, 2007; Lopez-Reyes et al., 2010; Krisch et al., 2011) as well as other phytopathogens (Conte et al., 2007; Camele et al., 2012). These volatile products may be employed to storeroom air and protect, perhaps better than fungicides commonly used for this purpose, stored fruits, crops or other type of plant material such as tubers or bulbs.

Regarding *Lavandula* essential oils some studies have been performed. For instance, *L. angustifolia* (= *L. officinalis*) was assessed against *Aspergillus* spp. (Thanaboripat, 2007) and food spoilage yeasts (*Torulasporadel brueckii*, *Zygosaccharomyces bailii*, *Pichia membranifaciens*, *Dekkera anomala* snd *Yarrowia lipolytica (*Araújo et al., 2003); *L. stoecha*s essential oils showed inhibitory effects on the growth of *Botrytis cinerea* (Soylu et al., 2010) and *Aspergillus candidus*, *A. niger*, *Fusarium culmarum* and *Penicillium* sp. (Magro et al., 2006). Additionally, several studies have also shown the effectiveness of lavender oils on the control of bacteria responsible for foodborne contaminations (Dadalioğlu and Evrendilek, 2004; Rota et al., 2004,).

Taking into account all the referred aspects and the lack of studies on the oils of *Lavandula* occurring in Portugal, it seemed opportune to evaluate their effect on different phytopathogenic strains, namely: *Alternaria alternata* (Fig. 1a; causal agent of leaf spot in many plant species), *Cladosporium cladosporioides* (Fig. 1b; mainly wheat contamination), *Fusarium circinatum* (Fig. 1c; listed as a quarantine species and causal agent of pitch canker disease), *F. verticillioides* (Fig. 1d; corn seedling blight, root rot, stalk rot and kernel rot) and *Penicillium itallicum* (Fig. 1e; *Citrus* fruits contamination).

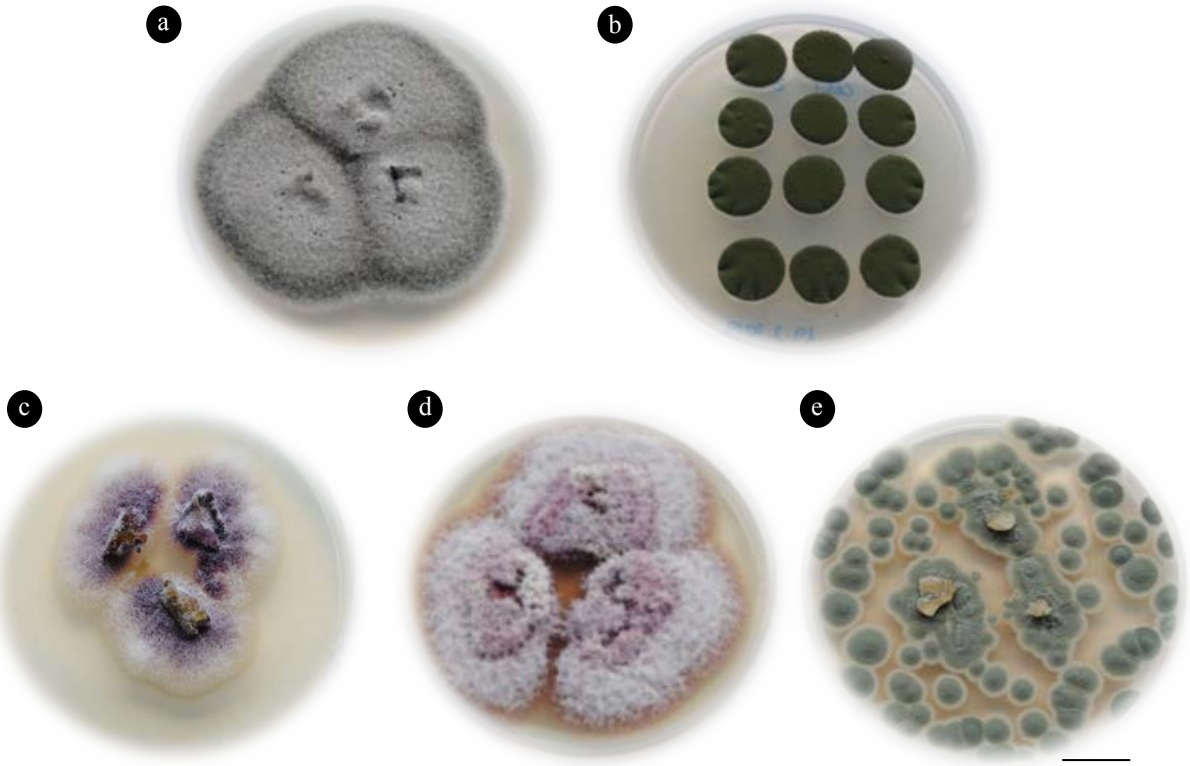

Figure 1. Phytopathogenic strains on potato or Sabouraud dextrose agar after 72 h of culture at 30 ºC; a) *Alternaria alternata*. b) *Cladosporium cladosporioides*. c) *Fusarium circinatum*. d) *Fusarium verticillioides*. e) *Penicillium itallicum*. Bar = 1 cm.

4.2 Material and methods

4.2.1 Essential oils

The oils selected for the antifungal activity are listed in table 1. The reason for the selection of these samples was previously referred in section 1 of this chapter, being sample C6 relaced by A0, with higher contents of camphor.

Table 1.Site of collection of *Lavandula* spp. used in the antifungal assays

Species	Region	Site of Collection	Sample
L. luisieri	Coimbra	Pióddão	D
	Algarve	Cabo de São Vicente	L
L. pedunculata	Guarda	Celorico da Beira	A0
	Bragança	Serra da Nogueira	B3
	Coimbra	Foz de Arouce	D7
L. multifida	Setúbal	Sesimbra	AI
L. viridis	Algarve	Barranco do Velho	A

4.2.2 Pure and reference compounds

Commercial authentic samples of α-pinene (Fluka, 99.0 % purity), carvacrol (Fluka, pure), 1,8-cineole (Merck, 99.5 % purity), *cis*-*β*-ocimene (SAFC, ≥ 90 % purity), linalool (Aldrich, 99.0 % purity), fenchone (Flucka, pure) and camphor (Extrasynthese) were used.

4.2.3 Fungal strains

The antifungal activity of the essential oils and their main compounds was evaluated against five fungi: three type strains from the Colección Espanõla de Cultivos Tipo (*Alternaria alternata* CECT 2662, *Cladosporium cladosporioides* CECT 2110 *Fusarium verticcillioides* CECT 2982 and *Penicillium itallicum* CECT 2294) and a quarantine species (*Fusarium circinatum*) kindly provided by Prof. António Portugal

(Department of Life Sciences, Faculty of Sciences and Technology of the University of Coimbra).

4.2.4 Antifungal activity

Broth macrodilution methods based on the CLSI reference protocol M38-A2 (CLSI, 2008), for filamentous fungi, were used to determine the MICs and MLCs of the essential oils and their major constituents. The experiments were performed as previously referred in section 1 of this chapter, being the test tubes incubated aerobically at 30 ºC for 72 h. All experiments were performed in triplicate and repeated whenever the results of each triplicate did not agree. A range of values is presented when different results were obtained.

4.3 Results and discussion

The present results show that in general *A. alternata* and *C. cladosporioides* are more susceptible to *Lavandula* essential oils than the other fungi tested (Table 2).

L. multifida and *L. luisieri* were the most active oils against all the tested strains with MIC values ranging from 0.16-0.64 µL/mL and 0.16-2.5 µL/mL, respectively (Table 2). *L. luisieri* with high amounts of necrodane derivatives (D) was, in general, more active than the oil with lower amounts of these compounds (Table 2). Regarding the main compounds (Table 3), carvacrol was the most active with MIC values ranging from 0.08-0.32 µL/mL, justifying the activity of *L. multifida* essential oil, rich in this compound. In fact the application of carvacrol as a new antimicrobial agent has been highlighted in a recent review showing both the published literature and recent patents claimed on this matter (Nostro and Papalia, 2012). Several mechanisms of action have also been proposed for carvacrol with a higher number of studies evidencing the increase in fluidity and permeability in membranes of the target organisms (Hyldgaard et al., 2012). Regarding *L. luisieri*, the evaluation of the antifungal activity of the necrodane derivatives and/or fractions rich in these compounds is being considered since 1,8-cineole (the other main compound of the oil assessed in the present study) was ineffective against the fungi tested.

Essential oils and/or their isolated compounds are natural, effective, and consumer-accepted tools against food and crop spoilage. However, to validate *in vitro* results further experiments using *in vivo* models are necessary and for uses as food or stored products preservatives, several limitations have to be considered, namely interactions with food matrix components, starch and proteins (Gutierrez et al., 2008; Cava-Roda et al., 2010; Rattanachaikunsopon and Phumkhachorn, 2010; Kyung, 2011) as well as negative organoleptic effects (Lv et al., 2011). Different strategies have been proposed to overcome these limitations namely encapsulation in polymers of edible and biodegradable coatings or sachets that provide a slow release to the food or storage products surface or to the headspace of their packages (Sánchez-González et al., 2011). These approaches also allow reduction in diffusion rates, maintaining the active compounds in the headspace or on the product surface for a longer time (Philips and Laird, 2011).

Table 2. Antifungal activity (MIC and MLC) of *Lavandula* spp. essential oils against food, crop and forest pathogens.

Strains	*L. luisieri* (L)		*L. luisieri* (D)		*L. multifida* (AI)		*L. pedunculata* (B3)		*L. pedunculata* (D7)		*L. pedunculata* (A0)		*L. viridis*	
	MIC*	MLC*	MIC*	MLC*	MIC*	MLC*	MIC*	MLC*	MIC*	MLC*	MIC*	MLC*	MIC*	MLC*
Alternaria alternata CECT 2662	0.32-0.64	0.32-0.64	0.16	0.16	0.16-0.32	0.16-0.32	0.32	0.32	0.32-0.64	0.32-0.64	0.64	0.64	064-1.25	1.25
Cladosporium cladosporioides CECT 2110	0.16-0.32	0.16-0.32	0.32-0.64	032-0.64	0.16	0.16	1.25	1.25	1.25	1.25	0.64	0.64-1.25	1.25	1.25
Fusarium circinatum	2.5	2.5	1.25	1.25-2.5	0.64	1.25	10	10	2.5-5	2.5-5	5.0	5	2.5	2.5
Fusarium verticillioides CECT 2982	2.5	2.5	1.25	1.25-2.5	0.32	0.32-064	10	10	2.5	2.5	2.5	2.5	2.5	2.5
Penicillium itallicum CECT 2294	2.5	5	1.25	5-10	0.64	1.25	10	20	5.0	10	5-10	5-10	2.5	2.5

Table 3. Antifungal activity (MIC and MLC) of *Lavandula* spp. essential oils main compounds against food, crop and forest pathogens

Strains	1,8-Cineole		Camphor		α-Pinene		Fenchone		Carvacrol		*cis*-β-Ocimene		Linalool	
	MIC*	MLC*	MIC*	MLC*	MIC*	MLC*	MIC*	MLC*	MIC*	MLC*	MIC*	MLC*	MIC*	MLC*
Alternaria alternata CECT 2662	10	10	>20	>20	1.2-2.5	1.25-2.5	5.0	5.0	0.08	0.08	2.5	2.5	0.64	0.64
Cladosporium cladosporioides CECT 2110	10	10	>20	>20	0.64	0.64	5.0	5.0	0.08	0.08	2.5	2.5	0.64	0.64
Fusarium circinatum	>20	>20	>20	>20	1.25	1.25	10-20	10-20	0.16-0.32	0.32	1.25-2.5	2.5	2.5	2.5
Fusarium verticcillioides CECT 2982	20	20	>20	>20	0.64-1.25	0.64-1.2	10-20	10-20	0.16	0.32	2.5	2.5	1.25-2.5	1.25-2.5
Penicillium itallicum CECT 2294	>20	>20	>20	>20	2.5	2.5	20	>20	0.16	0.32-0.64	5	5-10	2.5-5	2.5-5

*MIC and MLC were determined by a macrodilution method and expressed in µL/mL (v/v).

N.T- not tested. Results were obtained from three independent experiments performed in duplicate. When different MIC values were obtained a range of values is presented

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5. Nematicidal Activity

5. Nematicidal activity

5.1 Introduction

Nematodes are a group of vermiform animals that can be found in nearly all the environments and often as parasites of other organisms. Plant-parasitic nematodes cause important widespread pests, reducing the productivity due to both pathogenic effects and the wide range of hosts they can infect. These nematodes are responsible for around 11 % losses in agricultural production and are among the most difficult crop pests to control (Chitwood, 2002). The sedentary endoparasitic root-knot nematodes (RKN) belong to the genus *Meloidogyne* (Göeldi, 1892) and are obligate parasites of more than 3000 plant species (Abad et al., 2003). They normally reproduce and feed on modified root plant cells (Moens et al., 2009), causing damage on the root systems and, consequently, decreasing nutrient and water uptake and photosynthesis, leading to weak and poor-yield plants. Plant health is seriously affected, becoming more susceptible to other pathogenic agents such as fungi, bacteria and viruses (Abad et al., 2003; Moens et al., 2009). Symptoms caused by nematode infection in the aerial part of the plants (lack of vigor, chlorosis, stunting and wilting) are similar to those triggered by bacteria, fungi or viruses or even with those associated with mineral deficiencies making difficult to distinguish at the first glance the cause of the pest or disease (Whitehead, 1997).

The genus *Meloidogyne* includes more than 90 species, being *M. arenaria*, *M. incognita*, *M. javanica* and *M. hapla* the most common (Hussey and Jansen, 2002; Wesemael et al., 2011). In Portugal, *M. chitwoodi*, *M. hispanica* and *M. lusitanica* have also been reported associated with important agricultural crops (Abrantes et al., 2008; Conceição et al., 2009; Maleita et al., 2011).

