DEPARTAMENTO DE CIÊNCIAS DA VIDA

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Biological activities of *Lavandula angustifolia* Mill. essential oil

Filipa Antónia Mota Ferreira da Costa Meireles

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biodiversidade e Biotecnologia Vegetal, realizada sob a orientação científica do Professor Doutor Jorge Manuel Pataca Leal Canhoto (Faculdade de Ciências e Tecnologia da Universidade de Coimbra) e da Professora Doutora Lígia Maria Ribeiro Pires Salgueiro Silva Couto (Faculdade de Farmácia da Universidade de Coimbra)

Filipa Antónia Mota Ferreira da Costa Meireles

2012

I

por caminhos de lavanda e urze: raso,
o sangue sob a plaina dos dedos,

enquanto a mão aprende
toda a beatitude do mundo

a mão alçada sobre a lua dos olhos,
o gesto é conciso
como uma imagem impossível

II

depois, ameias entre os venenos,
os versos:

carótida, laringe, fuligem, falange

os versos: um secreto combate, os versos

tantas vezes não mais que sombras

entre a luz nocturna da lâmina
e a doçura da pálpebra

III

em verdade falo apenas do que há
dentro dos nomes

o que há dentro de um nome?

em verdade falo apenas de um imóvel caminho

um lentíssimo modo de rumar
ao silêncio

Luís Felício
Prémio Edmundo Bettencourt - Poesia 2011

ABSTRACT

Lavender (*Lavandula* spp.) essential oils have been used for thousands of years and these uses are in a work-in-progress for sustained scientific and clinical studies on their biological activities.

Currently used therapeutics on superficial mycoses treatment domain is reported for several disadvantages, including toxic side-effects and fungal resistance. Within plant-derived antimycotics, essential oils represent a good strategy in combination with commercial, synthetic antifungal drugs.

Within crop pests control, recent European Legislations have given strong impulse for biopesticides screening, including plant extracts.

The aim of the present study was to evaluate different biological activities of the essential oil of *Lavandula angustifolia* subsp. *angustifolia*: antifungal activity against *Candida*, *Cryptococcus*, dermatophyte and *Aspergillus* strains, and nematicidal activity against root-knot nematode *Meloidogyne javanica*.

Keywords: *Lavandula angustifolia*, essential oil, antifungal activity, nematicidal activity.

RESUMO

Os óleos essenciais de lavanda (*Lavandula* spp.) são usados há milhares de anos e a sua validação científica, assim como os testes clínicos, das suas actividades biológicas encontram-se num 'work-in-progress".

Entre as terapêuticas utilizadas actualmente no domínio das micoses superficiais têm sido referidos vários efeitos secundários e resistências. No âmbito dos antifúngicos de origem vegetal, os óleos essenciais representam uma boa estratégia na combinação com os medicamentos 'sintéticos'.

Noutro plano, o controlo de pragas que afectam sistemas agrícolas, de acordo com novas leis Europeias, a investigação de biopesticidas recebeu um novo impulso, incluindo os pesticidas derivados de plantas.

O objectivo do presente estudo foi avaliar diferentes actividades biológicas do óleo essencial de *Lavandula angustifolia* subsp. *angustifolia*: actividade antifúngica contra estirpes de *Candida*, *Cryptococcus*, dermatophyte e *Aspergillus*, e actividade nematicida contra o nemátode-das-galhas-radiculares *Meloidogyne javanica*.

Palavras-chave: *Lavandula angustifolia*, óleo essencial, actividade antifúngica, actividade nematicida.

TABLE OF CONTENTS

The present study was based on previous works on Essential Oils Investigation developed by Drug Discovery Group at the Laboratory of *Pharmacognosy, Faculty of Pharmacy of University of Coimbra* and by Nematology Group at the Laboratory of Nematology, Faculty of Science and Technology of University of Coimbra, with laboratory work supervised by PhD Student Mónica da Rocha Zuzarte.

I. INTRODUCTION

1. LAVENDER

1.1. Lavender, a scent throughout the centuries

The common name "lavender" (in Portuguese, "lavanda" [1]) and the name of genus *Lavandula* is more frequently attributed to the Latin *lavare*, which means "to wash", but some authors refer that is more likely *Lavandula* and "lavender" to be derived from the Latin *livere*, "to be livid or bluish", via the Medieval Latin *lavindula* (**Upson & Andrews, 2004**).

Lavender (*Lavandula* spp.) is a powerful aromatic and medicinal herb and appears to date as far back as the early Egyptians, who are reported to have wrapped their dead in lavender-dipped shrouds. Indeed, they used essential oils extensively, with medicinal, perfumery, cosmetic and religious purposes. Some authors say that Ancient Greeks used *lavender* to fight aching backs, insanity and insomnia and that Ancient Romans added *lavender* oil to the water in their famous *public baths, referring this plant to be Lavandula stoechas, whilst other authors say none of this was true and the "Celtic nard" of Sappho could actually be Asarum europaeum. Nevertheless, L. stoechas dried flowers might have traveled with the Roman Army to Britain and to North Africa.* "Lavenders" appear frequently chronicled as to be used in Medieval and Renaissance times for the storage of laundry. P*robable apocryphal stories refer* Louis XIV, *le Roi-Soleil*, demanding his shirts rinsed in a special perfume that included lavender, marjoram and nutmeg infused in orange water, as others tell lavenders to be commonly used as aphrodisiacs in Victorian times. In Britain, the golden era of herbs and herb gardens, was from the late 1400s to the mid 1600s, and then declined in favor of more general flower gardens. While lavender was traditionally grown as an herbal "household aid", it was not until the late 1700's that it was grown commercially.

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[1] In Portugal, lavender is also known as "alfazema"

Provence has been, until now, the world's largest lavender producing region. In the 1300's Marseille and Montpellier were prominent medical schools and are thought to have gathered wild lavender from that region as the local Provencal populace gathered wild lavender flowers to treat wounds. Toward the end of the 19th century inhabitants of Provence began to care for and maintain the wild lavender patches and, in this way, lavender entered the 20th century, from family to intensive cultivation.

The commercial products that are obtained today from the genus *Lavandula* include essential oils, fresh and dried flowers and inflorescences, and landscape plants. Distilled flowers yield an essential oil used in perfumes, colognes, skin lotions and other cosmetics, soaps, bath and talc powders. In culinary, small amounts are sometimes used to flavor teas and foods, such as dressings, fruit desserts, jellies, ice-cream, wines, and, *the well-known herbes de Provence*. Lavender is also a valuable meliferous plant. Scented sachets, also named "lavender pillows" (in Portuguese folk culture, "almofadinhas de cheiro" or "saquinhos de cheiro"), are known to give a fresh and sleep-inducing fragrance and also to deter moths and mosquitoes. Lavender (*Lavandula* spp.) is used in traditional and folk medicine of different parts of the world. (**Proença da Cunha *et al*., 2007**; **Upson & Andrews, 2004**).

Recently, aromatherapy is becoming increasingly popular, and lavender is used in aromatherapy as a relaxant (**Fakhari *et al*., 2005**).

The diluted essential oil, it has been rumored as antiseptic and anti-inflammatory for topical applications, with effectiveness in burns and insect bites (**Gattefossé, 1993**), and infusions of the plant are reported to be used as diuretic, carminative, antispasmodic, anti-epileptic, anti-rheumatic, antidepressive, sedative and pain reliever, as in nervous headache and migraine (**Chu & Kemper, 2001**; **Hajhashemi *et al*., 2003**).

The most economically important lavender has been *Lavandula angustifolia*, common lavender, also name "fine lavender" and "true lavender", used in fine perfumery. Lavandin, *Lavandula* x *intermedia*, is a hybrid of common and spike lavenders, and its oil is sold as a coarser and cheaper "lavender oil", because of its highest oil yield. *L. angustifolia* varieties are best for aromatherapy and cosmetic purposes, while lavandins and spike lavender, or broad-leafed lavender, *Lavandula latifolia*, are widely used in soap and candle production.

Common lavender – *Lavandula angustifolia* Miller subsp. *angustifolia* – is a woody perennial of the family Lamiaceae (or Labiatae), the taxonomy of which was recently reviewed by Upson and Andrews (**2004**).

1.2. The *genus Lavandula*

The present section is an abridgement of Upson and Andrews' taxonomy history of the genus *Lavandula*.