The interaction between RKN and the plants is quite complex since the nematodes establish an intimate relationship with their host, manipulating plant gene regulation and metabolism by releasing specific secreted effector molecules (Schaff et al., 2007). The nematodes hatch as second-stage juveniles (J2), the infective stage. They penetrate plant roots at the elongation zone with further migration to the root apex. Here they reach the vascular tissues and move back towards the differentiation zone, where the permanent feeding site is induced and maintained, a crucial step for nematode reproduction and development (Vanholme et al., 2004) After the establishment of a

host-parasite relationship, the J2 molt three times becoming adults. The females remain sedentary and lay eggs into a gelatinous matrix that keep the eggs together and protected from adverse environmental conditions and predation (Fig. 1).

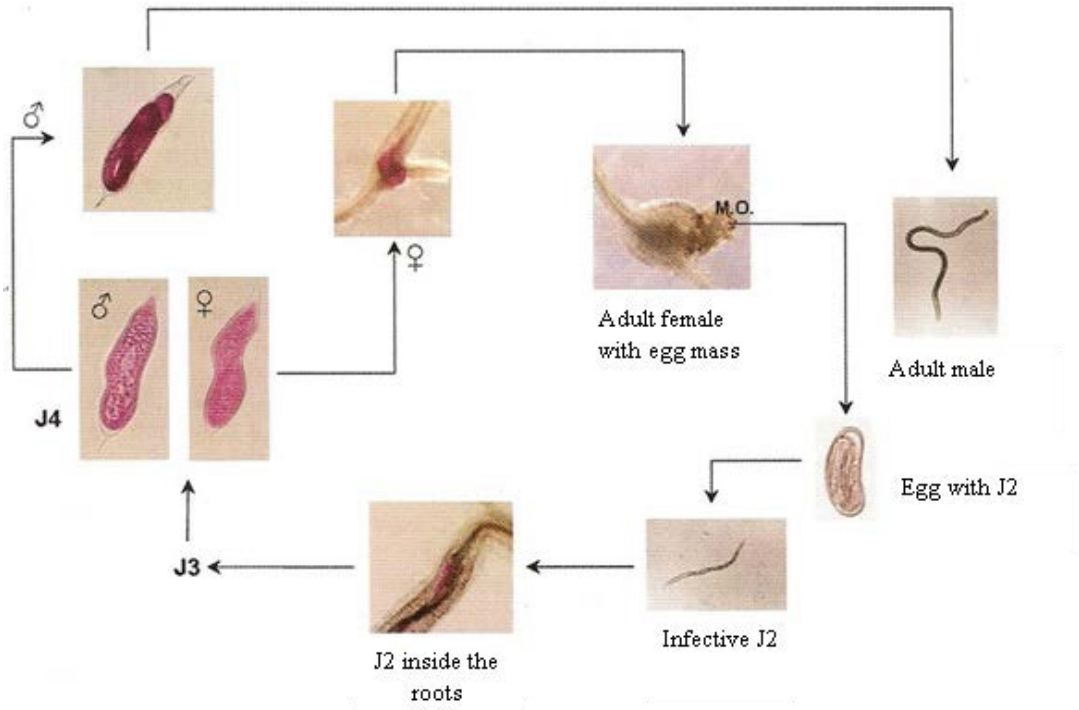

Figure 1. *Meloidogyne* spp. life cycle.
Adapted from: Abrantes et al. (2007).

Within the egg, embryogenesis proceeds to the first-stage juvenile (J1), which molts into the infective J2 (Moens et al., 2009). Second-stage juveniles need to locate a host as soon as possible since they depend on their own reserves stored in the intestine and their ability to invade roots (infectivity) is reduced after long periods in the soil (Karssen and Moens, 2006). Males are vermiform and migrate out of the root (Fig. 1), depending on their own intestine reserves to survive.

Plant cells infected by the nematodes undergo a differentiation process forming 5-7 multinucleated cells known as giant cells. Associated with cortical cell proliferation and hypertrophy this process leads to the formation of typical root galls.

Once nematodes have been established in the soil, it is very difficult to eliminate them. Therefore, management strategies have been mainly focused on attempts to reduce the nematode population in order to increase the crop yield with less economic

losses (Coyne et al., 2009). Quarantine measures have also been adopted to decrease the risk of spread and introduction of new species (Moens et al., 2009). In the past, many synthetic pesticides, such as carbamate, organophosphate, and organophthalide formulations, were used as chemical nematicides. However, due to adverse environmental effects and health concerns, their use has been drastically restricted, with many of them being banned or under revaluation (Ntalli et al., 2010). Nowadays, nematode control relies mostly on cultural practices, crop rotation, use of resistant cultivars and on the application of a few synthetic nematicides still authorized (Chitwood, 2002; Dufour et al., 2003). Nevertheless, crop rotation is of limited value for *Meloidogyne* spp., due to its wide host range (Trudgill, 1997), and although resistant cultivars have proven to be successful in the management of *Meloidogyne* on tomato, the presence of virulent biotypes challenge these resistant cultivars (Castagnone-Sereno, 2002). Therefore, the increasing awareness of producers and consumers on the risks of chemical nematicides together with the ineffectiveness of alternative methods, have stimulated the development of new bio-nematicides, environmentally safe, inexpensive, and agronomically useful (Haydock et al., 2006).

Plants and their secondary metabolites are valuable sources of bioactive compounds which can be used as biocides or as models for the synthesis of effective ecofriendly chemicals (Akhtar and Mahmood, 1994; Chitwood, 2002). In particular, plant essential oils from the *Lamiaceae* family have been explored in agriculture, based on their efficacy against crop pathogens, including bacteria (Karamanoli et al., 2005; Hyldgaard et al., 2012), fungi (Ćosić et al., 2010) and insects (Papachristos et al., 2004; Isman, 2006; Ebadollahi et al., 2010). Regarding RKN, some studies have reported the nematicidal activity of *Lamiaceae* essential oils and/or its isolated compounds mainly against *M. incognita* (Echeverrigaray et al., 2010; Ntalli et al., 2011) and *M. javanica* (Sangwan et al., 1990; Oka et al., 2000; Oka, 2001; Onifade et al., 2008). Moreover, many of these essential oils are included in the Generally Recognized as Safe (GRAS) list fully approved by the FDA and EPA since minimal adverse effects on humans and on the environment occur.

The aim of this study was to evaluate the effect of lavender essential oils on hatching, mortality and infectivity of *M. javanica*, in order to develop a potential lavender-based nematicide for agricultural purposes.

5.2 Material and methods

Preliminary experiments were performed in order to select the most promising lavender essential oils with nematicidal properties, being *L. viridis* the most active oil and, therefore, selected to conduct the hatching, mortality and infectivity bioassays.

The species *L. multifida* and *L. latifolia* were not included in these assays since the oil of the former reacted with the solvent used, interfering with the final results, and due to limitations in getting enough plant material of the latter, as referred in the previous chapters.

5.2.1. Essential oil

The oil of *L. viridis* (A) used in the nematicidal experiments was obtained from plants collected at Barranco do Velho region, at the South of Portugal.

5.2.2. Nematode isolate

A RKN isolate, obtained from infected *Solanum tuberosum* L. roots collected in Guarda, centre of Portugal, and identified as *M. javanica*, was maintained on tomato, *S. lycopersicum* L. cv. Easypeel. Plants were reared and maintained in a growth chamber at 25±3 ºC, with a 12 h photoperiod at the Nematology Laboratory at the University of Coimbra. The species identification was confirmed by esterase phenotype analysis (Pais et al., 1989).

5.2.3. Hatching bioassay

The concentrations of the essential oil (0.64-20.0 µL/mL) were prepared by serial dilution with 5000 ppm Triton X-100. RKN egg masses were handpicked from infected tomato roots and transferred to Petri dishes with tap water. Eggs were released with the help of a needle and 15 eggs with J2 were handpicked and transferred to glass-staining bocks with 1 mL of each oil concentration (Fig 2a-c) and maintained in a moist chamber, in the dark, at 25 ºC. All treatments and controls (tap water and Triton X-100) were replicated 5 times and hatched J2 recorded every 24 h, up to 360 h, using a stereoscope (Leica Zomm 2000). Data was converted to percentage cumulative hatching inhibition, corrected by Abbott's formula (Abbott, 1925) with reference to Triton X-100

control. Data derived from the 288 h observation were subjected to probit analysis (Finney, 1971) using IBM SPSS Statistics v.19 for Windows.

5.2.4. Mortality bioassay

Egg masses, handpicked from infected tomato roots, were transferred to a 200 µm pore muslin sieve in a Petri dish containing tap water, and placed in a growth chamber at 25 ºC. After 24 h, the suspension containing hatched J2 was discarded and only the second hatched J2 in the following 24 h were used in the assay. Fifteen J2 were handpicked and placed into 1 mL of each essential oil concentration (Fig. 2a, b and d). The experiments were performed as referred in hatching bioassay. Nematode mortality was recorded at 1, 3, 6, 9, 12, 18, 20, 22 and 24 h and then every 24 h, up to 360 h, being immobilized J2 transferred to water to confirm mortality. Data was converted to percentage cumulative mortality, corrected by Abbott's formula (Abbott, 1925) with reference Triton X-100 control. Data derived from the 216 h observation were subjected to probit analysis (Finney, 1971) using IBM SPSS Statistics v.19 for Windows.

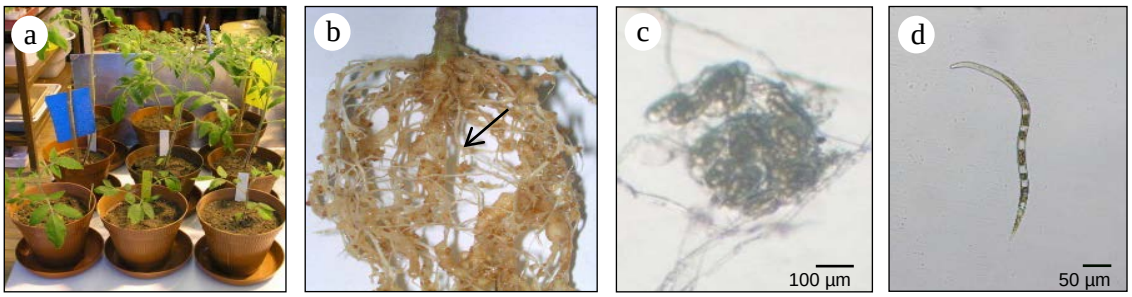

Figure 2. a) *Meloidogyne javanica* isolate culture on tomato (*Solanum lycopersicum*, cv Easypeel). b) Tomato roots with galls and egg masses (arrow). c) Embryonated eggs separated from the gelatinous matrix. d) *M. javanica* second-stage juvenile.

5.2.5. Infectivity/development/reproduction bioassay

For this experiment tomato plants were reared in 100 cm^3 plastic plots, with a mixture of autoclaved sandy loam soil and sand (1:1), in a growth chamber at 25±3 ºC for 3 weeks. The essential oil concentration (2.5 µL/mL) was chosen on basis of the J2

behavior in the mortality assay. Although this concentration did not cause J2 mortality, mobility was affected and, therefore, it seemed reasonable to test its ability to affect J2 infectivity and reproduction. Recently 200 hatched J2 were exposed to 2.5 µL/mL essential oil and controls for 2 h and transferred to tap water immediately before they were inoculated near the roots of tomato plants. In this way, the plants were never in contact with the essential oil. All treatments and controls were replicated 5 times. The plants were maintained in the growth chamber at 25±3 ºC, under a 12 h photoperiod. To study the penetration, post-embryogenic development and reproduction, observations were done every 5 days until 15 days and then at 30 and 50 days. The plants were uprooted and the roots, washed with water, were stained with acid fuchsin (Byrd et al., 1983) and visualized under a stereomicroscope. Infectivity was expressed, in comparison to control plants, as the number of J2 that penetrated the roots, number of galls, egg masses and adult females recorded in the root systems. To evaluate the reproduction, eggs were extracted from the roots, 50 days after inoculation (DAI), with 200 mL of 0.5 % hypochlorite sodium (Hussey and Barker, 1973).

5.3 Results

5.3.1 Hatching bioassay

Hatching was 87.40 % in tap water and 85.82 % in TritonX-100. In general, in the presence of *L. viridis* essential oil, the inhibition of hatching increased in time and was dose-dependent. Hatching was significantly reduced in eggs (Fig. 3a) exposed to 20 µL/mL (ca. 91 %) and 10 µL/mL (ca. 70 %) essential oil concentrations, when compared to Triton X-100 control. The oil concentration 6.591 µL/mL inhibited hatching by 50 % (IC50), after 288 h of exposure (Fig. 3b).

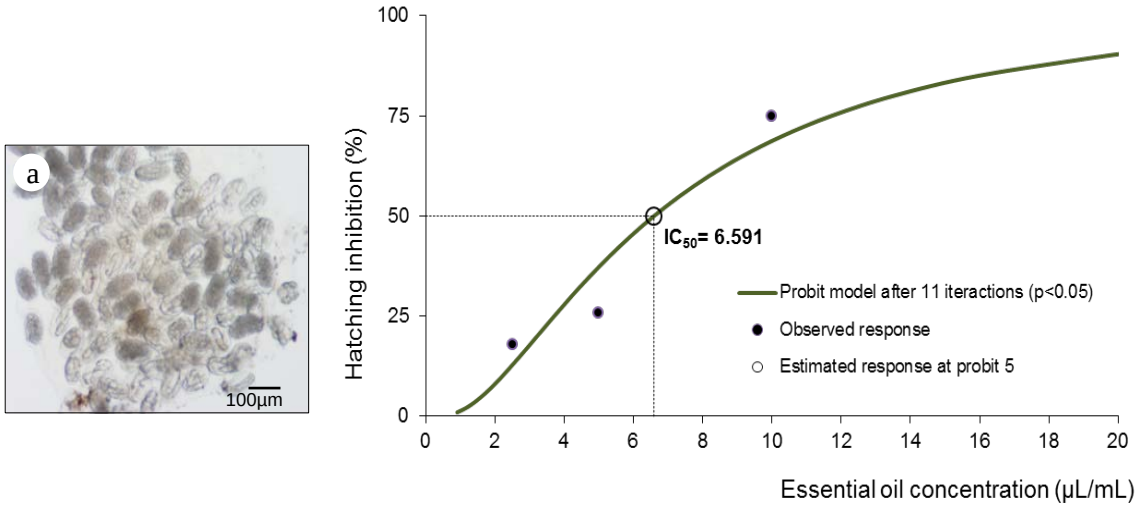

Figure 3. a) *Meloidogyne javanica* eggs. b) Probit plot of the effect of exposure for 288 h to different concentrations of *Lavandula viridis* essential oil on second-stage juvenile hatching.

5.3.2 Mortality bioassay

The tap water and solvent control (5000 ppm TritonX-100) did not affect J2 (Fig. 4a) mortality (Fig. 4b). On the other hand, 100 % of mortality was registered for J2 exposed to 20 µL/mL and 10 µL/mL of *L. viridis* essential oil after a 3 h and a 216 h of exposure, respectively. The essential oil concentration 6.271 µL/mL induced J2 mortality by 50 % (IC50), after 216 h of exposure (Fig. 4b).

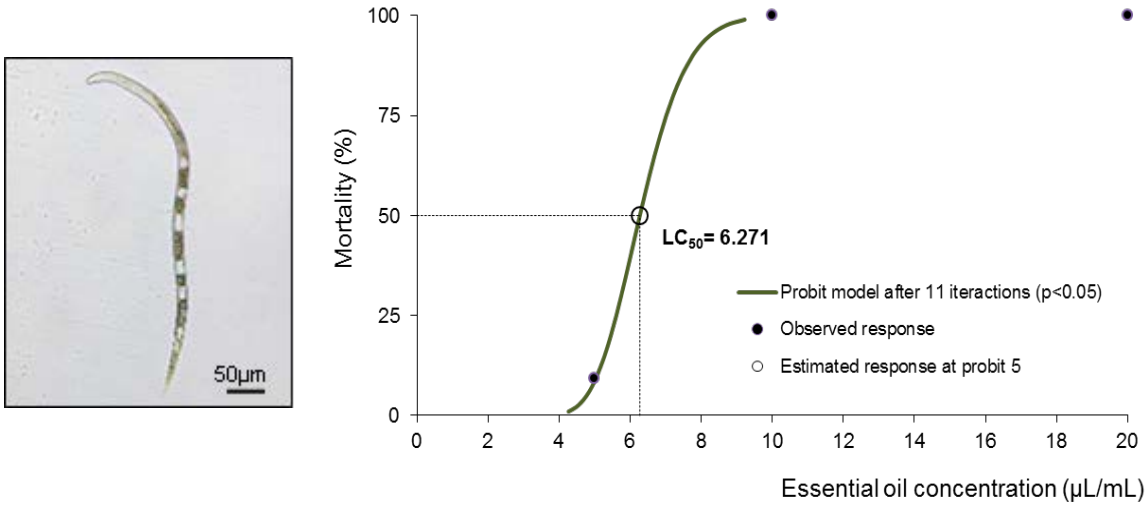

Figure 4. a) *Meloidogyne javanica* second-stage juvenile (J2). b) Probit plot of the effect of exposure for 216 h to different concentrations of *Lavandula viridis* essential oil on J2 mortality.

5.3.3 Infectivity/development/reproduction bioassay

The exposure of J2 to 2.5 µL/mL of *L. viridis* essential oil for 2 h inhibited its infectivity on a susceptible host. The J2 penetration significantly decreased after 3 (73.73 %) and 5 days (82.96 %) of inoculation (Fig. 5a and 5b). Moreover, 30 DAI, the number of galls (Fig. 5c and 5d) and females (Fig. 5e and 5f) were lower in tomato roots treated with the essential oil (decreases of 34.01 % for galls and 60.69 % for females). Fifty DAI, the number of egg masses (Fig. 5g and 5h) and eggs (Fig.5i and 5j) also decreased 37.47 % and 58.33 %, respectively.

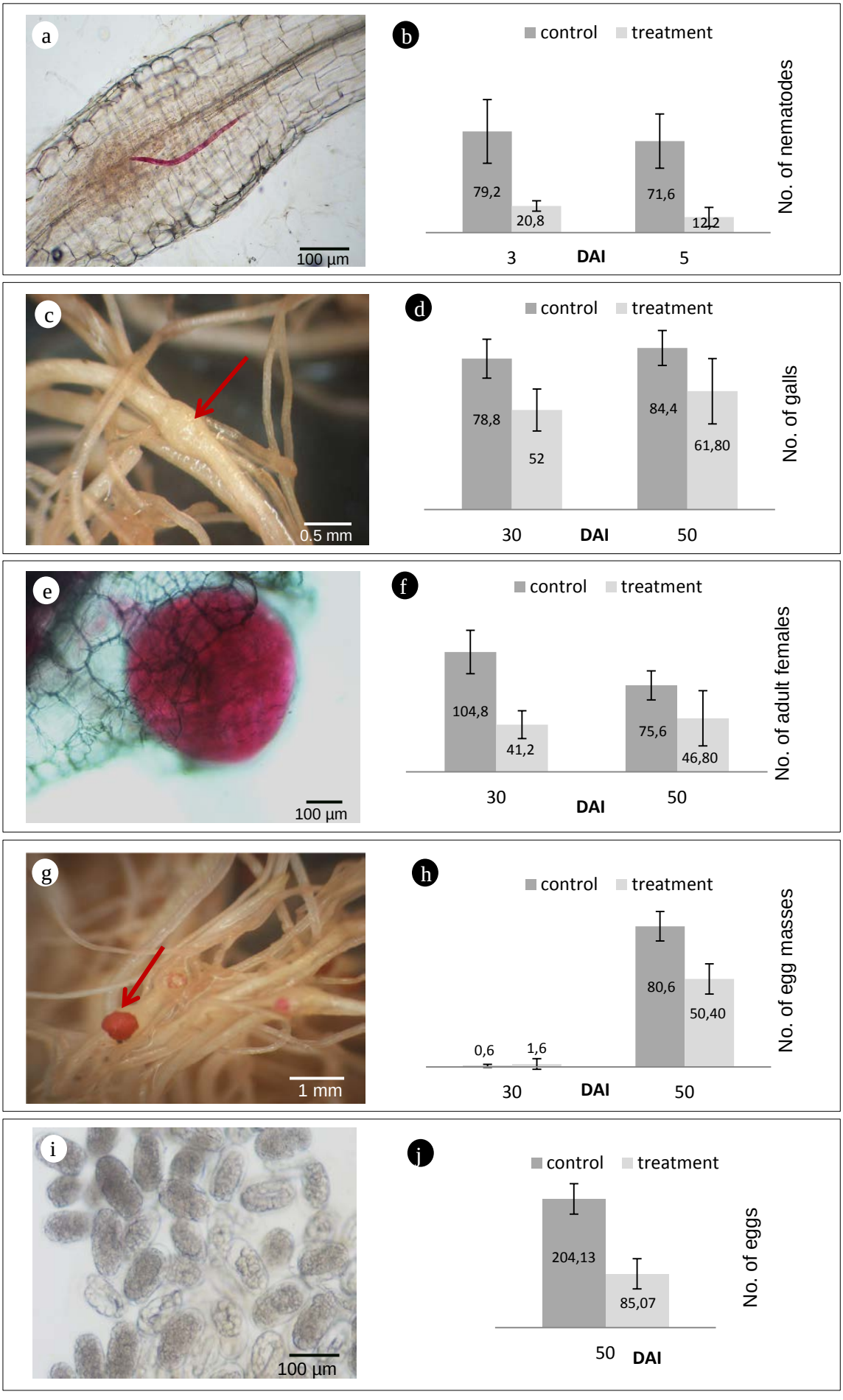
a
100 µm
b
control
treatment
No. of nematodes
79,2
20,8
71,6
12,2
3
DAI
5
c
0.5 mm
d
control
treatment
No. of galls
78,8
52
84,4
61,80
30
DAI
50
e
100 µm
f
control
treatment
No. of adult females
104,8
41,2
75,6
46,80
30
DAI
50
g
1 mm
h
control
treatment
No. of egg masses
0,6
1,6
80,6
50,40
30
DAI
50
i
100 µm
j
control
treatment
No. of eggs
204,13
85,07
50
DAI

Figure 5. Effect of *Lavandula viridis* essential oil on second-stage juveniles penetration 3 (a and b) and 5 days after inoculation (DAI) (b); number of galls (arrow) (c and d); adult females 30 (e and f) and 50 DAI (f); number of egg masses (arrow) 30 (h) and 50 DAI (g and h) and number of eggs 50 DAI (I and j). Data are means of five replicates.

5.4. Discussion

Few studies on the nematicidal activity of essential oils against RKN have been reported (Sangwan et al., 1990; Leela et al, 1992; Oka et al., 2000; Oka 2001; Pérez et al., 2003; Ibrahim et al., 2006; Wiratno et al., 2009).

Regarding *M. javanica*, the effect of 27 essential oils, including *L. officinalis* oil, *in vivo* and in pot experiments, was evaluated (Oka et al., 2000). The oils of *Carum carvi*, *Foeniculum vulgare*, *Mentha rotundifolia* and *M. spicata* were highly effective, causing J2 immobilization and hatching inhibition at concentrations of 800 µL/L and 600 µL/L, respectively, while *L. officinalis* oil (1 mL/L) was one of the less effective, causing 11.8 % of J2 immobility. Moreover, hatching was 23.1 % in the presence of this oil, against 32.5 % in control. In the pot experiments, the effect of the oil was only evaluated on root galling (Oka et al., 2000), being the best results obtained with the oils of *Coridothymus capitatus*, *Mentha rotundifolia*, two chemotypes of *Origanum vulgare* (carvacrol and thymol types) and *O. syriacum* (carvacrol type) at a concentration of 100 mg/kg.

In this study, the *L. viridis* oil was more effective than that of L. *officinalis*, since it was able to cause J2 mortality (IC50 = 6.27 µL/mL) whereas for *L. officinalis* only immobilization was registered. Regarding the hatching inhibition assay, 50 % of inhibition was obtained in the present study (IC50 = 6.591 µL/mL) but higher concentrations of the oil were used, in comparison to *L. officinalis*. On the other hand, the results of the infectivity assay with J2 treated with *L. viridis* oil for 2 h, were based on the evaluation of several parameters, J2 penetration, root galling and number of females, egg masses and eggs. The results showed that a lower concentration (2.5 µL/mL) was able to significantly affect all the parameters.

According to Onifade et al. (2008), *in vitro* and greenhouse experiments performed with *M. javanica*, showed that the most effective results were obtained with a mixture of *Haplophyllum tuberculatum* and *Plectranthus cylindraceus* (1:1) oils. The mixture (12.5 µg/mL) was very toxic to *M. javanica* causing 100 % of hatching

inhibition and J2 mortality (*in vitro* assays) and formation of less root galls (pot assays), suggesting that the J2 infectivity was also affected. These results also indicate that a synergistic effect among the essential oil components may occur, resulting in an increased effect when both oils are present. The analysis of the effect of monoterpenoids usually present in essential oils proved that eugenol, linalool and geraniol exhibited a non-specific activity against *M. javanica* and other nematodes (Sangwan et al., 1990). Later, the effect of other essential oil components against *M. javanica* were also evaluated, showing that the main compound of *Foeniculum vulgare*, *trans*-anethole, had the highest nematicidal activity *in vitro* whereas carvacrol, the main compound of several *Thymus* and *Origanum* species, was the most active in reducing gall formation (Oka et al., 2000). Other compounds related to *trans*-anethole also showed effective nematicidal activity against *M. javanica* (Oka, 2001). Moreover, aldehydes like cinnamaldehyde (present in cinnamon oil) and citral (present in *Cymbopogon* spp.) exhibited a very high nematicidal activity and were the most active compounds tested against nematodes (Oka, 2001; Tsao and Yu, 2000). Considering the chemical composition of *L. viridis*, oxygenated monoterpenes are the most abundant being 1,8-cineole the main compound (34.5 %). In a preliminary experiment, 1,8-cineole was less active on the hatching inhibition than the oil (data not shown), suggesting a synergistic effect among the components of *L. viridis* oil.

The mechanism of action of the essential oil in nematodes is not clear. Possible mechanisms include the interference with the neuromodulator octopamine (Kostyukovsky et al., 2002) or GABA-gated chloride channels as reported for insect pests (Priestley et al., 2003); changes in protein structure of the nematode surface (Bargmann et al., 1993) and/or disruption of biological membranes, as suggested for antifungal activities (Vale-Silva et al., 2010). In a previous study, on the mode of action of *L. viridis* oil on *Candida albicans* (see section 1 of the present chapter), the fungal cells became metabolically inactive before the oil caused cell death. A mechanism of action starting from damage to mitochondrial membranes, changes in mitochondrial permeability, generation of free radicals followed by the damage of essential biomolecules, including lipids, proteins and nucleic acids, cytoplasmic membranes disruption and cell death was proposed (Zuzarte et al., 2011). These findings and the asymmetry observed in the present study between the concentration of *L. viridis* oil

necessary to cause J2 mortality (IC50=6.27 µL/mL, 216 h of exposure) and the concentration responsible for compromising infection (2.5 µL/mL, 2 h of exposure), suggest that a potential mechanism of action relying on primary leakage of cytoplasmic contents, due to direct damage to cell membranes, can be excluded.