The first written document on lavender can be traced to the Greek Dioscorides, a military physician under the Roman Emperor Nero, who collected medicinal plants from around the Mediterranean. Dioscorides described these plants and provided information about their medical uses in a 5-volume work *De Materia Medica*, published around AD 65, on which wrote about *L. stoechas* medicinal value. The **De Materia Medica** served as the foundation for Arab physicians who read Syrian and Old Persian translations. In the Middle Ages, the Persian Avicenna, mentioned the healing uses of lavender on his "Book of Healing". The Abbess Hildegard of Bingen works' mention medicinal properties of lavender (probably referring to *L. angustifolia* and *L. latifolia*). In the 15th and 16th centuries, given the attributes associated with lavender, species we know name as *L. angustifolia*, *L. latifolia*, *L. multifida*, *L. dentata* and *L. pedunculata* started to be reported by herbalists [2]. In the beginning of the 17th century, interest in plants crossed the boards of medicinal uses and they started to be studied for their intrinsic and scientific value, with the early taxonomists, when *L. viridis* and *L. canariensis* were recognized. An important work of this period was *Instituitiones Rei Herbarie* by Tournefort in 1700. In the middle of 17th century, Linnaeus introduced the modern botanical nomenclature with *Species Plantarum* (1753). At that time, seven species of *Lavandula* were recognized in European and Mediterranean floras. The first monograph of the genus *Lavandula* was a dissertation made by Linnaeus' son. The second monograph on the genus was *Histoire Naturelle des Lavandes* by the Baron

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[2] The Abbes Hildegard recommended lavender water for swollen eyelids. In his *Dictionnaire des drogues simples* (1798), Lémery summarized much of the information from previous ages, including lavender properties (**Gattefossé, 1993**).

Gingins in 1826. Of major importance was the work of George Bentham in his *Labiatarum genera et species* published between 1832 and 1836, where he established the generic classification of the family Labiatae. During late 19th and early 20th centuries many species were described, often as a result of colonial exploration of Africa and Arabia.

The genus *Lavandula* is native to the Canary Islands, Cape Verde and Madeira, the Mediterranean Basin, the Near-East, North Africa, North-Eastern tropical Africa, Arabian Peninsula, South–West Asia and India. In Portugal, occur spontaneously species of *L. latifolia, L. viridis, L. pedunculata*, *L. multifida* and *L. luisieri*.

1.3. *Lavandula angustifolia* Miller

Lavandula angustifolia was published by Philip Miller in the 8th edition of his Gardeners Dictionary (Miller, 1768). The subgenus *Lavandula* comprises all the species with multi-flowered cymes and are woody shrubs with narrow usually entire leaves. Other diagnostic characters are reticulate-veined bracts, the fusion of calyx veins typically giving a 13-veined calyx (8-veined in one species), and the upper middle calyx lobe modified into an appendage. The nutlets bear only a small basal scar (**Upson & Andrews, 2004**).

L. angustifolia Mill. subsp. *angustifolia* description according to The European Garden Flora **(Cullen, 2011)**:

Shrub to 80 cm. Leaves 2-5 cm x 3-5 mm, mainly linear-lanceolate, entire, with grey felted hairs when young, becoming greener with age. Inflorescence-stalk 10-25 cm, unbranched, usually with compact spike 2-5 or 8 cm, some lower flower clusters distant from main spike; whorls 5-7-(sometimes 9-)flowered; minute bracteoles present. Bracts broadly ovate-diamond-shaped to obovate. Flowers stalked. Calyx 1 3-veined, with appendage, cylindric in shape, with a woolly indumentum. Corolla strongly bilaterally symmetric, nearly twice the length of calyx with prominent lobes, shades of blue-mauve, white, rarely violet pink in colour.

Synonyms are: *L. spica* L., *L. minor* Garsault, *L. officinalis* Chaix ex Vill., *L. vulgaris* Lam., *L. angustifolia* Moench, *L. fragrans* Salisb., *L. vera* DC., *L. spica* Loisel., *L. fragrans* Jord. ex Billot, *L. delphinensis* Jord. ex Billot, *L. officinalis* var. *delphinensis* (Jord.) Rouy & Fouc., *L. spica* var. *delphinensis* (Jord.) Briq., *L. angustifolia* var. *delphinensis* (Jord.). The epithet for the typical species is the same as for the species. Common names are: common lavender, true lavender or fine lavender; in Portuguese, "lavanda" or "alfazema".

Is native to France, in the Southern Alps and the Cevennes region of Languedoc on the High Causse, and to Italy, North-East and South. This species grows on calcareous soils amongst open vegetation, often very arid habitats from (250-)500 to 1800(-2000) m. Mediterranean countries south orientations are regarded as more suitable for development of aromatics due to higher temperatures and lower precipitation that north orientated lands, which reflects in oil yield.

Their pollinators include: *Apis mellifera*, *Vanessa cardui*, *Bombus lucorum*, *Bombus terrestris*, *Anthidium* sp., *Iphiclides podalirius* (**Hassiotis *et al*., 2010**; **Upson & Andrews, 2004).**

L. angustifolia Mill. subsp. *pyrenaica* occurs not only in the Pyrenees but extends to north-eastern Spain. *L. angustifolia* Mill. subsp. *angustifolia* produces the finest oil, colourless and sweet, with no camphor, while *L. angustifolia* Mill. subsp. *pyrenaica* oil is reputed to be inferior to subsp. *angustifolia* due to much higher percentage of borneol and camphor (**Upson & Andrews, 2004**).

Lavender for oil production is mainly grown in Australia, New Zealand, Bulgaria, China, England, France, Russia, Moldova, Ukraine and the former Yugoslavia. Upson & Andrews (**2004**) describe more than 150 cultivars and field varieties of *L. angustifolia* Mill. subsp. *angustifolia*, such as "Hidcote (30 cm, deep lilac flowers), 'Munstead' (45 cm, blue-lilac flowers), 'Rosea' (45 cm, pink flowers), and 'Nana Alba' (20 cm, dwarf, white).

The word 'variety' has a definite botanical use and it is therefore confusing when it is used occasionally in horticulture to refer to any plant, whatever its rank, as when people speak of a plant nursery full of 'interesting varieties'. Where the distinguishing characteristics are confined to a particular geographic area the subspecies is generally used. When the minor differences are local or ecological, variety is mostly used (**Spencer *et al*., 2007**)

2. ESSENTIAL OILS

2.1. Essential oils – preamble

2.1.1. Secondary metabolites and biosynthetic pathways

Plant primary products refer to the chemical families of carbohydrates, proteins, lipids and nucleic acids, and their functions are related to structure, physiology and genetics, with main role in plant development. Secondary metabolites normally occur as minor compounds in low concentrations. They are involved in signaling mechanisms, plant defense and show characteristic patterns in their relationship to plant family, genus or species, and, thus, underscore their importance for chemotaxonomical and systematic determinations (**Rohloff, 2004**).

Higher plants have an especially diverse number of volatile secondary compounds of paramount ecological importance because, although most animals rely on their mobility to obtain food and escape predators, plants are usually immobile and, thus, must use alternative strategies. The enormous variety of metabolites emitted by plants suggests that volatile compounds may provide a detailed language for communication with organisms across distances (**Pichersky & Gershenzon, 2002**; **Qualley & Dudareva, 2010).**

Plant secondary metabolites can be divided into three chemically distinct groups: terpenes, phenolics, and nitrogen-containing compounds (**figure 1**). The terpenes - or terpenoids - from the German *terpentine* [3] (turpentine) represent the largest class of plant secondary metabolites (**Taiz & Zeiger, 2010**).

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[3] Kekulé, in 1880, was the first scientist to name $C_{10}H_{16}$ compounds as "terpenes", because of their existence in turpentine.
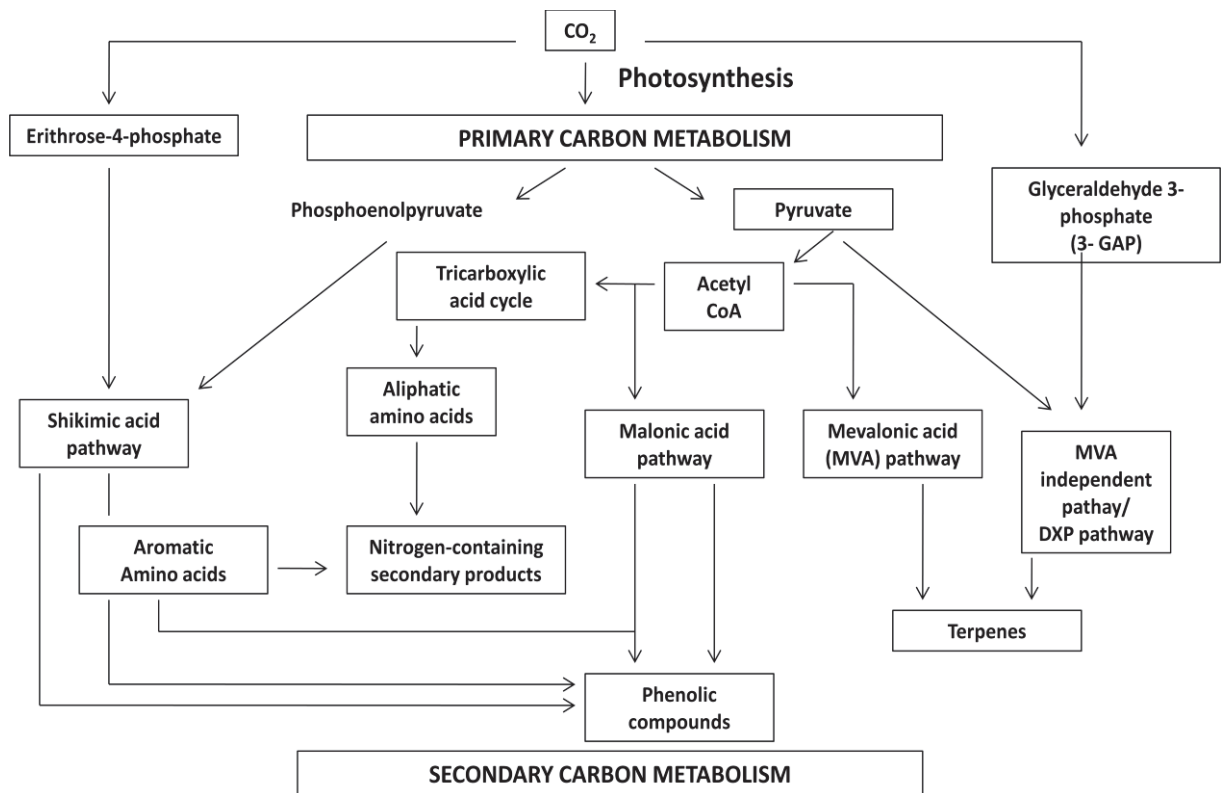
Figure 1. Major pathways of secondary-metabolite biosynthesis and their relationships with primary metabolism (modified from **Taiz & Zeiger, 2010**).