In conclusion, it was demonstrated that *L. viridis* essential oil has a nematicidal activity against RKN and has potential to develop a novel bio-nematicide eco-friendly and environmentally safe. It offers a good non-chemical alternative for the RKN management. The essential oil was not phytotoxic to tomato plants, but further experiments are required to evaluate the effect of the oil on the management of RKN in field trials and on the non-target organisms that live in soil.

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6. Insect Repellency

6. Insect Repellency

6.1 Introduction

Repellents normally act by creating a vapor barrier (Brown and Hebert, 1997) that prevents arthropods from flying to, landing on or biting a given surface (Choochote et al., 2007). Over the last years research over repellent compounds has increased with many synthetic repellents being used to protect storage products (e.g. grains, fruits, tubers), control vectors that transmit parasites and pathogens to humans and animals and also to manage agricultural and forest pests (Isman, 2006; Nerio et al., 2010). However, the use of many synthetic repellents is associated to health and environmental risks, justifying the search for effective natural and ecofriendly alternatives. Essential oils have proven to be an excellent choice and some of them have already been incorporated in commercial repellant formulations (Maia and Moore, 2011). These compounds have shown high repellency activity against arthropods with most of the research focusing on Diptera species related to malaria, yellow fever, dengue and viral encephalitis (Nerio et al., 2010). Some studies on Coleoptera and Lepidoptera species that contaminate storage products have also been reported (e.g. Bazzoni et al., 2002; Papachristos and Stamopoulos, 2002; Hori, 2003; Chaubey, 2007; Ayvaz et al., 2008; Pérez et al., 2010;) as well as studies focusing on agricultural pests (Munneke et al., 2004; Sampson et al., 2005). However, the protective effect of essential oils usually dissipates very quickly due to their high volatility (Bernard, 2000). Therefore, the development of new formulations (e.g. Moretti et al., 2002; Nentwig, 2003; Chang et al., 2006; Maji et al., 2007), fixative additives (e.g. Blackwell et al., 2003; Yang and Ma, 2005; Choochate et al., 2007) or the production of combined repellents (Kamsuk et al., 2006) are strategies that should be considered to improve essential oils repellent effectiveness.

Lamiaceae species are widely known as a source of bioactive essential oils, being some of them efficient insecticides on a wide range of pests (Regnault-Roger et al., 1993; Maia and Moore, 2011). The insecticidal activity of the oils can be related to their repellent potential or other effects, namely larvicidal, growth inhibition and /or antifeedant properties.

Regarding *Lavandula* spp. essential oils, some studies have reported antifeedant effects of *L. angustifolia* (Regnault-Roger and Hamraoui, 1993), *L. luisieri* (González-

Coloma et al., 2006; González-Coloma et al., 2011) and *L. stoechas* (Ebadollahi et al., 2010); as well as repellent activity of *L. angustifolia* (van Tol et al., 2007), *L. officinalis* (= *L. angustifolia*) (Choi et al., 2002) and *L. hybrida* (Papachristos and Stamopoulos, 2002). Also domestic uses of lavender oils are known to combat mites, grain weevils, aphids and clothes moth (Hori, 1998).

One of the most important pathogens affecting the coniferous forests worldwide is the nematode *Bursaphelenchus xylophilus* (Steiner and Buhrer, 1934) commonly known as pine-wood nematode (PWN), the causal agent of pine wilt disease (PWD) (Mota and Vieira, 2008) and a quarantine species in the European Union. *B. xylophilus* was detected for the first time in Portugal in maritime pine tree (*Pinus pinaster)* in 1999 (Mota and Vieira, 2008). The life cycle of the PWN involves both a propagative and a dispersal cycle (Fig. 1). The later requires the presence of an insect vector, *Monochamus* spp.

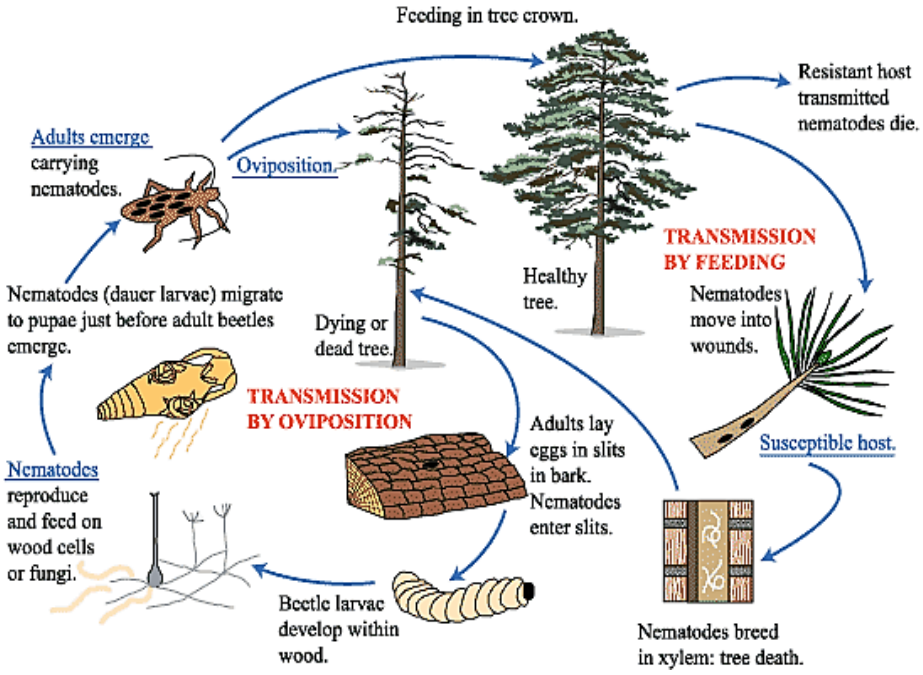

Figure 1. Life cycle of *Bursaphelenchus xylophilus*. The propagative cycle involves 6 life stages (egg, four larval stages and adult) and occurs in the sapwood. At the third larval stages two modifications may occur: the larvae can change into fourth stage and consequently into adults, remaining in the tress or turn into a nonfeeding dispersal stage. The later requires the presence of *Monochamus* spp. pupae in the wood. The third stage larvae are able to aggregate on the wall of the pupal chamber of the beetle and molt to a nonfeeding larval stage that survives the transport phase. These fourth stage larvae are transported to new hosts in the beetles respiratory system. Within 48 h, after transmission to a conifer, adults of *B. xylophilus* emerge.
Adapted from http://www.forestry.gov.uk/fr/GGAE-5RHJYD.

The genus *Monochamus* comprises ca. 150 species. In Europe 5 native species can be found, being *M. galloprovincialis* (pine sawyer) the most alarming species in Portugal. Until the introduction of *B. xylophylus* in Europe, the pine sawyer was considered a secondary forest insect (Naves et al., 2008). Nowadays, it is look upon as one of the most threatening species in coniferous forests due to its involvement in the dispersal of PWN. Control of PWD depends primarily on fumigation of infested trees with metham-sodium, aerial application of synthetic pesticides (thiacloprid) to control the insect vector and trunk injection with nematicides (e.g. abamectin, emamectin benzoate) (Kishi, 1995; Anon, 2003; Lee et al., 2003). However, many of the chemicals used are highly toxic, very expensive and can accumulate in the soil and water. For this reason and also due to European Union tight restrictions on the use of a large number of pesticides, it is urgent to develop new natural control strategies. Phytochemicals, namely essential oils have been suggested as excellent alternatives, with some studies showing their effectiveness on *B. xylophilus* (e.g. Pérez et al., 2003; Elbadri et al., 2008; Barbosa et al., 2010; Ntalli et al., 2010). Regarding the insecticidal activity of essential oil against the insect vector, *Monochamus* spp., studies are very scarce (e.g. Li and Zhang, 2007). Taking into account this information and the urgent need for effective alternatives, a preliminary study on the repellency effect of *Lavandula* spp. oils was conducted against *M. galloprovincialis*.

6.2 Material and methods

6.2.1 Essential oils

The oils of four *Lavandula* species (*L. luisieri*, *L. multifida*, *L.pedunculata* and *L. viridis*) were used in the insect repellent assay (Table 1). The chemical composition of these oils is shown in section 3 of the previous chapter.

Table 1. Site of collection of *Lavandula* spp. used in insect repellent assays

Species	Region	Site of Collection	Sample
L. luisieri	Coimbra	Pióda̋o	D
L. multifida	Setúbal	Sesimbra	AI
L. pedunculata	Bragança	Serra da Nogueira	B3
L. viridis	Algarve	Barranco do Velho	A

6.2.2 Synthetic chemicals

Authentic samples of *α*-pinene (99 % purity) and ethanol (99% purity) were used as positive controls.

6.2.3 Insect population

Dead pine trees from Herdade da Comporta were felled during 2011 late winter early spring, and *M. galloprovincialis* colonized tree parts (trunk and branches) were brought to Instituto Nacional de Recursos Biológicos Laboratory (INRB, Oeiras, Portugal) where last instar post-diapause larvae were isolated from the pupation chambers and placed individually inside plastic Petri dishes, at 10 ºC to stop development until it was necessary for further studies. Following this step the stored larvae were placed at 25 ºC under a 12:12 (light/dark) photoperiod at the Entomology Laboratory of INRB to fasten development into adult forms.

Newly formed adults were kept at room temperature with males and females kept apart. After about 5 days of emergence still immature females and males were

paired and used in repellency tests. A total of 20 couples were chosen for each set of experiments (Figure 2).

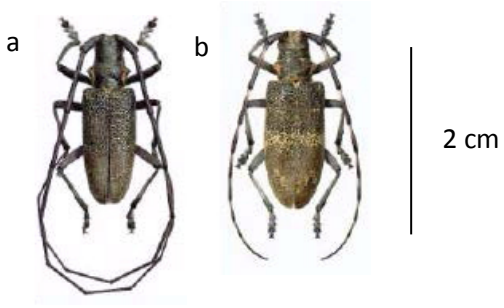

Figure 2. *Monochamus galloprovincialis* adults. a) Male with black antennae, 2-3 times longer than the body. b) Female with whitish rings on the antennae, 1/3 or 1/2 times longer than the body.

6.2.4. Repellency test

The repellency tests were performed at the INRB laboratories under supervision of Dr. Luís Bonifácio. To test the effects of the essential oils on the behavior of *M. galloprovincialis* a wind-tunnel assay was used (Fig. 3 and 4). The wind tunnel (2 m long x 1 m wide) was divided into four sections (I, II, III, IV) each 25 cm long (Fig. 3a and 3b). Twenty couples of *M. galloprovincialis* (Fig. 3c and 3d) were tested for each essential oil (Fig. 3e) and the experiments repeated 3 times. A mixture of α-pinene and ethanol were used as control. In each test run, one individual was released in the center of the tunnel (Fig. 3f), 50 cm downwind from the chemical source. The wind velocity was adjusted to 0.5 m/s and the behavior of each insect was recorded for 5 min. The temperature of the wind-tunnel during the experiments was around 20 ºC and the experiments were performed in the dark, since light caused insects immobility. Also, the test room was constantly ventilated avoiding overload of volatiles.

The behavioral responses of the insects were grouped as:

1. Immobilized
2. Running time
3. Initiating flight

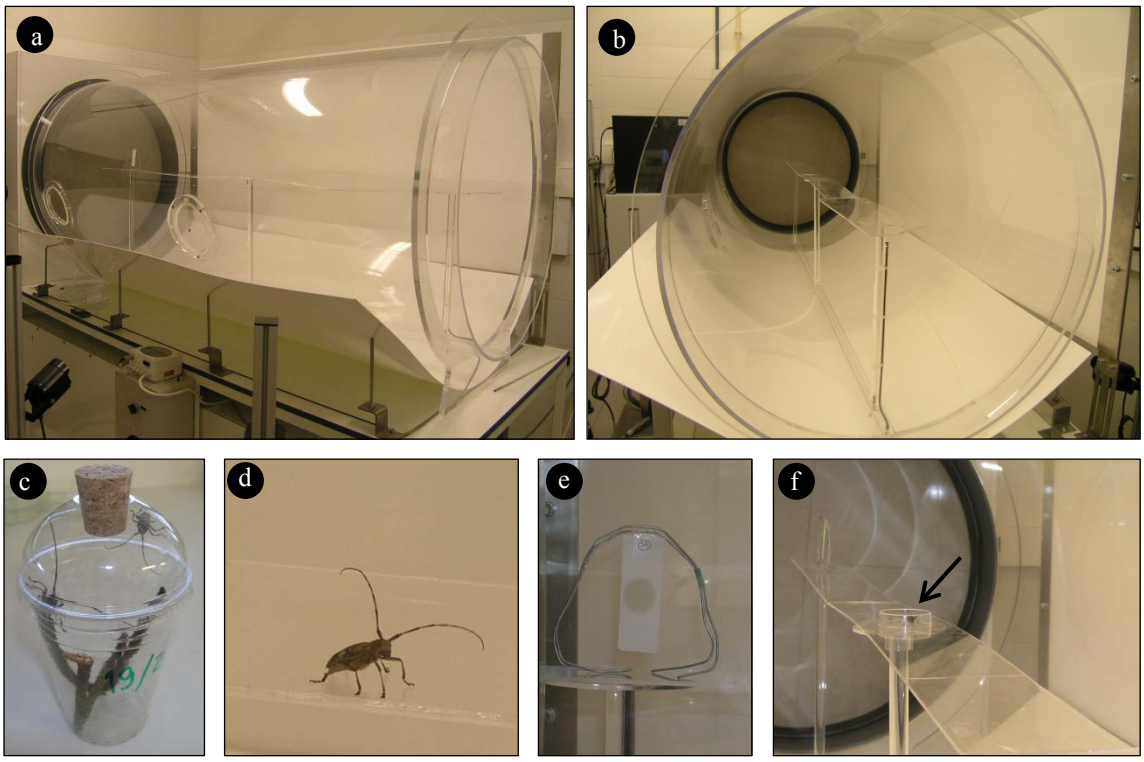

Figure 3. Wind-tunnel assay. a) Lateral view of the wind tunnel. b) Front view of the wind tunnel. c and d) *Monochamus galloprovincilais* coupled up before the assay (c) and during the test (d). e) Filter paper with 20 µL/mL of essential oil. f) Placement of the insect in the middle of the tunnel (arrow).

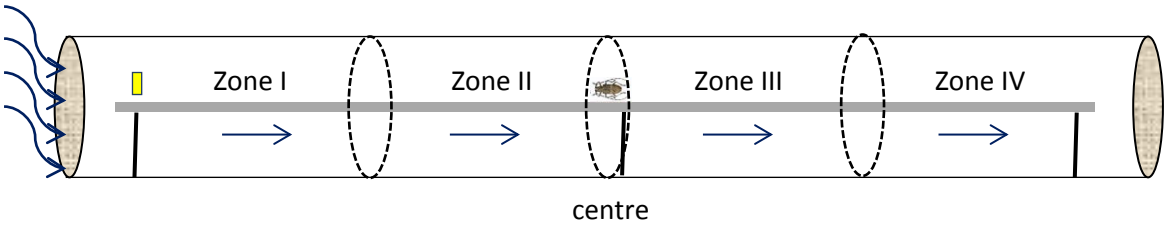

Figure 4. Schematic drawing of the wind tunnel showing its division into 4 sections (I, II, III, IV) each 25 cm long. At the beginning of each experiment, the insect was placed in the center of the tunnel and its behavior registered for 5 min.
▯ Chemical source (essential oil or control).

6.2.5 Statistical analysis

The analysis of insects behavior was based on Fettköther et al. (2000) with results expressed as percentage of insects responding to each volatile from total tested insects. Results were compared to control by Fisher's exact test using Statistica Software.

6.3 Results and discussion

The use of *α*-pinene and ethanol as a control simulates a weakened host plant that insects choose to feed on and also for egg posture. *α*-pinene is the main compound of *Pinus* essential oil and ethanol is produced by plant cells in decline. This mixture is therefore an attractant for mature insects (Bonifácio, 2009). In the lab, the insects did not show complete attractant behavior since food was provided until and during the experiments. In this way, the behavior observed in the presence of these compounds was insects wellbeing, i.e., the insects did not show any pattern of agitation and many times remained motionless. On the other hand, some of the oils tested disturbed the insects, causing agitation and, in some cases, insects flew away from the scent odor, demonstrating the repellent effect of the odor.

The essential oils of *L. luisieri*, *L. pedunculata* and *L. viridis* caused a higher disturbance (repellant activity) since a significant lower percentage of insects remained immobile (Table 2; Fig. 5a) and a higher number flew away from the scent odour (Table 2; Fig. 5b), in comparison to control. The essential oils from *L. luisieri* and *L. viridis* were the most effective, showing an higher repellent behavior (Fig. 5b). On the other hand, *L. multifida* essential oils showed an opposite effect, with and higher percentage of insects remaining immobilized (Table 2; Fig 5a) and a lower number flying away (Table 2; Fig. 5b) suggesting an attractant effect of the oil. Curiously, this attractant effect (insect wellbeing) was higher than that shown in the control.

Besides immobility and flying, the number of insects arriving at each zone of the tunnel (I, II, III, IV; Fig 4) during the 5 m in assay, was also recorded. The results showed that in the presence of *L. luisieri* or *L. viridis* oils, the insects passed through the zones III and IV (away from the odor scent) more times, confirming the repellant potential of these oils (Fig. 6).

Table 2. Behavioural response of insects to *Lavandula* spp. essential oils.

Scent source	Immobility (%)	Flying (%)
Control (α-pinene + ethanol)	20	10
Oils		
Lavandula luisieri	5***	25***
Lavandula multifida	35*	5*
Lavandula pedunculata	5**	15**
Lavandula viridis	10***	30***

Results are expressed as percentage of total insects testes and analysed using Fisher´s exact test (* $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.0001$, compared to control)

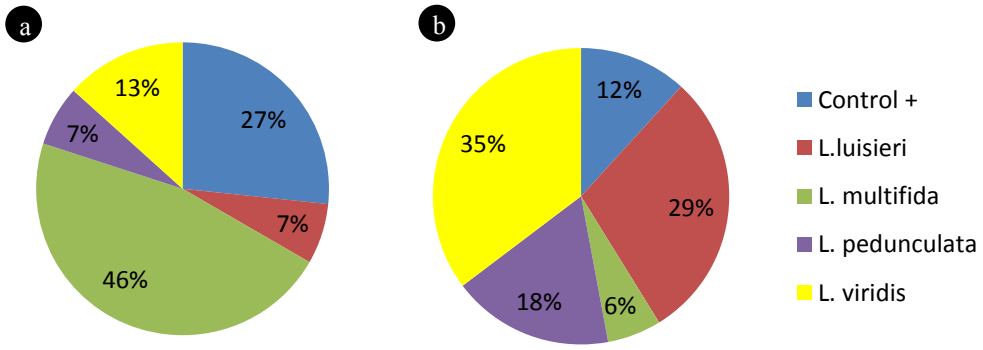

Figure 5. Percentage of insects that a) remained immobilized during the assay and b) flew away from the scent source during the assay.

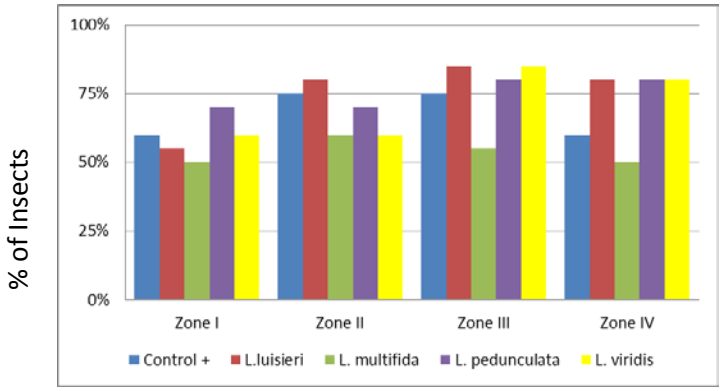

Figure 6. Insect behavior during the wind-tunnel assay. Bars indicate the percentage of total insects for each condition tested, arriving at the several zones of the tunnel during the 5 min assay.

Taken together, these preliminary results indicate that the essential oils of *L. luisieri* and *L. viridis* have potential to be further used in the development of biological repellents against *M. galloprovincialis*.

L. luisieiri essential oil (sample D) is characterized by high amounts of necrodane derivatives (see table 3, section 3, chapter II). These compounds have previously been found in the defensive secretions of the beetle *Necrodes surinamensis* (Eisner et al., 1986; Roach et al., 1990) suggesting their potential defensive role. In fact, the oils of *L. luisieri* have previously shown antifeedant effects against *Spodoptera littoralis*, *Myzus persicae* and *Rhopalosiphum padi* (González-Coloma et al., 2011). Moreover, the necrodane derivatives in combination with camphor (effect estimated through linear regression) increased the antifeedant effects against *R. padi* (González-Coloma et al., 2011).

Regarding *L. viridis*, this is the first report on the effect of its oil against insects. *L. viridis* essential oils are very homogenous being characterized by high amounts of 1,8-cineole and camphor (see table 6, section 3, chapter II). These compounds have been tested against several insects, For example, 1,8-cineole showed acaricidal activity against *Pediculus humanus* (Toloza et al., 2006), insecticidal effects on *Blatella germanica* (Jang et al., 2005), repellent activity against *Triboleum castaneum* and fumigant toxicity to *Sitophilus zeamais* (Wang et al., 2009) whereas camphor showed high toxicity to stored products insects due to acetylcholinesterase inhibition (Abdelgaleil et al., 2009; Lopez and Pascual-Villalobos, 2010). Therefore, the repellant activity of *L. luisieri* and *L. viridis* in the present study may be related to their main compounds, justifying further experiments with these compounds, in order to identify the active compounds responsible for the activity of the oils. Furthermore, field experiences are being considered however, problems related with essential oil volatility, poor water solubility and aptitude for oxidation, have to be resolved prior to their addition to any alternative control system (Moretti et al., 1998).

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IV. In Vitro Culture

1. Proliferation of axillary meristems

1.1 Introduction

The economic exploitation of aromatic and medicinal species has increased due to a renewed interest in the use of natural compounds (Kala et al., 2006). These metabolites can be obtained from wild field-growing plants or from cultivated plants. However, for environmental reasons the gathering of large amounts of plants growing in the wild must be avoided since this can threat the species and reduce biodiversity. Moreover, essential oils quality strongly depends upon environmental factors that may interfere in plant yield (Figueiredo et al., 2008). Also chemotypes and ecotypes make it difficult to obtain high quality and uniform essential oils for very competitive markets. In recent years micropropagation techniques have emerged cloning tools for the multiplication of selected genotypes with interesting chemical profiles (Chaturvedi et al., 2007; Sidhu, 2010; Verma et al., 2012).

Lavandula spp. essential oils are strictly regulated by ISO standards (e.g. ISO TC 54 - ISO/CD 8902, 2007; ISO TC 54 N - ISO/WD 4719, 2009) that require high-quality of the oils. As in other species, *Lavandula* spp. may be propagated sexually or asexually. However, reproduction through seeds is usually slow and the plants display a great variation in a n umber of characteristics such as growth rate and essential oil composition. Therefore, techniques of vegetative propagation are the most effective to produce a large number of individuals displaying a genotype of interest. However, multiplication through cuttings is slow, cumbersome and poor rooting is often observed (Segura and Calvo, 1991; Chawla, 2009; Canhoto, 2010). Moreover, due to repeated cycles of propagation, modifications in morphological and chemical characteristics have been reported among the regenerated plants (Moutet, 1980; Panizza and Tognoni, 1992). Considering all these aspects, micropropagation is assuming an increased importance in plant cloning used for the extraction of natural compounds. Micropropagation can assure a large-scale production in controlled conditions, in a short period of time, without negative impacts on habitats (Canhoto, 2010). From the three techniques of micropropagation usually used, proliferation of axillary meristems is the most suitable for plant cloning (Gahan and George, 2008) since propagation by organogenesis or by somatic embryogenesis may induce variability among the

regenerated plants (Deverno, 1995; Firoozabady and Mo, 2004; Jin et al., 2008), specially when an intermediate callus phase occurs.

Tissue culture techniques have been applied to several *Lavandula* species. For instance, axillary shoot proliferation was reported for *L. dentata* (Jordan et al., 1998; Sudriá et al., 1999, 2001; Echeverrigaray et al., 2005; Machado et al., 2011), *L. latifolia* (Sánchez-Gras and Calvo, 1996), *L. stoechas* (Nobre, 1996), *L. vera* (Andrade et al., 1999) and *L. viridis* (Dias et al., 2002; Nogueira and Romano, 2002). Direct shoot formation from different tissue explants was achieved in *L. latifolia* (Calvo and Segura, 1989a) and shoot formation from *callus* was reported for *L. angustifolia* (Quazi, 1980; Ghiorghita et al., 2009), *L.* x *intermedia* (Dronne et al., 1999), *L. latifolia* (Calvo and Segura, 1988, 1989b; Jordan et al., 1990), *L. officinalis* x *L. latifolia* (Panizza and Tognoni, 1988) and *L. vera* (Tsuro et al., 1999, 2000). A preliminary study on somatic embryogenesis of *L. vera* was carried out by Kintzios et al. (2002).

The health and agricultural potential of the essential oils of *Lavandula* spp. growing wild in Portugal justifies the development of efficient protocols for the rapid and successful *in vitro* propagation of these species. Previously, a reliable protocol for large scale propagation of *L. pedunculata* was established for the first time (Zuzarte et al., 2010). Now, a protocol for *in vitro* propagation of *L. multifida* is proposed, for the first time. This species occurs in confined regions of Portugal and due to its interesting chemical characteristics (high amounts of phenolic compounds) alternative propagation protocols need to be developed, avoiding excessive plant gathering in the wild and assuring plant uniformity. The development of effective protocols for *in vitro* culture is a crucial stage to achieve a more ambitious long-term objective which is to develop end-products of high-quality with a well-defined chemical profile for clinical and agricultural applications. Besides, *in vitro* propagation can be used to rapidly propagate interesting genotypes selected on the basis of the secondary metabolites they produce.

1.2 Material and methods

1.2.1 Plant material

Aerial parts of *L. multifida* (sample AI) were collected at Sesimbra (see table 1, chapter I) and maintained for a few days in the lab at room temperature with the basal part of the stems dipped in water to promote axillary shoot development and to facilitate the further isolation of axillary buds.

1.2.2 Micropropagation

1.2.2.1 Establishment of *in vitro* cultures

The developing axillary buds were dipped in 70 % ethanol for 30 s before surface disinfection (5 min) in a 7 % (w/v) calcium hypochlorite solution containing a drop of Tween 20. Following rinsing in sterile distilled water, the material was cultured on MS medium (Murashige and Skoog, 1962) supplemented with 3 % sucrose. Ascorbic acid (10 mg/L) was added to the culture media to prevent tissue oxidation. The pH of all media was adjusted to 5.75 prior to the addition of 6 g/L agar. Media were sterilized by autoclaving at 121 ºC for 20 min at 1.1 atm. Explants were cultured in test tubes (15 mL culture medium per test tube) and maintained in a growth chamber at 25 ºC ± 1 ºC with a photoperiod of 16 h light/8 h dark. Cultures were transferred to fresh culture medium every four weeks until enough stock material was available to perform further experiments.