The chemical structure of the terpenoids is based on up to five C_5-*isoprene units* [$CH_2=C(CH_3)-CH=CH_2$], which lead to higher molecular structures of the *isoprenoids* through elongation and cyclization. The mono- and sesquiterpenes (the C_{10} and C_{15} isoprenoids, respectively) are produced by all plants, at least in small quantities, as the major contributors to the aroma and scent of fruits and flowers (**Rohloff, 2004**).

All terpenoids are derived through the condensation of the universal isoprenoid precursor isopentenyl diphosphate (IPP) and its allylic isomer, dimethyl allyl diphosphate (DMAPP). In plants, IPP and DMAPP are produced via two independent pathways found in plastids and cytosol/peroxisomes, respectively. One pathway – the 1-deoxy-D-xylulose-5-phosphate (DXP) pathway – is localized in plastids, and supplies IPP and DMAPP for the production of monoterpenes, diterpenes, and tetraterpenes (beyond 20 *Carbon* atoms, compound volatility is lost). Flux through this pathway is controlled in part by 1-deoxy-D-xylulose-5-phosphate synthase (DXS), the first enzyme of the pathway (**figure 2**). Overexpression of *DXS* results in an elevated production of total terpenoids in plants. The other pathway – the classical mevalonic acid (MVA) pathway – operates in the cytosol and produces precursor for the biosynthesis of sesqui- and triterpenes (**Qualley & Dudareva, 2010; Lane *et al.*, 2010**).

16
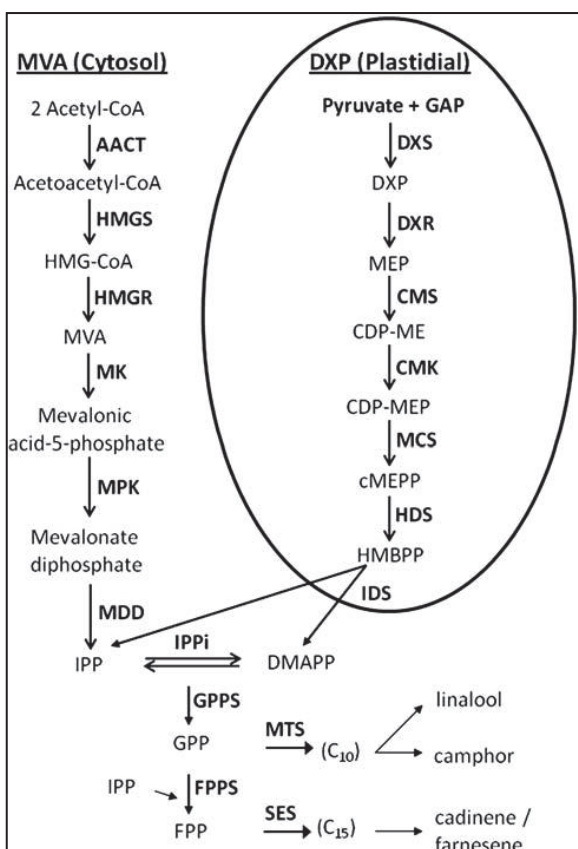
Figure 2. The MVA and DXP pathways of isoprenoid synthesis in plants (modified from **Lane *et al*., 2010**).

2.1.2 Secretory structures in plants

The storage of EOs in higher plants is not restricted to specialized plant parts. EOs occur in both roots, stems, leaves, flowers and seeds, or in the plant as a whole. Both epidermal or mesophyll tissue can function as the site of terpene biosynthesis in general, whereas typical storage cells or cell structures characterize the taxonomic group of aromatic plants (**Rohloff, 2004**). They are particularly abundant in several species of the Lamiaceae (e.g., lavenders) as the main constituents of EOs. In these plants, mono- and sesquiterpenes are produced in secretory cells, and stored in sub-cuticular storage cavity of the glandular trichomes (or oil glands) present on the surfaces of leaves and floral tissues (**Lane *et al*., 2010**).

Histochemical analyses conducted in *L angustifolia* (**Iriti *et al*. 2006** *apud* **Guitton *et al*., 2010**) demonstrated that terpene secretion mainly takes place in peltate glandular trichomes. In *L. angustifolia*, the greatest density of multicellular peltate

trichomes is found in the depressed sinuses of the adaxial calyx epidermis (**Guitton *et al*., 2010**).

2.1.3 Ecological aspects of essential oils

The isoprenoids comprise a family of over 25,000 structurally and functionally diverse secondary metabolites with ecological and physiological functions in plant–environment interactions (e.g., pollinator attraction and defense) and in growth and development (e.g., as plant growth regulators) (**Lane *et al*., 2010**). Whereas some volatiles are common to almost all plants, others are specific to only one or a few related taxa. Floral volatiles serve as attractants for species-specific pollinators, whereas the volatiles emitted from vegetative parts, appear to protect the plant against herbivory (**Pichersky & Gershenzon, 2002**). Against herbivorous insects, plants use both direct and indirect mechanisms. Direct defense mechanisms affect negatively the physiology of the herbivore with toxic or anti-nutritional compounds, or by interfering with the behavior of the herbivore with repelling or deterring compounds, while indirect defense mechanisms promote the effectiveness of natural enemies of the herbivore by producing volatiles that can attract predators or parasitoids of the herbivore - plants synthesize and emit blends of volatile compounds from their damaged and undamaged tissues, which act as important host-location cues for parasitic insects. Furthermore, certain volatile monoterpenes have been found to inhibit the growth of competing plants (a process called allelopathy) (**De Moraes, 1998**; **Oppenheim & Gould, 2002**; **Pare & Tumlinson, 1997; Qualley & Dudareva, 2010**; **Van Poecke *et al*., 2001**; **Wagner, 1991**).

Volatile compounds from plants, especially essential oils, are known to have antimicrobial and insecticidal activities (**Oka *et al*., 2000**).

2.1.4. Factors influencing production and composition of essential oils

Essential oil composition and yield varies not only between different species of the same genus, but also within different specimens of the same species by physiological and environmental factors. The first include plant organ, age and life-

stage of the plant (pre- or post-flowering) – in several plant species, it has been demonstrated that volatile organic compound composition and content are subject to changes during the ontogenic development of the whole plant or of some of its organs (**Sangwan *et al*., 2001** *apud* **Guitton *et al*., 2010**). Within environmental factors, soil composition, hydric stress and pests play an important role; nevertheless, the most influential is that of geo-climatic location, as it gives rise to different *chemotypes* of EOs. Here, infraspecific and intervarietal differences can be observed in both morphology and chemical structures, but, must be noted, with no correlation between morphological and chemical characters (**Bakkali *et al*., 2008**; **Bowles, 2003**; **Rohloff, 2004**).

2.1.5. Human use of essential oils

Whilst the chemical structures of terpenoids can be found beyond plant kingdom- mosses, liverworts, seaweeds, sponges and fungi have also been shown to contain essential oils - the occurrence of essential oils (**EOs**), also known as volatile oils or essences, is restricted to over 3000 plant species from about 60 different families – the so-called *aromatic plants* – but only about 300 species are the basis for the economically important production of EOs in the world. Well-known families rich in EO bearing species are Apiaceae, Asteraceae, Cupressaceae, Hypericaceae, Lamiaceae, Lauraceae, Myrtaceae, Pinaceae, Piperaceae, Rutaceae, Santalaceae, Zingiberaceae and Zygophyllaceae. Except for the conifers, the majority of the aromatic plants and EO commodities in terms of world trade are related to the families of Labiatae, Umbelliferae and Compositae (**Adorjan & Buchbauer, 2010**; **Baser & Demirci, 2007**; **Bowles, 2003**; **Rohloff, 2004**).

The major producers of EOs are developing or emerging countries (Brazil, China, Egypt, India, Mexico, Guatemala and Indonesia), while the major consumers are the industrialized countries (USA, Western Europe and Japan) (**Koroch & Juliani, 2007**).

EOs and some of their components are commercially important in the pharmaceutical, agronomic, food (as flavouring and preservatives), sanitary, cosmetic and perfume industries. Furthermore, the budding aromatherapy sector is expected to expand the market in coming years (**Bakkali *et al*., 2008**).

Many EOs have been used for centuries in folk medicine and in recent years they have been studied with regard to their biological activities. EOs have been reported in literature for antimicrobial, antiviral, anti-inflammatory, anti-oxidative, anti-nociceptive, anti-cancer, penetration enhancing properties and also insect-repellent, insecticidal and even herbicidal activities (**Adorjan & Buchbauer, 2010**; **Rohloff, 2004**).

2.1.6. International standards, extraction and analytical techniques for essential oils

According to International Standard Organization (ISO) standards on "Aromatic natural raw materials" ISO 9235(1997) of the ISO/TC, the concept of *essential oil* is restricted to the products obtained exclusively by distillation of raw plant material, with or without water steam (hydrodistillation, steam distillation or dry distillation) or by expression, a mechanical process without heating that is applied to *Citrus* fruits.

2.1.6.1. Extraction techniques of essential oils: steam distillation and hydrodistillation

An EO is classically obtained using equipment based on the circulatory distillation approach introduced by Clevenger. Apparatus and operation modes to obtain EO are well established and are described in several pharmacopoeias (**Rubiolo *et al.*, 2010**). Figure 3 shows a diagram of the apparatus reported in the *European Pharmacopoeia*.
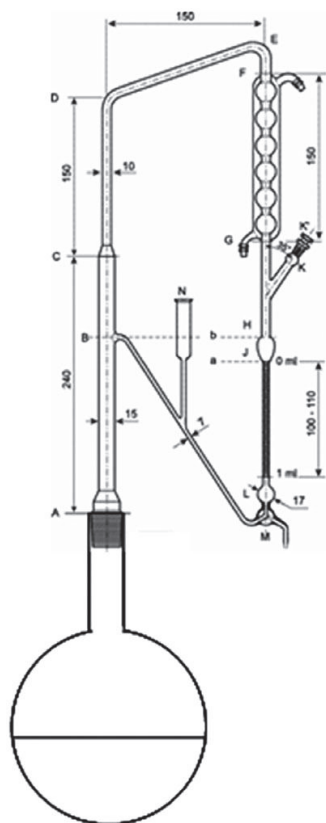
Figure 3. Clevenger circulatory distillation apparatus reported in the *European Pharmacopoeia*. Dimensions are indicated in millimeters (modified from **Rubiolo *et al.*, 2010**).

Extracts of aromatic plant or animal materials obtained using organic solvents or fluidized gasses are not considered as essential oils. Concretes, absolutes, spice oleoresins, *etc.* are classified as aromatic extracts (**Baser & Demirci, 2007**).

2.1.6.2. Analysis of Essential Oils

In general, techniques of fractionation of EOs are chromatospectral techniques and the most used one is gas chromatography (GC). GC consists in a mobile gaseous phase and a stationary liquid phase retained in an inert support and separation occurs due to different distribution between both phases; different compounds elute with different speeds, according to their volatilities and polarities. For characterization of EO constituents, GC can be coupled with spectroscopic detectors that identify the constituents, based on their structure (a quadrupole mass detector or an ion-trap

detector) - this is mass spectrometry (MS). The combination of these two techniques is commonly called GC-MS (**Baser & Demirci, 2007**; **Proença da Cunha *et al*., 2007**).

Most of the commercialized EOs are chemotyped by GC-MS analysis. Analytical monographs have been published (*European Pharmacopoeia* and ISO) to ensure good quality of EOs (**Bakkali *et al*., 2008**).

Natural oils generally contain constituents of homogeneous chirality, whereas synthetic constituents will be a mixture of chiralities (**Bowles, 2003**). The pattern of distribution of enantiomers may serve as fingerprints to prove the authenticity of a certain EO or its adulteration. As high ratio of stereospecificity is achieved in enzyme-catalyzed reactions, high enantiomeric purity is expected in chiral natural products. EOs generally possess chiral compounds with high enantiomeric purity (**Baser & Demirci, 2007**).

Generally, the major components are found to reflect the biophysical and biological features of the EOs from which they were isolated. However, many studies have shown that the activity of the main components may be modulated by other minor molecules, in a synergistic effect (**Bakkali *et al*., 2008**; **Koroch & Juliani, 2007**).

2.2. Lavender essential oils

2.2.1. *Lavandula angustifolia* essential oil chemical composition

Lavandula angustifolia Mill. essential oil (LAEO) is a complex mixture of mainly monoterpenes - linalool, camphor, 1,8-cineole and borneol - and a few sesquiterpenes (e.g., [*E*]-*β*-farnesene) as minor oil constituents. Linalool and its corresponding acetate ester, linalyl acetate, are the most abundant. Other lavender oils such as *L. x intermedia* (lavandin), *L. stoechas* and *L. x allardii* are generally viewed as having less commercial value than *L. angustifolia* oils because camphor generally contributes an undesirable odor and LAEO has a high linalool to camphor ratio. However, *L. angustifolia* has a low overall oil yield in contrast with certain lavandins (*L.*x *intermedia,* e.g., Grosso lavender), which accumulate relatively high levels of camphor (**Lane *et al*. 2010**; **Moon *et al*., 2007**).

LAEO composition has been extensively investigated by using gas chromatography–mass spectrometry (GC–MS) (**Hassiotis *et al*., 2010**) and, according to ISO 3515:2002, LAEO has the following chromatographic profile:

Table 1. Chromatographic profile of *Lavandula angustifolia* Mill. essential oil (modified from **ISO 3515:2002**).

| Component | Spontaneous lavender | | Clonal lavenders (principal origins) | | | | | | | | | |
| | France | | France "Maillette" | | Bulgaria | | Russian Federation | | Australia | | Other origins | |
	min. %	max. %	min. %	max. %	min. %	max. %	min. %	max. %	min. %	max. %	min. %	max. %
Limonene	—	0,5	—	0,3	—	0,6	—	1	—	0,5	—	1
1,8-Cineole [a]	—	1	—	0,5	—	2	—	2,5	—	1	—	3
β-Phellandrene [a]	Traces	0,5	—	0,2	—	0,6	—	1	—	0,5	—	1
cis-β-Ocimene	4	10	—	2,5	3	9	3	8	3	9	1	10
trans-β-Ocimene	1,5	6	—	2	2	5	2	5	0,5	1	0,5	6
3-Octanone	Traces	2	1	2,5	0,2	1,6	—	0,6	2	5	—	3
Camphor	Traces	0,5	—	1,2	—	0,6	—	0,6	—	0,5	—	1,5
Linalol	25	38	30	45	22	34	20	35	25	38	20	43
Linalyl acetate	25	45	33	46	30	42	29	44	25	45	25	47
Lavandulol	0,3	—	—	0,5	0,3	—	0,1	—	0,3	—	—	3
Terpinen-4-ol	2	6	—	1,5	2	5	1,2	5	1,5	6	—	8
Lavandulyl acetate	2	—	—	1,3	2	5	1	3,5	1	—	—	8
α-Terpineol	—	1	0,5	1,5	0,8	2	0,5	2	—	1,0	—	2

Both cyclic and acyclic monoterpenes and sesquiterpenes often possess various functional groups, which increase their volatility. The alcohol linalool is an acyclic monoterpene and lynalyl acetate it's his acetate ester (**Baser & Demirci, 2007**).
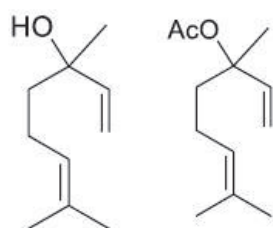

Figure 4. Linalool (left) and linalyl acetate (right) molecular structures (modified from **Baser & Demirci, 2007**).

As heat and water vapour can cause molecular rearrangements, a common rearrangement reaction that occurs during distillation is the de-esterification of esters to their component alcohols and carboxylic acid. An example of this is during the distillation of lavender oil, where the levels of linalool and linalyl acetate are key determinants of the overall fragrance of the oil. Linalool is sweeter, whereas the ester, linalyl acetate, is sharper and more refreshing in odour. Depending on the length of distillation, you can alter the ratio of linalool to linalyl acetate and thus affect the end odour of the oil (**Bowles, 2003**).

The extraction product can vary in quality, quantity and in composition according to climate, soil composition, plant organ, age and vegetative cycle stage. So, in order to obtain essential oils of constant composition, they have to be extracted under the same conditions from the same organ of the plant which has been growing on the same soil, under the same climate and has been picked in the same season (**Bakkali *et al*., 2008**).

Adulteration with synthetic linalool and linalyl acetate in lavender and lavandin oils can be determined with chiral gas chromatography or with gas chromatography-pyrolysis-isotope mass spectrometry (GC-P-IRMS) (**Upson & Andrews, 2004**).