1.2.2.2 Shoot multiplication

After three months in culture, nodal segments of the established shoots (≥ 0.5 cm) were subcultured for shoot proliferation on MS medium containing 3 % sucrose and different concentrations of benzyladenine (BAP: 0.0, 0.1, 0.2, 0.5 and 1.0 mg/L) or zeatin (ZEA: 0.0, 0.1, 0. 2, 0.5 and 1.0 mg/L). The media were prepared as described above and the nodal segments were cultured in test tubes and maintained in a growth chamber at 25 ºC ± 1 ºC with a photoperiod of 16 h light/8 h dark. To evaluate the

influence of the cytokinin concentration on shoot multiplication, both the number of explants producing shoots and the number of shoots obtained (≥0.5 cm) were recorded. Results were registered after 30 days of culture. Also, the number of explants developing *callus* was registered.

1.2.2.3 Shoot elongation

Following the proliferation stage, the shoots (≥0.5 cm) were transferred to MS medium with 3 % sucrose without plant growth regulators, in order to promote shoot elongation. The media were prepared as described above and the shoots were cultured in test tubes and maintained in a growth chamber at 25 ºC ± 1 ºC with a photoperiod of 16 h light/8 h dark. Results were taken after 30 days in culture and both the shoot length and the number of nodes per shoot were registered.

1.2.2.4 Shoot rooting

Shoots from the previous phase were cultured on MS medium with 3 % sucrose and supplemented with indol-3-butyric acid (IBA: 0.0; 0.5 and 1.0 mg/L). The media were prepared as described above and the shoots were cultured in test tubes and maintained in a growth chamber at 25 ºC ± 1 ºC with a photoperiod of 16 h light/8 h dark. The number of roots per plant as well as the length of the longer and shorter root in each plant was recorded after 30 days of culture. Then the shoots were placed in MS culture medium without growth regulators an allowed to grow for one month, before acclimatization.

1.2.2.5 Acclimatization

Rooted plantlets were placed in plastic pots containing a mixture of garden soil and perlite (1:1) (v/v). The pots were maintained in an acclimatisation chamber at 25±2 ºC with 70 % of humidity and a 16 h photoperiod for 3 weeks.

1.2.2.6 Statistical analyses

All experiments were conducted under a randomized block design including three replicates with 15 explants per treatment. Data were processed by analysis of

variance (ANOVA) and comparisons between the mean values of treatments were made using Fisher's test at the confidence level of $P \leq 0.05$ using Statistica 6.0 for Windows.

1.2.2.7 Light microscopy and histochemistry

To assure the uniformity of the *in vitro* propagated plants, the type of glandular trichomes in both the micropropagated plantlets and the respective field-growing parent plant were compared using LM and histochemical analysis.

For LM observations, sections (ca. 50-80 µm) of fresh leaves were obtained using razor blades. The sections were mounted on glass slides using the histomount mounting solution and observed unstained. Observations were made with a Nikon Eclipse E400 light microscope equipped with a Nikon Digital Sight DS-U1 photographic camera, using the Act-2U software.

The histochemical tests were performed in 30-50 µm thick sections of fresh leaves, obtained with a Sorvall® tissue sectioner. Terpenes were identified using NADI reagent (David and Carde, 1964). Observations were made in a Nikon Eclipse E400 light microscope equipped with a Nikon Digital Sight DS-U1 photographic camera, using the Act-2U software.

1.3 Results and discussion

The present study reports for the first time a simple and efficient protocol for the *in vitro* establishment and multiplication of *L. multifida* (Fig.1).

Figure 1. Micropropagation of *Lavandula multifida*. a) Adult plant. b) Establishment phase: inoculation of the axillary buds in test tubes containing culture medium. c) Multiplication phase: shoots formed after 30 days of culture in medium with cytokinins. d) Elongation phase: shoots after 30 days of culture in medium without growth regulators. e) Rooting phase: roots formed spontaneously or due to the addition of IBA to the culture medium. f) Acclimatization phase: transfer of plants to soil. g) Transfer of acclimatized plants to the greenhouse. Bars = 1cm.

1.3.1 Shoot establishment

The methodology used for explants disinfection was very efficient, allowing the establishment of more than 80% of aseptic cultures (Table 1). During the establishment phase the addition of ascorbic acid to the culture medium was irrelevant since necrotic explants did not occur (Table 1) on media without this antioxidant agent.

Table 1. Influence of ascorbic during the establishment phase of *Lavandula multifida*.

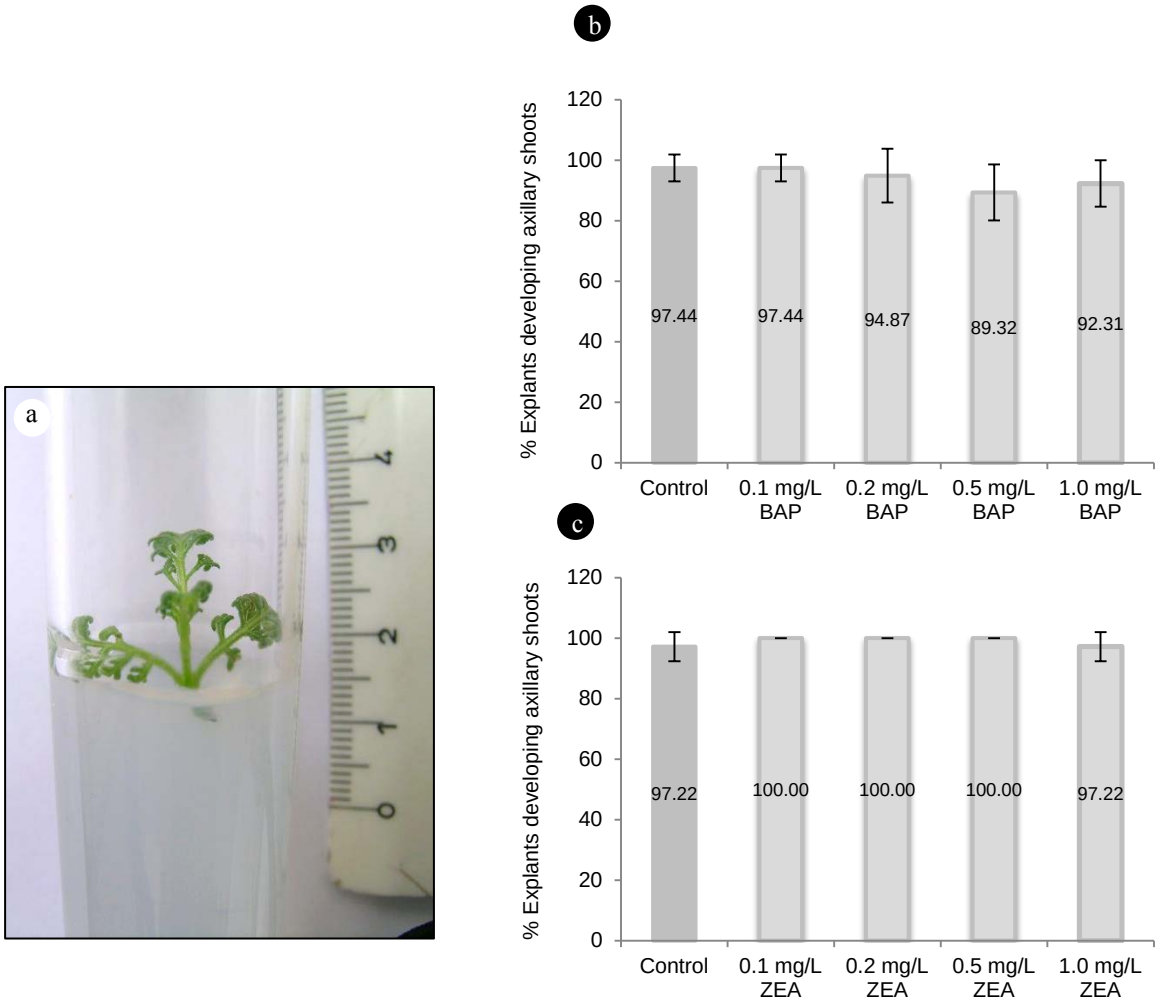
Culture Medium	No. inoculated explants	1 week of culture		
		Healthy	Contaminated	Necrotic
MS	60	52 (86,67%)	8 (13,33%)	0
MS + 10 mg/L ascorbic acid	60	49 (81,67%)	11 (18,33%)	0

1.3.2 Shoot proliferation

During the proliferation phase, the different concentrations of BAP and ZEA did not significantly interfere with the percentage of explants showing axillary shoot development (Fig 2a-2c). However, the number of shoots (≥0.5 cm) per explant increased with the addition of higher concentrations of ZEA to the culture medium (Fig. 3a-3c). The highest multiplication rate (6.14 shoots per explant) was obtained using MS supplemented with 1.0 m g/L zeatin (Fig. 3c). The efficiency of cytokinines was also shown on shoot multiplication of other lavenders: *L. dentata* (Jordan *et al.* 1998; Echeverrigaray et al., 2005), *L. officinalis* x *L. latifolia* (Panizza and Tognoni, 1992), *L. pedunculata* (Zuzarte et al., 2009), *L. stoechas* (Mesquita et al., 1990; Nobre, 1996) and *L. vera* (Andrade et al., 1999).

Some shoots displayed hiperhydricity. This is quite common during *in vitro* culture and has also been reported for other *Lavandula* species such as *L. dentata* (Echeverrigaray et al., 2005), *L. stoechas* (Nobre, 1996), *L. pedunculata* (Zuzarte te al., 2009) and *L. vera* (Andrade et al., 1999). Although several factors may induce the appearance of hiperhydricity (Debergh et al., 1992), the excess of cytokinins in the culture medium has been referred as one of the most important in promoting this

phenotype (Debergh et al., 1981; Leshem et al., 1988; Kataeva et al., 1991; Williams and Taji, 1991). In *L. multifida* a positive relationship also seemed to occur between the increase of hiperhydricity and the raise of cytokines in the culture medium. However, this phenotype did not impair plant regeneration in this species since hyperhydricity disappeared following plantlet transfer to MS medium lacking growth regulators.



Figue 2. a) *Lavandula multifida* after 10 days of culture in MS medium with cytokinnins. b and c) Effect of different concentrations of benzyladenine (b) and zeatin (c) on the percentage of explants developing axillary shoots. Each value represents the mean ± SEM from three independent experiments.

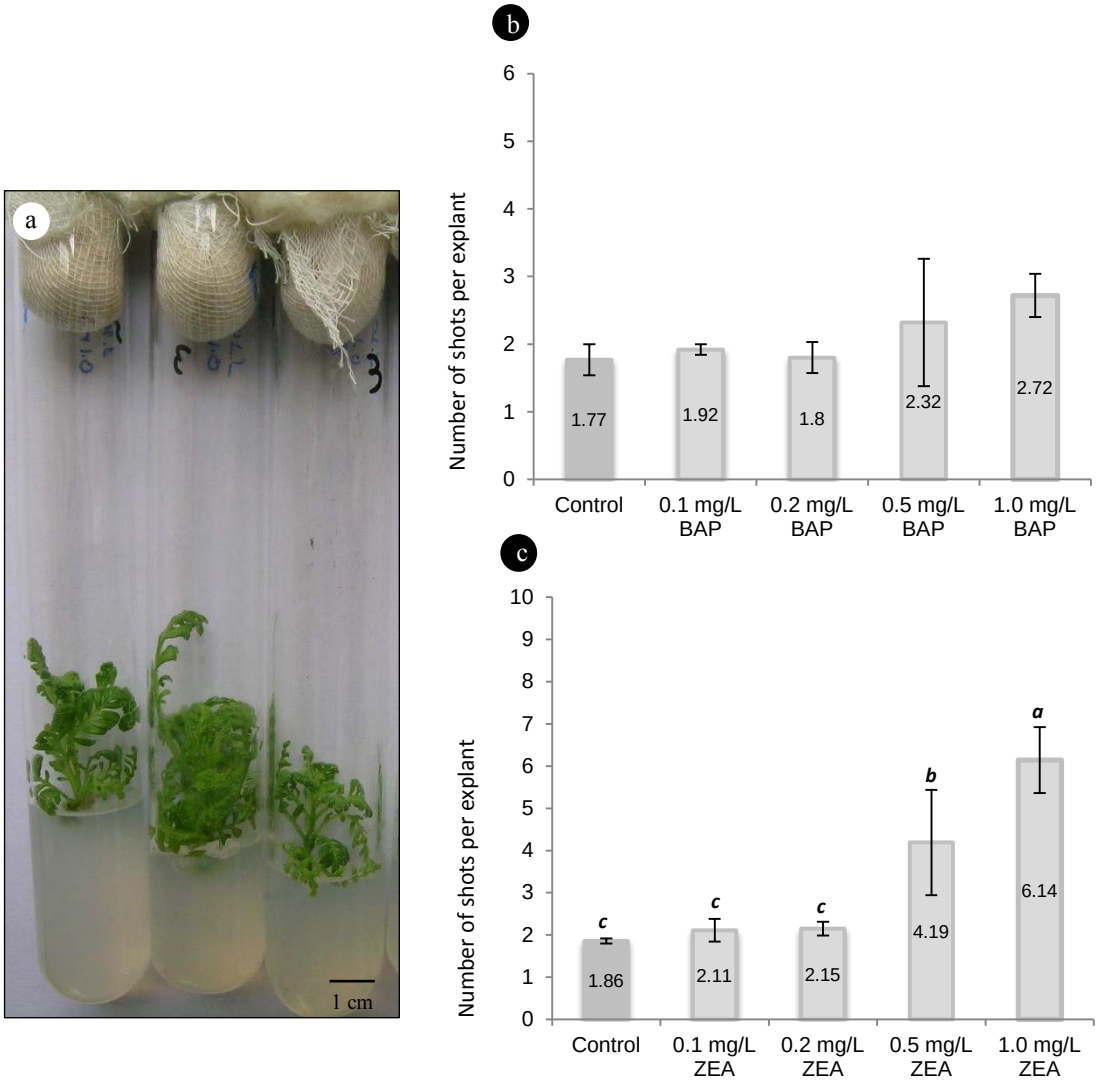
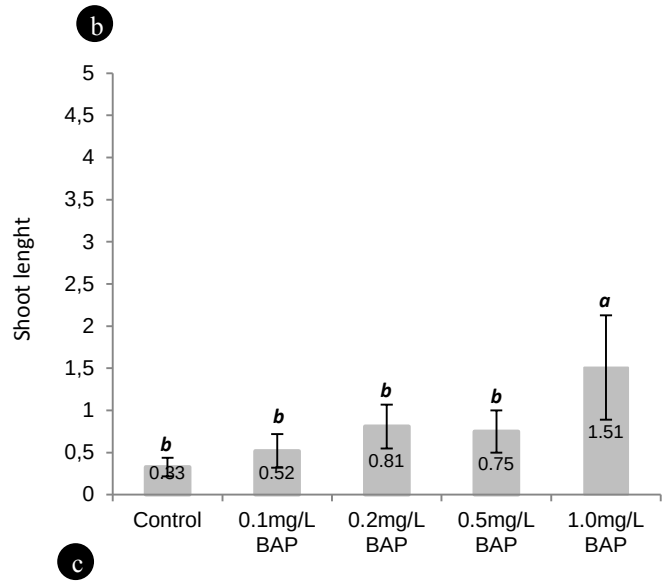


Figure 3. a) Multiplication phase of *Lavandula multifida*, 30 days after culture. b and c) Effect of different concentrations of benzyladenine (b) and zeatin (c) on the number of shoots per explant. Each value represents the mean ± SEM from three independent experiments; ***a***, ***b*** and ***c*** represent statistic differences for $p < 0.05$.