2.2.2. Lavender oils production, *in vitro* culture and genetic improvement

Pakistani Hanif Quazi was a pioneer in lavenders' tissue culture with "*In vitro* multiplication of *Lavandula* spp." published in Annals of Botany in 1980, which included *L. angustifolia* and *L. latifolia*. Knowing from start that both plants could be vegetatively propagated from woody stem cuttings but it was a slow and uncertain process, and also that, when grown from seed, plants of both species exhibited too much variation in growth rate for commercial plantings, he investigated the possibility of *in vitro* propagation and reported successful callus culture and leaf bud multiplication in the two *Lavandula* species (**Quazi, 1980**). Indeed, propagation by seed enables variation in oil composition and propagation by stem cuttings results in poor rooting of several clones and risks of modification of morphological and chemical characteristics by repeated vegetative propagation. Thus, micropropagation through axillary budding became an alternative for vegetative propagation, allowing for the multiplication of

selected genotypes and chemiotypes (**Moutet**, **1980** *apud* **Andrade** *et al.***, 1999**; **Panizza and Tognoni, 1992** *apud* **Andrade** *et al.***, 1999**).

Larkin and Scowcroft (**1981**), like many authors before in the 1970's, observed that *in vitro* culture techniques could induce genetic variability and that it could provide a valuable add-on to plant improvement. They proposed the general term we now use *ad lib*, 'somaclonal variation', for a phenomenon that they argued to be widespread and not an artefactual noise confined to vegetatively-propagated plants. At present time, we know that tissue culture-derived somaclonal variation is likely a reflection of response to cellular stress and many reports refer to the usefulness of understanding the mechanism of tissue culture variation in defining cellular mechanisms acting in the process of evolution and in elucidating the mechanism by which plants respond to stress (**Jin** *et al.***, 2008**).

Propagation by organogenesis or by somatic embryogenesis has been reported to induce variability among the regenerated plants. In plant propagation, the most crucial aspect is to retain genetic integrity with respect to the mother plants. For nuclear DNA content analysis, the use of flow cytometry has been reported as an excellent mean of assessing clonal fidelity (**Jin** *et al.***, 2008**; **Zuzarte** *et al.***, 2010**).

Commercially important cultivars of *L. angustifolia* are relatively small, low yielding plants compared to *L.* x *intermedia*. Lavandin cultivars, including "Provence" and "Grosso", are generally larger plants with larger flowers yielding up to 5 times more oil than *L. angustifolia* cultivars (**Urwin** *et al.***, 2007**; **Falk** *et al.***, 2010**).

The world demand for lavender EO is still increasing. It is estimated that over 200,000 ha are being cultivated in Europe and the quality of produced EO is important especially for medicinal, pharmaceutical and aromatherapy uses (**Hassiotis** *et al.***, 2010.**

Several lavender EO quality standards have been established such as those of the AFNOR (Association Française de Normalisation) standards in France. Meeting these standards, while maintaining high crop yields, is a key issue for the lavender industry. Traditional and current approaches are based on the selection of cultivars within the rich genetic diversity of wild populations, or of somaclones or the use of laboratory-generated autotetraploids, as did Urwin *et al*. (**2007**) with *L. angustifolia* using colchicine, which has long been used in plant breeding programs (**Urwin** *et al.***, 2007**; **Guitton** *et al.***, 2010**).

Woronuk *et al*. (**2010**), in an attempt to improve EO yield and composition, regenerated *L. angustifolia* cultivated plants through tissue culture in the presence of the

mutagen ethyl methanesulfonate (EMS). EMS treatment of the *L. angustifolia* cultivar resulted in the production of high yielding plants that maintained a preferable linalool to camphor ratio (; **Woronuk et al., 2010).**

Falk *et al.* (**2010)** reported an improved method for regeneration (particularly rooting) of lavender plants through somatic organogenesis (**Falk et al., 2010**).

Hassiotis *et al.* (**2010)** produced a synthetic variety of *L. angustifolia* with improved essential oil production and composition from natural selected genotypes growing in Greece. The name of this synthetic variety is *L. angustifolia* var. *etherio* and the major EO compounds are linalool 26.9% and linalyl acetate with 22.8%. and oil yield, under laboratory extraction, having reached, 2.6% (w/fw) (**Hassiotis et al., 2010**).

2.2.3. Biological activities of *Lavandula angustifolia* essential oil

Lavender oil has a history of use in wound healing and although it was reported to be particularly effective during World War I there is little or no scientific evidence that lavender accelerates wound healing or reduces scarring (**Cavanagh & Wilkinson, 2002).**

René-Maurice Gattefossé, a French chemist, was the first person to coin the word "aromatherapy" before World War II. He burnt both his hands in a laboratory explosion. Involved or not in some mysticism, Gattefossé reported that when his burns became infected with gas gangrene, he saved his hands from amputation (the treatment for gas gangrene at that time) by using EO of lavender. Gattefossé discussed the widespread use of EOs on burns by French physicians dating back to 1915 in his classic book on aromatherapy, which was translated into English by Robert Tisserand and published as *Gattefosse's Aromatherapy* in 1993 (**Buckle, 2002**). However, even nowadays there is little published evidence that lavender accelerates wound healing or reduces scarring (**Cuttle et al., 2009**).

Recently, aromatherapy is becoming increasingly popular, and lavender is used in aromatherapy as a relaxant (**Hassiotis et al. 2010).**

The potency of lavender oil has long been a matter of speculation and, mostly by the arousing interest on EOs uses, its biological activities have been subject of an increasing number of scientific investigations for validation of its acclaimed properties and for safety testing. As mentioned earlier, *Lavandula* spp. oils are traditionally

believed to be antiseptic, anti-inflammatory, diuretic, carminative, antispasmodic, anti-epileptic, anti-rheumatic, antidepressive, sedative and pain reliever, but these uses have only very recently started to be sustained by scientific or clinical studies.

3. BIOLOGICAL ACTIVITIES OF *LAVANDULA ANGUSTIFOLIA* ESSENTIAL OIL - OVERVIEW

Lavandula angustifolia essential oil has been reported in the scientific literature with the following biological activities:

- anti-oxidant activity (**Adorjan & Buchbauer, 2010; Blazekovic *et al*., 2010; Meftazide *et al*., 2011**; **Spiridon *et al*., 2011**);
- anti-inflamatory activity (**Hajhashemi *et al*., 2003**);
- anti-nociceptive activity (**Hajhashemi *et al*., 2003**; **Sakurada *et al*, 2009**; **Dobetsberger & Buchbauer, 2011**);
- antispasmodic activity (**Lis-Balchin & Hart, 1999**);
- local anaesthetic activity (**Ghelardini *et al*., 1999**);
- anti-mutagenic activity (**Evandri *et al*., 2005**);
- anxiolytic effects (**Cruz & Park, 2011**; **Dobetsberger & Buchbauer, 2011**);
- possible adjuvant role on treatment of depression (**Dwyer *et al*., 2011**);
- anti-Acari activities (**Perrucci *et al*., 1996**; **Mansour *et al*., 2008**; **Pirali-Kheirabadi & Silva, 2010**);
- anti-parasitic activities against human protozoan pathogens *Giardia duodenalis* and *Trichomonas vaginalis* and the fish pathogen *Hexamita inflata* (**Moon *et al*., 2006**);
- antibacterial activity (**Hawrelak *et al*., 2009**; **Gómez-Estaca *et al*., 2010; Sokovic *et al*., 2010**; **Budzynska *et al*., 2011**; **Djenane *et al*., 2011**; **Hussain *et al*., 2011**; **Stanković *et al*., 2011**);
- insect-repellent and insecticidal activities (**Koschier & Sedy, 2003**; **Mauchline *et al*., 2008**; **Conti *et al*., 2010**);
- herbicide activity (**Haig et al., 2009**; **Rolim de Almeida et al., 2010**);
- and antifungal activity which will be referred in issue **4.2**.

We did not find any published or other available previous studies on nematicidal activity of EOs from genus *Lavandula*, except for Oka *et al.* (**2000**).

4. FUNGAL DISEASES AND ESSENTIAL OILS AS PROMISING ANTIMYCOTICS

4.1 Currently used therapeutics and plant-derived antimycotics

Scientific and *medical literature have widely been reporting that* the incidence of invasive, opportunistic fungal infections increased significantly since the 1980's due to the increasing numbers of patients who are at risk for the development of serious fungal infections, including immunocompromised patients (receiving parenteral hyperalimentation and/or broad-spectrum antibiotics, and intravascular catheter users), patients undergoing transplantation and major surgery, patients with neoplastic disease and advanced age. These groups pose considerable therapeutic challenges and the mortality and morbidity of these infections is quite substantial (**Giordani *et al.*, 2004**; **Pfaller *et al.*, 2006**; **Espinel-Ingroff, 2009**; **Pozzatti *et al.*, 2010**).

Currently used therapeutics are reported for several disadvantages including drug-drug interactions and rapid development of fungal resistance with prolonged treatment. Furthermore, some existing antimycotics like azoles may sometimes cause allergic reactions or other side-effects (**Shin, 2003**; **Shin & Lim, 2004**; **Pawar & Thaker, 2006**; **Pozzatti *et al.*, 2010**).

Plant-derived antimycotics have increasingly been attracting the attention of botanists and mycologists because they are natural, cheaper, safer and eco-friendly. EOs are characterized by a broad-spectrum of activity, including antifungal properties, as food and feed preservatives, as inhibitors of mycotoxin production, and as antimycotic agents. They are one of the most promising groups, from which a new prototype of antifungal agents may be developed. Nevertheless, they appear to have relatively mild activities compared to commercial, synthetic antifungal drugs and, consequently, they have been evaluated for synergism after administration in combination with synthetic drugs. Combinations with amphotericin B or ketoconazole have already indicated a

potentiation of these drugs leading to the possibility of decreasing their necessary quantity and, therefore, to a decrease or elimination of toxic side-effects (**Shin, 2003**; **Giordani** *et al***., 2004**; **Shin & Lim, 2004**; **Pawar & Thaker, 2006**; **Pyun & Shin, 2006**; **Pozzatti** *et al***., 2010**).

The main mechanism of the antimicrobial activity of EOs is associated with their lipophilicity and consequent interaction with the microbial cell membrane. The inhibition of chain respiration, as a result of EOs interactions with mitochondrial membranes, has also been demonstrated. Although no specific interaction of EOs with human membranes limits their clinical use as antimicrobial agents in the treatment of systemic diseases, EOs clinical application has been reported for potential harsh or caustic effects when used at high concentrations and also for malabsorption from the human intestin. Studies that have shown the most promising clinical efficacy are in the treatment of superficial mycoses domain (**Inouye** *et al***.**, **2000**; **Shin, 2003**; **Pozzatti** *et al***., 2010**).

4.1.1 Common invasive fungal infections

4.1.1.1 Major types of infectious agents

The most common invasive fungal infections are caused by the species *Candida* and *Aspergillus*. *Aspergillus* infections are becoming more frequent, resulting in significant morbidity and mortality in developing countries. *A. fumigatus* is the usual cause of invasive aspergillosis. The increasing population at risk for developing fungal disease has also increased the incidence of infection by dermatophytes. Chronic infections of skin carry considerable morbidity and can become serious in immunocompromised patients resulting in invasive infections. Dermatophytosis is also one of the top common and widespread infectious disease worldwide. In immunocompromised individuals, cryptococcosis is also a major fatal infection and is an AIDS defining illness (**Isham, 2006**; **Pawar & Thaker, 2006**; **Espinel-Ingroff, 2009; Johnston & May 2010**; **Khan & Ahmad, 2011**; **Pereira** *et al***., 2011**).

4.1.1.2. Candidiasis.

Candida is harmless commensal yeast-like fungus in healthy humans, which can cause superficial as well as life-threatening systemic infections under immune compromised situations. In healthy individuals, *Candida* infections are usually due to impaired epithelial barrier functions and occur in all age groups, although they are most common in the newborn and the elderly. They usually remain superficial and respond readily to treatment. Systemic candidiasis is usually seen in patients with cell-mediated immune deficiency, and those receiving aggressive cancer treatment, immunosuppression, or transplantation therapy. Various species of *Candida* are capable of causing infection in humans, but *C. albicans* predominates, being generally responsible for 90% - 100% of mucosal infections and for 50% - 70% of episodes of candidemia (bloodstream infection). Approximately 95%–97% of all *Candida*-associated candidemia are caused by 5 species: *C. albicans, C. glabrata, C. parapsilosis, C. tropicalis,* and *C. krusei.* The remaining 3% - 5% include *C. lusitaniae, C. guilliermondii,* and *C. rugosa,* among others. Non-*albicans Candida* species are emerging more frequently and often are resistant to fluconazole (**Pfaller *et al*., 2006**; **Bouza & Munoz, 2008**; **Pozzatti *et al*., 2010**).

Azole drugs and their derivatives continue to dominate as the antifungal agents on *Candida* infections, either as topical applications or as oral drugs. Recently developed therapeutic options include a new triazole, voriconazole, and a new class of antifungal agents, the echinocandins, which inhibit the synthesis of a fungal cell wall component (1,3-beta-D-glucan). However, the treatment of *Candida* infections has led to a rise in the number of species offering secondary resistance towards azoles, namely fluconazole, in previously susceptible species. Furthermore, the echinocandins have demonstrated less activity against strains of *Candida parapsilosis* and *Candida guilliermondii* (**Isham, 2006**; **Horn *et al*., 2009**; **Agarwal *et al*., 2010**).

4.1.1.3. Aspergillosis

Aspergillus conidia are ubiquitous in air, soil, and decaying matter. Exposure to *Aspergillus* species in the environment may cause allergic reactions in hypersensitized hosts or destructive, invasive pulmonary and disseminated disease in highly immunosuppressed individuals. In healthy individuals, though, only inhalation of a very

high quantity of conidia would cause infection. The majority of infections are caused by *A. fumigatus, A. flavus, A. niger,* and *A. terreus. A. fumigatus* is the usual cause of invasive aspergillosis (**Pfaller *et al*., 2006**). *A. niger* and *A. flavus* commonly cause food poisoning by aflatoxin formation and can also cause aspergillosis. Non-*fumigatus Aspergillus* species are emerging more frequently and often are resistant to amphotericin B. Combinational therapy of EOs and synthetic antibiotics amphotericin B or ketoconazole may be particularly useful against *Aspergillus species,* especially A. *flavus* (**Shin, 2003**).

4.1.1.4. Dermatophytosis (tinea)

Dermatophytes are a group of closely related fungi that have keratinase and can therefore cause infections in keratinised human and animal tissues (skin, hair and nails), leading to a disease known as dermatophytosis. This group is composed by the genera *Epidermophyton*, *Trichophyton* and *Microsporum*, forming an approximated total of 40 species.

Drugs used orally in the treatment of dermatophytoses include griseofulvin, terbinafine, and several azole antifungals, namely, fluconazole, ketoconazole and itraconazole (**Trevor *et al*., 2010**; **de Diego, 2011**).

4.1.1.5. Cryptococcosis

C. neoformans is a yeast-like fungus ubiquitous in the soil, where it grows as a saprophyte. It is common in pigeon nests and around pigeon droppings. It can also be isolated from numerous environmental sources including vegetables and fruit, house dust, air conditioners, air and sawdust. Cryptococcosis is a chronic, subacute to acute pulmonary, systemic or meningitic disease, initiated by the inhalation of basidiospores or yeast cells of *C. neoformans*. Primary pulmonary infections have no diagnostic symptoms and are usually subclinical. On dissemination, the fungus usually shows a predilection for the central nervous system, however skin, bones and other visceral organs may also become involved. *C. neoformans* is a major cause of fatal infections of the central nervous system in HIV-positive patients and is an AIDS defining illness. Worldwide, 7-10% of patients with AIDS are affected. Dissemination to the brain and meninges is the most common clinical manifestation of cryptococcosis and includes

meningitis, meningoencephalitis or expanding cryptococcoma. Meningitis the most common clinical form, however the clinical signs are rarely dramatic. Meningoencephalitis due to invasion of the cerebral cortex, brain stem and cerebellum is an uncommon, rapid fulminate infection, often leading to coma and death within a short time. Cryptococcoma is a rare entity, characterized by localized, solid, tumor-like masses, usually found in the cerebral hemispheres or cerebellum, or more rarely in the spinal cord. Other T cell deficiencies such as organ transplantation and bone marrow transplantation are especially at high risk of suffering from cryptococcosis.

It is *worth mentioning* that *Cryptococcus gattii* (formerly *Cryptococcus neoformans* var. *gattii*) has recently been responsible for an ongoing outbreak of cryptococcosis in apparently immunocompetent humans and animals (**Perfect *et al.* 2010**).

Current antifungal agents such as amphotericin B, fluconazole, itraconazole and flucytosine are usually prescribed for the treatment of *C. neoformans* infections. Mortality rate has been reduced, however, fungal resistance and serious side-effects are growing problems. Within the echinocandins anidulafungin, caspofungin, and micafungin, the latter have no *in vivo* activity *versus Cryptococcus* species (**Zhou & Murphy, 2006**; **Perfect *et al.* 2010**).