1.3.3 Shoot elongation

Following separation and transfer to MS medium without growth regulators, the shoots from all media elongated (Fig 4 and 5). During the elongation phase, the possible influence of BAP and ZEA present in the previous multiplication medium was evaluated with respect to shoot length, and number of nodes *per* shoot, after 30 days in culture in MS medium lacking growth regulators. Regarding shoot length higher concentration of both cytokinines in the initial multiplication medium induced longer shoots (Fig. 4) but only higher concentrations of BAP were responsible for a higher

number of nodes per shoot (Fig. 5a). Longer shoots (1.5 cm; Fig. 4b) and a higher number of nodes per shoot (3.73; Fig. 5b) developed in explants from MS medium with 1.0 mg/L BAP. These results indicate that in *L. multifida* the BAP concentration used in the multiplication medium has further influence in the elongation phase, namely on the length of the shoots and the number of nodes per shoot. This is similar to what has been reported for *L. pedunculata* (Zuzarte et al., 2009) and *L. stoechas* (Nobre, 1996) but different from what has been described for *L. latifolia* (Calvo and Segura, 1989) and *L. dentata* (Jordan et al., 1998). The number of nodes per shoot depends on the plastochron (Reinhardt and Kuhlemeier, 2002) and the present results seem to indicate that in *L. multifida* this parameter is strongly influenced by the concentration of BAP present on the medium with a certain level of the growth regulator reducing the plastochron, therefore increasing the number of nodes per shoot and the potential for multiplication.



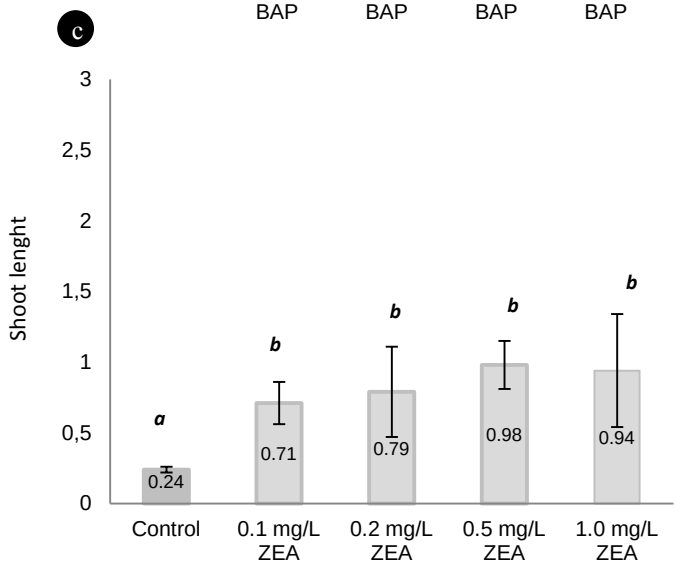


Figure 4. *Lavandula multifida* after 30 days of culture in MS medium. b and c) Influence of different concentrations of benzyladenine (b) and zeatin (c) in the previous multiplication medium on shoot length during the elongation phase. Each value represents the mean ± SEM from three independent experiments; ***a***, ***b*** and ***c*** represent statistic differences for p <0.05.

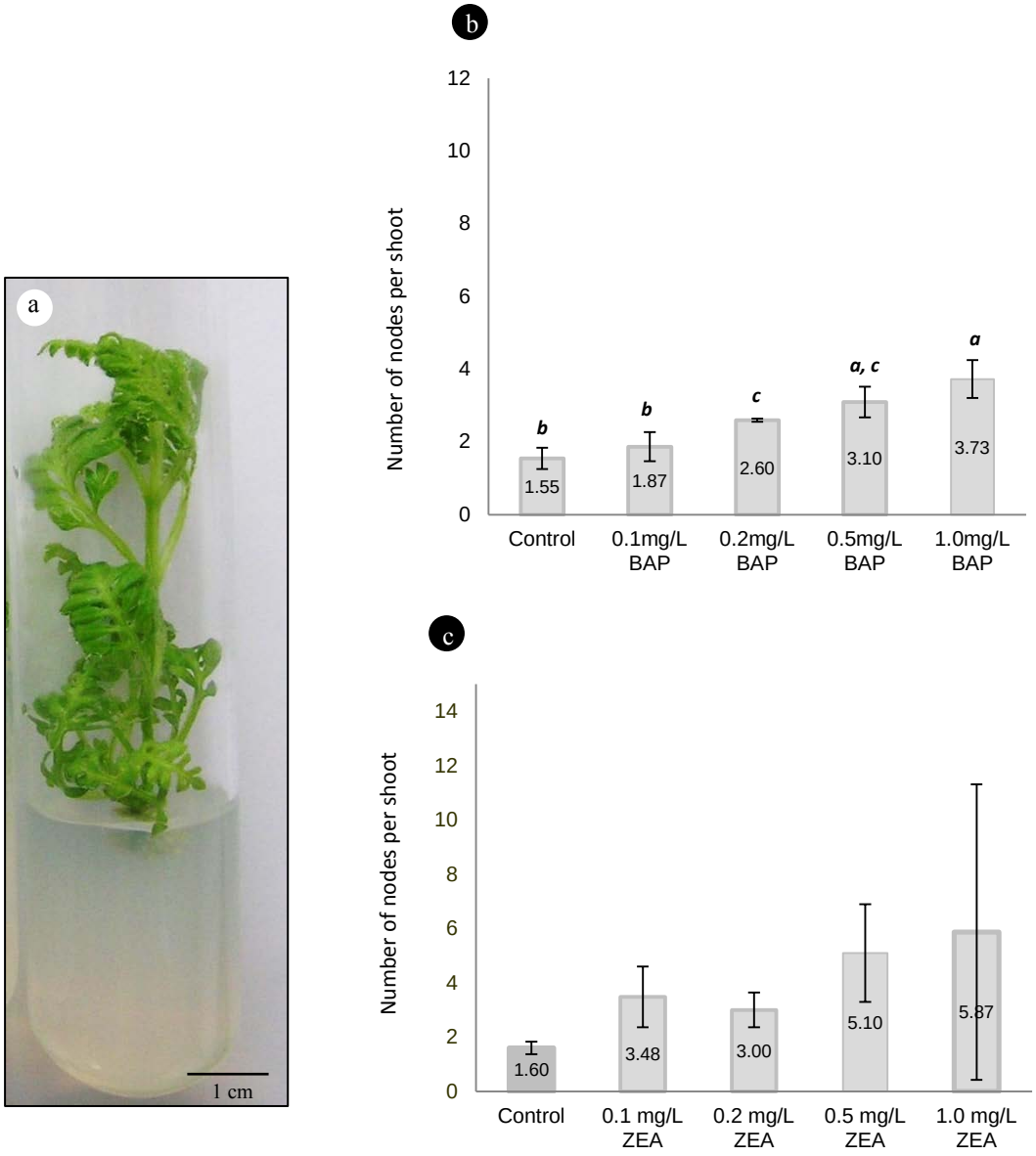
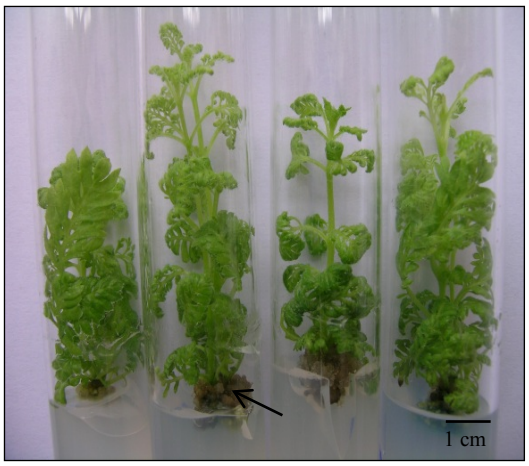


Figure 5. *Lavandula multifida* 30 days after culture in MS medium (a). Effect of different concentrations of benzyladenine (b) and zeatine (c) in the previous multiplication medium on the number of nodes per shoot during the elongation phase. Each value represents the mean ± SEM from three independent experiments; ***a***, ***b*** and ***c*** represent statistic differences for p <0.05.

Once the pre-existing buds started to develop, *callus* proliferated mainly at the cut end of the explants (Fig. 6). Shoot organogenesis from basal *callus* is likely to occur and has been reported in several *Lavandula* species such as *L. angustifolia* (Quazi, 1980), *L.* x *intermedia* (Dronne et al., 1999), *L. latifolia* (Calvo and Segura, 1988; Jordan et al., 1990), *L. officinalis* x *L. latifolia* (Panizza and Tognoni, 1988) and *L. vera* (Tsuro et al., 2000). Organogenesis may be an alternative to shoot proliferation but a careful analysis of the propagated material must be performed to confirm the uniformity of the regenerated plants.



Figue 6. *Lavandula multifida* with *callus* (arrow) at the base of the explant, after 30 days of culture on MS medium with cytokinins.

1.3.4 Shoot rooting

During the elongation phase 41.75 % of the shoots rooted spontaneously (Fig 7a). Shoots that did not root spontaneously were induced to root using IBA. The effect of two concentrations of IBA on root formation was evaluated through the number of roots per shoot and roots length (highest and lowest root length per shoot). Spontaneous rooting is important for *in vitro* propagation as it allows a considerable gain of time in the regeneration process and avoids the use of auxins that are known to induce *callus* and anomalous vascular connections between shoots and adventitious roots (Németh, 1986; Ziv, 1986). Also, it was shown that the addition of IBA decreased the essential oil concentration in *L. dentata* plantlets by 31 % in comparison to control and inhibited its secretion capacity (Sudriá et al., 2001). In the present study, the two concentrations of IBA used to promote rooting in *L. multifida*, did not show significant differences

concerning the number of roots formed (Fig. 7b) but longer roots occurred in the absence of auxins (control) (Fig. 7c and 7d).