4.2 Previous studies on antifungal activity of EOs

4.2.1 Previous studies on antifungal activity of EOs against *Candida* species

While screening antifungal activity of various commercial EOs, including LAEO, Giordani *et al*. (**2004**) concluded that LAEO has a weak antifungal activity towards *C. albicans* compared to *Thymus vulgaris* and *Origanum vulgare* EOs, also Lamiaceae, because of the absence of thymol and carvacrol and so presuming that the antifungal activity of the EOs is dependent on the quantity of phenolic alcohols. Carvacrol is well known for its antimicrobial activity. In Giordani *et al*., *Thymus vulgaris* EOs thymol chemotype revealed a potential role of as an antifungal agent and, furthermore, exhibited potentiation of amphotericin B. Souza *et al*. (**2007**), aiming for natural antimicrobials in food preservation, also demonstrated *O. vulgare* EO

effectiveness to inhibit the growth of *C. albicans* and *C. krusei.* Pozzatti *et al.* (**2010**) studied the activities of EOs (*Rosmarinus officinalis*, *Origanum vulgar*e, *Thymus vulgaris*, *Lippia graveolens*, *Zingiber officinale*, *Ocimum basilicum*, *Salvia officinalis* and *Cinnamomum zeylanicum* - unreported provenance of plant material) in the inhibition of *C. albicans* and *C. dubliniensis* germ tube formation. Germ tube formation from blastoconidia by *Candida* species has been shown as a potential virulence factor in their pathogenesis, since it is the first stage of true hyphae development. Furthermore, inhibition of the dimorphic transition alone has been suggested to be sufficient to treat disseminated candidosis. Pozzatti *et al.* reported carvacrol to be one of the major components directly related to germ tube inhibition, but underlining that previous studies had reported the EOs to be more effective than the purified compound, thus showing the synergism between carvacrol and other minor components. Gonçalves *et al.* (**2011**) found *C. guillermondii* isolated from recurrent cases of vulvovaginal candidosis to be sensitive to *Thapsia minor* EOs, where oxygenated monoterpenes represented the main fraction with geranyl acetate as the major constituent (**Giordani *et al*. 2004**; **Marwah *et al*., 2007**; **Souza *et al*., 2007**; **Gonçalves *et al*., 2010**; **Pozzatti *et al*., 2010**; **Gonçalves *et al*., 2011**).

On the genus *Lavandula*, Zuzarte *et al.* (**2009**) refer a low activity of Portuguese lavender oils from *L. pedunculata* against *Candida* species, while studies with the *L. viridis* EOs proved to have fungicidal activity against the three tested *C. albicans* strains (ATCC 10231 and clinical D5 and M1) and also to inhibit filamentation at concentrations lower than those required for yeast inhibition. Hanamanthagouda *et al.* (**2009**) studied the EOs of an Indian *Lavandula bipinnata* reporting moderately inhibitory activity against *C. albicans*. D'Auria *et al.* (**2005**) investigated the antifungal effect of LAEO of commercial provenance and also commercial linalool and linalyl acetate (97% pure), against 50 clinical isolates of *C. albicans* and reported LAEO to have both fungistatic and fungicidal activity against the *C. albicans* strains tested as well as inhibition capacity for germ tube formation at concentrations lower than those required for yeast inhibition. Furthermore, linalool proved to be more effective than EO (**D'Auria *et al*., 2005**; **Hanamanthagouda *et al*., 2009**; **Zuzarte *et al*., 2009**; **Zuzarte *et al*., 2011**).

4.2.2. Previous studies on antifungal activity of EOs against *Aspergillus* species.

Shin (**2003**) evaluated the antifungal activities of several EOs (*Cedrus atlantica, Styrax tonkinensis, Juniperus communis, Melaleuca alternifolia, Pelargonium graveolens, Pogestemon patchouli, Rosmarinus officinalis* and *Lavandula angustifolia* - unreported provenance of plant material) and their main components against *A. niger* and *A. flavus*. All the EOs, except for *C. atlantica, J. communis,* and P. *patchouli* EOs*,* significantly inhibited growth of *A. niger* but to a lesser extent that of *A. flavus*. While combining EOs and synthetic drugs, combination of amphotericin B or ketoconazole with *P. graveolens* EO or with one of its two major components, geraniol and citronellol (which are well-known antimicrobial aliphatic primary alcohols), all of them caused a significant decrease in the MIC of each compound against *Aspergillus*, compared to their individual MIC values (**Shin, 2003**).

Commercial origanum oil (Lamiaceae) has been shown to delay or inhibit the growth of *A. flavus* (**Manohar *et al.*, 2001**). Pawar & Thaker (**2006**) conducted a study with 75 different EOs of commercial provenance, including LAEO, for the inhibition of hyphal growth and spore formation in *A. niger*. The top five EOs which demonstrated marked inhibitory effect against hyphal growth and spore formation were *Cinnamomum zeylanicum, Cinnamomum zeylanicum, Cinnamomum cassia, Syzygium aromaticum* and *Cymbopogon citratus*; LAEO showed a moderate activity (**Pawar & Thaker, 2006**). Tullio *et al*. (**2006**), also with commercial EOs (*Foeniculum vulgare, Eugenia caryophyllata, Pinus sylvestris*, and the Lamiaceae *Thymus vulgaris, Salvia officinalis, Melissa officinalis* and *Lavandula angustifolia*), studied antifungal activity against filamentous fungi, which included clinical isolates from bronchial alveolar lavage of *Aspergillus*: *A. niger, A. flavus, A. flavus* var. *columnaris*, and *A. fumigatus*. LAEO showed a weak activity, which was attributed to its low water solubility, against its previously proven good activity when assayed by vapor contact (**Tullio *et al.*, 2006**). Angioni *et al*. (**2006**) studied antifungal activity of *Lavandula stoechas* EOs from Sardinia, but it exibited mild activity against the tested *Aspergillus, A. flavus* (**Angioni *et al.*, 2006**). Moon *et al*. (**2007**) screened lavender oils (all from Australian growers/distillers, except for LAEO, with UK provenance) antifungal action against fungi of both medical and agricultural importance, including an *Aspergillus* species, with *A. nidulans*. The oils tested were obtained from *L. angustifolia, L. x* allardii, three

varieties of *L. x intermedia*, *L. x heterophylla* and one variety of *L. stoechas*, and oils assayed inhibited the growth of *A. nidulans*. Moon *et al*. referred that LAEO had already been demonstrated by Inouye *et al*. (**2001**) to be effective against a range of fungi including *A. fumigatus*. Moon *et al*. reported a lack of correlation between major oil components and antifungal activity, and hypothized that the different susceptibilities of the fungi may be related to either the minor components of the oil or differences in the cell wall/cell membrane of the fungi themselves, aiming for a further research to determine which component(s) of the oils are responsible for the antifungal effect (**Moon *et al*., 2007**). Hanamanthagouda *et al*. (**2009**) studies already cited revealed *L. bipinnata* EOs to have inhibitory activity against *A. niger*, also. Zuzarte *et al*. (**2009, 2011**) refer a moderate activity of lavender oils from *L. pedunculata* and *L. viridis* EOs against *Aspergillus*. (**Hanamanthagouda *et al*., 2009**; **Zuzarte *et al*., 2009**; **Zuzarte *et al*., 2011**).

4.2.3. Previous studies on antifungal activity of EOs against dermatophytes.

Pandey *et al*. (**2010**), on antidermatophytic activity of Indian *Curcuma longa*, refer previous studies reporting antimicrobial activity of *Leptospermum petersonii* and *Syzygium aromaticum* EOs against *T. mentagrophytes, T. rubrum, E. floccosum* and *M. gypseum* by Park *et al*. (**2007**), and also by Silva *et al*. (**2005**) with *Ocimum gratissimum* EOs (Lamiaceae), which had proved effective against *M. canis, M. gypseum, T. rubrum* and *T. mentagrophytes*. Pandey *et al*. found *C. longa* EOs to be effective against *E. floccosum M. gypseum, M. nanum, T. mentagrophytes, T. rubrum* and *T. violaceum* (**Pandey *et al*., 2010**). Pyun & Shin (**2006**) studied antifungal effects of the EOs from South Korean grown *Allium* plants against *Trichophyton* species (*T. erinacei*, *T. rubrum* and *T. soudanense*) and synergism of the oils with ketoconazole. They reported *A. sativum* oil activity to have 12.5–25% of the activity of ketoconazole, finding it a remarkable level of activity for a natural product. Furthermore, the combined effects resulted in a significant synergism of ketoconazole with *A. sativum* oil, and also with allicin (**Pyun & Shin, 2006**).

It may be *worth mentioning* Guerrini *et al*. (**2009**) found Ecuador grown *Piper aduncum* EOs to be active against *T. mentagrophytes* (**Guerrini *et al*., 2009**). Marwah *et al*. (**2007**) studied antimicrobial activity and the major components of the EO of *Plectranthus cylindraceus* (a naturally grown Lamiaceae in Oman) with carvacrol

chemotype. The antifungal activity of the oil was assessed against dermatophytic fungi including *M. canis*, *M. gypseum*, *T. rubrum* and *T. mentagrophytes*. The results showed broad spectrum antimicrobial activity of the EO and that such bioactivity could be attributed in part to carvacrol (**Marwah *et al.*, 2007**).

The higher susceptibility of dermatophytes to some EOs has been reported. Salgueiro *et al*. (**2006**) while studying antifungal activity of *Thymus capitellatus* oils (an endemic Portuguese Lamiaceae) including *M. canis*, *M. gypseum*, *T. rubrum*, *T. mentagrophytes* and *E. floccosum*, concluded that the EOs exhibited antifungal activity for the dermatophyte strains and that the highest antifungal activity of the oil could be associated with the contribution of the linalyl acetate (**Salgueiro *et al*, 2006**). Also, Gonçalves *et al*. with Portuguese *Mentha cervina* plants containing lower amounts of pulegone, with *Thymus zygis* subsp. *sylvestris* (especially carvacrol chemotype) and with *Thapsia minor* (**Gonçalves *et al*. 2007, 2010 & 2011**). Also, Tavares *et al*. (**2010**) with *Distichoselinum tenuifolium* with the Iberian endemic *Distichoselinum tenuifolium*, being myrcene the main compound (**Tavares et al., 2010**).