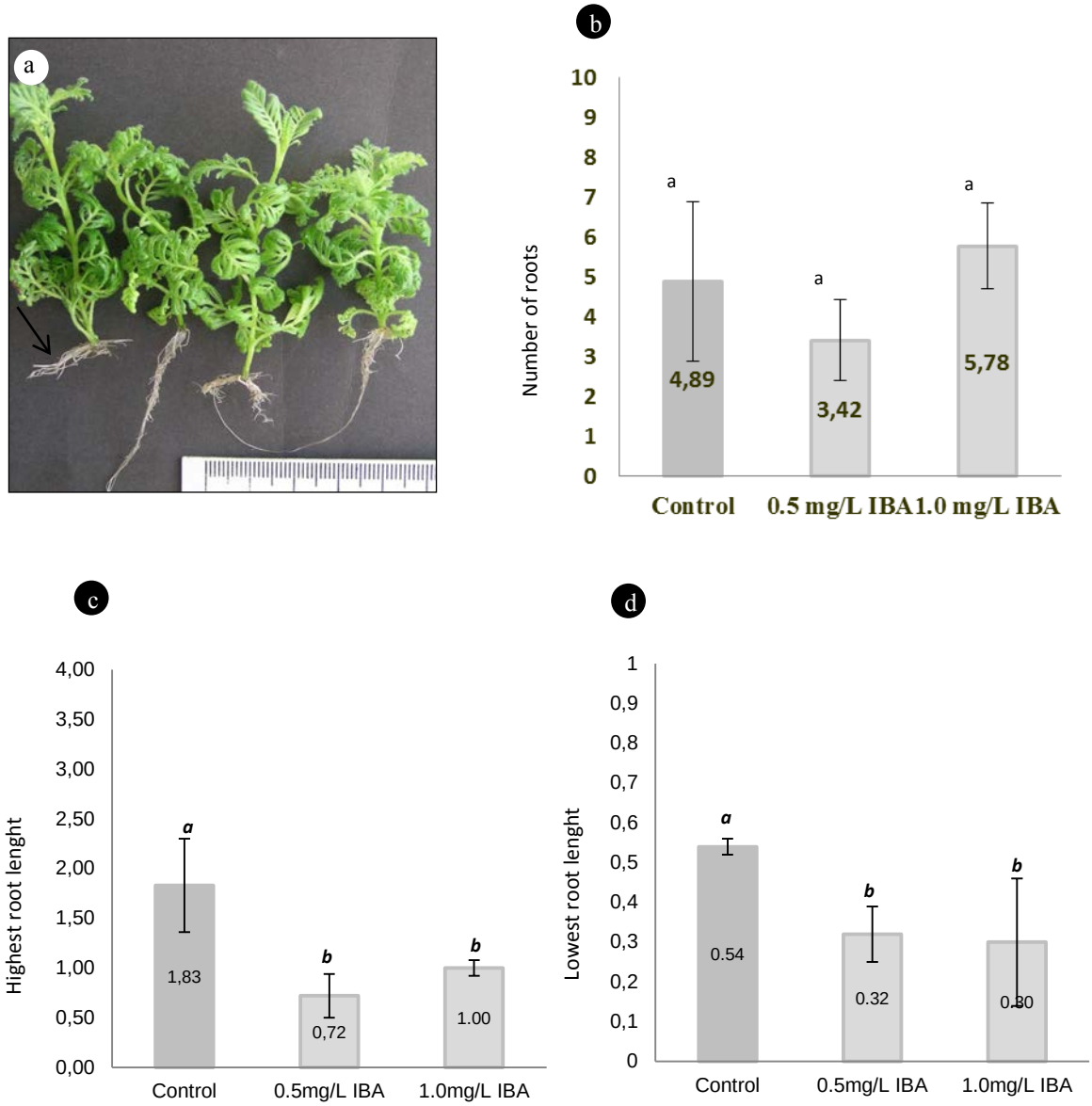


Figure 7. a-d) Rooting of *Lavandula multifida*. a) Roots formed spontaneously during the elongation phase. b - d) Effect of different concentrations of IBA on the number of roots (b), highest root length (c) and lowest root length (d). Each value represents the mean ± SEM from three independent experiments; ***a***, ***b*** and ***c*** represent statistic differences for $p<0.05$.

1.3.5 Acclimatization

In vitro rooted plantlets were successfully transferred to soil, exhibiting a normal development with high degree of homogeneity and no evidences of somaclonal variation. Also, flowering was observed during the acclimatization phase of the micropropagated plants (Fig. 8).

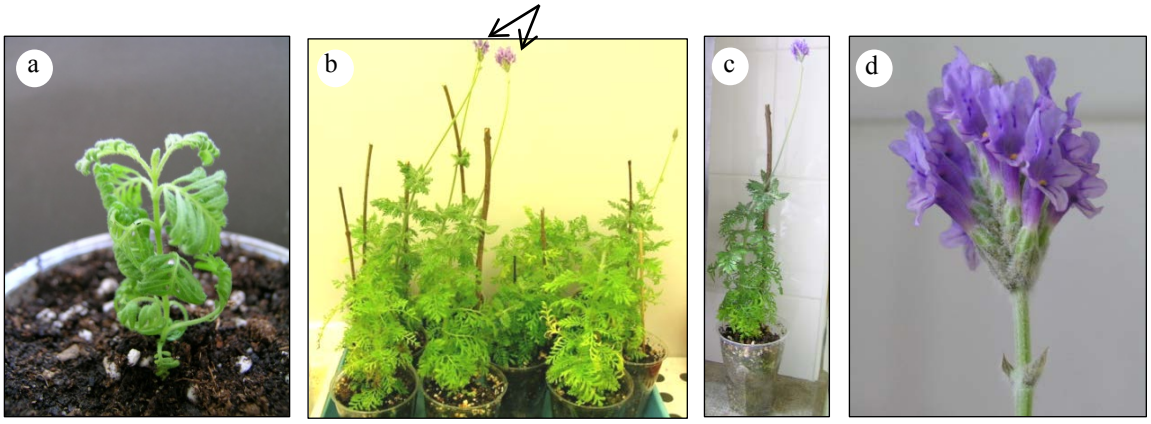


Figure 8. a-d) Acclimatization phase of *L. multifida*. a) Transfer of rooted plants to soil, after 30 days after root formation. b) Plants maintained in the acclimatization chamber for 30 days. The arrows point to the inflorescences formed still during the acclimatization phase. c) Fully developed and acclimatized plant. d) Flowering of a *L. multifida* plant obtained through axillary shoot proliferation.

The present results show that micropropagation through axillary shoot proliferation is a reliable method for the rapid multiplication of *L. multifida* allowing plant conservation without damage of the natural resources, which are limited in Portugal. Moreover, a rapid multiplication for large-scale plantations of selected high-yielding plants could also be achieved applying the referred protocol.

Previous experiments showed that the essential oils of both *in vitro* shoot-cultures and micropropagated plants of *L. viridis* had the same main compounds as the original field-grown parent plant, without remarkable compositional variations (Nogueira and Romano, 2002). Also, the analysis of the volatiles emitted directly (headspace solid phase microextraction) from *in vitro* shoot cultures, and micropropagated plants of *L. viridis* when compared with those from the field-growing parent plant, showed the same major compound (1,8-cineole), although monoterpenes and sesquiterpene hydrocarbons occurred in higher amounts in the *in vitro* shoot cultures and micropropagated plants (Gonçalves et al., 2008). In *L. pedunculata*, the

trichomes and essential oils of *in vitro* plantlets of two selected chemotypes were identical to those occurring in field-growing plants (Zuzarte et al., 2009).

Microscopical observations showed the presence of the same types of trichomes in *in vitro* plantlets and in field-growing adult plants, including the characteristic peltate trichome referred in section 2 of chapter II (Fig. 9a-9c). Also histochemical tests using Nadi reagent (David and Carde, 1964) (see chapter II, section 2) detected essential oils in the trichomes of *in vitro* shoots, at very early stages of plant development (Fig. 9c). Further experiments are being conducted to evaluate the essential oils composition of these *in vitro* plantlets, as well as those from acclimatized and fully adapted *ex-situ* plants. Comparisons with the oils obtained from the parent-plant will also be included.

Micropropagation under controlled conditions could be an interesting alternative for field production of *L. multifida*, since higher amounts of plants can be obtained in less time. For example, by using only MS with 1.0 mg/L of ZEA as a multiplication medium, it is able to obtain around 360 acclimatized plantlets, after 7 months of culture.

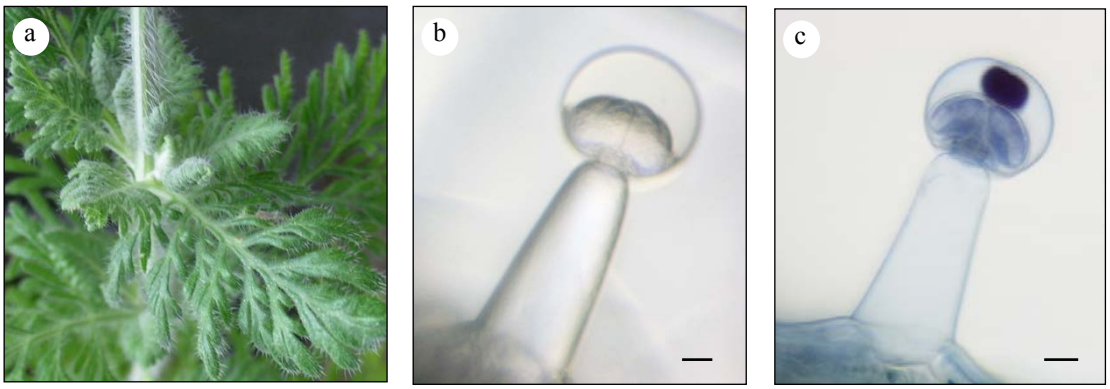


Figure 9. a) Leaf detail of a fully acclimatized plant. b) Peltate trichome occurring in the leaves of *in vitro* shoot cultures. c) Detection of essential oils in a peltate trichome in *in vitro* shoot cultures using Nadi reagent. Bars = 10 µm.

Currently, at the Laboratory of Biotechnology of the Department of Life Sciences of the University of Coimbra, *in vitro* cultures of the 5 lavender species included in the present work are being maintained and are available for further investigations.

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V. Conclusions and Future Perspectives

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In this study morphological, chemical and biological studies were performed on lavenders (*Lavander* spp.) growing spontaneously in Portugal. The results improved the scientific knowledge on the genus and contributed to the valorisation of these species opening the way to a further economic exploitation, mainly in the health and agricultural sectores. The main conclusions of this work as well as the directions for further required research are pointed out below:

1. a taxonomical clarification, mainly for *L. lusieri* and *L. stoechas,* was purposed based on morphological and chemical data (presence of necrodane derivatives in *L. luisieri*) justifying the classification of these *taxa* as independent species, instead of the actual classification that considers *L. luisieri* a subspecies of *L. stoechas*. Moreover, the polymorphism observed in *L. pedunculata* does not support the division in subspecies, since no correlations between morphological variability, chemical polymorphism and distribution were found.
2. A detailed morphological characterization of the secretory structures showed the presence of different types of trichomes, including uncommon bifurcated trichomes and ramified-mixed trichomes, the later reported for the first time in Lamiaceae, Moreover, for *L. multifida* a distinct peltate trichome with a long stalk was found. The high number of glandular trichomes in *L. viridis* justifies the higher essential oil yield in this species. Contrarily, *L. multifida* showed a very low density of these trichomes explaining its low oil yield. The differences observed including type and density of the trichomes allowed a clear identification of these species. Besides the taxonomical importance, this aspect is very relevant for quality control in the market of medicinal and aromatic plants.
3. A chemical profile for *L. luisieri*, *L. multifida*, *L. pedunculata* and *L. viridis* essential oils was established allowing the identification of these species based on chemical features: *L. luisieri* showed a very distinct chemical composition, unique in the plant kingdom, due to the presence of necrodane derivatives; *L. multifida* was characterized by high amounts of carvacrol and *cis*-*β*-ocimene, the

later reported for the first time in this species; *L. pedunculata* showed a high chemical polymorphism mainly in the amounts of 1,8-cineole, fenchone and camphor; *L. viridis* exhibited a very homogeneous chemical composition with high amounts of 1,8-cineole.

4. The antifungal activity of *Lavandula* spp. oils was evaluated against yeast, dermatophytes and *Aspergillus* strains responsible for human and animal diseases. In general, the oils were more active against dermatophytes and *Cryptococcus neoformans*, with MIC values ranging from 0.16 to 1.25 µL/mL. Moreover, significant inhibitions in the germ tube formation of *C. albicans* were also observed, at concentrations quite below the respective MIC, suggesting a possible application of the oils in the treatment of disseminative candidosis. For *L. multifida* and *L. viridis* oils a mechanism of action related to damage of mitochondrial membranes followed by disruption of cytoplasmic membrane and cell death was suggested. Further experiments including electron microscopy analyses and physiological studies should be considered to validate the mechanism of action suggested. Regarding the main compounds of the oils, carvacrol was the most active but its cytotoxicity limits its application. Further experiments using oil fractions should be considered to identify the most active fractions in order to identify other compounds responsible for the activity of the oils.
5. Cytotoxicity assays allowed the identification of the most effective oil concentrations without or with very low detrimental effects on different cell lines (keratinocytes, alveolar epithelial cells and macrophages). In general, *L. viridis* was the safer oil justifying further *in vivo* validations.
6. The anti-inflammatory potential of *L. viridis* oil was demonstrated by the selective inhibition of NO production through modulation of pro-inflammatory signalling cascades ERK ½ and NF-κB as well as inhibitions at translational (iNOS, IL-1β and IL-6) and protein levels (iNOS and COX-2). Further experiments to identify which compound or compounds are responsible for this activity will be carried out. Furthermore, the anti-inflammatory potential of *L. luisieri* and *L. pedunculata* oils should also be deeply explored considering the interesting results obtained for NO and iNOS inhibition. The most promising oil,

fraction or active compound without cytotoxicity should then be considered for validation in *in vivo* models.

7. *L. multifida* and *L. luisieri* oils showed interesting antifungal activities mainly against the phytopathogenic strains *Alternaria alternata* and *Cladosporium cladosporioides*. Moreover, carvacrol proved to be the most active compound against all the tested phytopathogenic fungi, showing a potential use in food and/or storage products preservation and justifying practical validations in these type of products. The evaluation of the antifungal activity of necrodane derivatives is also being considered in order to identify which compound or compounds are responsible for the activity of *L. luisieri* oil.
8. *L. viridis* essential oil showed interesting nematicidal potential against *Meloidogyne javanica*. Nevertheless, further experiments to evaluate the effect of the oil on the management of the nematode in field trials and on non-target organisms that also live in the soil are necessary for a further development of a bio-nematicide. The essential oils of *L. luisieri* and *L. viridis* showed potential to be further used in the development of a natural repellent against *Monochamus galloprovincialis* and, therefore, contribute to the management of *Bursaphelenchus xylophilus*, a plague affecting maritime pine in Portugal and other countries. Nevertheless, several aspects concerning essential oil volatility, solubility and oxidation must be carefully checked before a practical application.
9. An efficient protocol for a rapid multiplication of *L. multifida* was established and can be applied for large-scale propagation, avoiding plant harvest in the wild and, therefore contributing to the preservation of this species that occurs only in limited regions of Portugal. Although SEM observations showed the presence of the same type of trichomes in *in vitro* plantlets and in field-growing adult plants and histochemical analysis detected essential oils in early stages of *in vitro* development, further experiments regarding the comparison of essential oil composition of *in vitro* and acclimatized plantlets as well as fully adapted plants to *ex-situ* conditions with the oils obtained from the parent-plant, are warranted. Furthermore, different micropropagation techniques should be applied to *L. latifolia* as a conservation strategy, since this species in highly endangered in Portugal.