The works of Zuzarte *et al*. on antifungal activities of *L. pedunculata* (**2009**) and *L. viridis* (**2011**), the latter an Iberian endemism, cited above for *Candida* and *Aspergillus*, both revealed a significant antifungal activity of the EOs against dermatophyte strains. Zuzarte *et al*. concluded also that the higher activity of the oils of *L. pedunculata* compared to that of the major compounds assayed individually (1,8-cineole, fenchone, and camphor) was presumably due to a synergistic effect among the different compounds present in the oils, and that the activity of *L. viridis* EO was mainly due to the presence of α-pinene in the oil (**Zuzarte *et al.*, 2009**; **Zuzarte *et al.*, 2011**). According to Moon *et al*. (**2007**), Inouye *et al*. (**2001**) had demonstrated that LAEO was also effective against a *T. rubrum*. All oils tested by Moon *et al*. inhibited the growth of *T. mentagrophytes* (**Moon *et al.*, 2007**).

4.2.4. Previous studies on antifungal activity of EOs against *Cryptococcus neoformans*.

Some of the literature already cited for EOs activities against the yeast-like fungus *C. albicans* includes tests with the *C. neoformans*. Noteworthy are the results of Zuzarte *et al*. (**2009**, **2011**), where *C. neoformans* exhibited sensitivity to *L. pedunculata*

and *L. viridis* EOs similar to dermatophyte strains tested, as did Tavares *et al.* (**2010**) and Gonçalves *et al.* (**2010**, **2011**), working with the same *C. neoformans* strain as Zuzarte *et al.* (CECT 1078).

Marongiu *et al.*, (**2010**) found two varieties of *Calamintha nepeta* subsp. *nepeta* (Lamiaceae) to have a relatively good activity against *C. neoformans* (EOs from Italian varieties had piperitenone oxide and piperitenone as main components, while Portuguese *C. nepeta* was predominantly composed of isomenthone, 1,8-cineole and trans-isopulegone (**Marongiu *et al.*, 2010**). Also in Lamiaceae, Viljoen *et al.* (**2006**) found two varietes of *Mentha longifolia* subsp. *polyadena* in southern Africa (the ones whose oils were dominated by *cis*-piperitone oxide and piperitenone oxide) to be effective against *C. neoformans* (**Viljoen *et al.*, 2006**).

The *following* are *few interesting studies. Daucus carota* subsp. *carota* EO, spontaneous in Portugal and Sardinia showed significant activity against *C. neoformans* (the main components in the Sardinian EO were β-bisabolene and 11-α-(H)-himachal-4-en-1-β-ol; the oils from Portuguese samples were predominantly composed of geranyl acetate and α-pinene (**Maxia *et al.*, 2009**). Lopes-Lutz *et al.* (**2008**)*, w*hile screening for antimicrobial activities of *Artemisia* EOs, found wild growing Canadian *Artemisia biennis*, containing alpha-pinene, 1,8-cineole, artemisia ketone and camphor as the main components, to be active against *C. neoformans* (**Lopes-Lutz *et al.*, 2008**). Other active EOs recently reported include *Pteronia incana*, a common plant in southern Africa, with β-pinene, limonene, 1,8-cineole, myrcene, spathulenol, *p*-cymene and methyleugenol as main compounds (**Hulley *et al.*, 2010**).

5. CROP PESTS CONTROL AND ESSENTIAL OILS

5.1. Current control strategies against plant-parasitic nematodes and plant-derived nematicidal compounds

For decades, plant–parasitic nematodes in intensive crop-production systems have been controlled mainly by chemical soil fumigants and nematicides, such as methyl bromide, ethylene dibromide, dibromochloropropane, but, in the last two decades, they have been progressively withdrawn from the market due to concerns about environmental safety, human and animal health, especially with consumer demand for safe food. Currently, only a small number of nematicides, which are organophosphates and carbamates, and soil fumigants are available for nematode control in most countries. Among pesticides, nematicides are the most problematic because they are likely to contaminate groundwater, and some of them are also absorbed by plants (**Oka, 2010**). European Legislations (Reg. CE 396/2005; 1095/2007; 33/2008, 299/2008 and 1107/2009) have revised and restricted the use of pesticides on agricultural crops and research on low environmental impact alternatives to chemicals has received a strong impulse (**Maistrello *et al*., 2010**).

A wide range of options has been considered, including physical methods such as soil solarization, steam and ozone treatments, which have been tested. Focusing on agronomic strategies, amendments/preparations have been commercially released as nematicides, although their control efficacies are generally low, or even non-existent, and, in addition, nitrate leaching to the groundwater is a major potential damage (**Maistrello *et al*., 2010**; **Oka, 2010**). Noteworthy is the induced resistance in plants caused by chemicals from microorganisms. Rhizobacteria that increase plant growth and/or plant resistance are termed 'plant growth-promoting rhizobacteria' (PGPR) and induced resistance caused by such bacteria is termed 'systemic acquired resistance'. Root galls caused by *M. incognita* were reported to be reduced by some strains of PGPR (**Kokalis-Burelle *et al*., 2002 *apud* Oka, 2010**), and by endophytic species of *Bacillus*, such as *B. pumilus* and *B. mycoides* (**Mekete *et al*., 2009 *apud* Oka, 2010**). Similar to bacterial inducers, fungal endophytes, increase plant resistance to nematodes (**Sikora *et al*., 2008 *apud* Oka, 2010**). *Trichoderma harzianum* peat preparations reduced root

galling and increased top fresh weight of tomatoes grown in *M. javanica* infested soil. The fungus showed the ability to infect *M. javanica* eggs and juveniles in the laboratory (**Sharon *et al*., 2001 *apud* Oka, 2010**).

As referred in section 2.1.3, secondary metabolites are involved in the plant-defense mechanisms. They represent a large reservoir of chemical structures with biological activity against plant pests. Natural and synthetic analogs based on natural compounds in plants have been developed for use in pest control, e.g. the use of pyrethrin and pyrethroids as insecticides (**Oka, 2010**). Many plant species are known to be highly resistant to nematodes, including many members of the family Asteraceae, within which several nematicidal compounds have been isolated. The most studied of these are the polythienyls, especially, the photodynamic compound α-Terthienyl and its analogs have been isolated from *Tagetes* spp. and found to be highly effective nematicides in roots (**Duke, 1990**; **Oka, 2000**). However, only a small number of plants known to contain such compounds have been used as soil amendments for nematode control in commercial fields. Utilization of volatile toxic compounds, such as isothiocyanates generated from the glucosinolates in Brassicaceae crops for soilborne disease control is generally termed biofumigation, but not suitable for *Meloidogyne* spp. control because most Brassicaceae are their hosts (**Oka, 2010**). Phenols released from neem oil cake into amended soil were suggested to increase resistance of tomato plants to *M. javanica* due to the higher phenol content in their roots (**Sitaramajah and Singh, 1978 *apud* Oka, 2010**). It is still not clear whether application of organic amendments with a high content of phenolic compounds directly affects nematodes or increases plant resistance, because several phenolic compounds possess nematicidal activity (**Chitwood, 2002 *apud* Oka, 2010**).

5.2 Root-knot nematode: *Meloidogyne javanica*

The most wide-spread and economically important plant-parasitic nematodes in agriculture worldwide are root-knot (*Meloidogyne* spp.) and cyst (*Heterodera* spp. and *Globodera* spp.) nematodes (**Sasser and Carter, 1985 *apud* Galhano, 1997**; **Oka *et al*., 2000c**).

Nematodes from the genus *Meloidogyne* Goeldi, 1887, *commonly referred to as* root-knot nematodes, are sedentary endoparasitic animals found in *temperate, tropical,*

and subtropical climates. The most common species are M. arenaria (Neal, 1889) Chitwood, 1949, M. hapla Chitwood, 1949, M. incognita (Kofoid & White, 1919) Chitwood, 1949 and M. javanica (Treub, 1885) Chitwood, 1949. Apart from these, two more species were found in Portugal, M. hispanica Hirschmann, 1986 and M. lusitanica Abrantes & Santos, 1991. Phytoparasitic nematodes need plant cells as nutrient source to be able to complete their life cycle. During parasitic phase, morphological and physiological changes occur both in nematode and plant. Their life cycle has four juvenile stages (J1 to J4) and two adult stages with *marked sexual dimorphism (the female is round-shaped and the male is vermiform). Juvenile stage J1 and part of J2 occur within the egg.* When second-stage juvenile (J2) emerges from the egg, the infective stage begins. At this point, they are able to penetrate roots and cause morphological, physiological and cytogenetic changes in host plant cells. Hyperplasia and hypertrophy result in *galls* or "knots". After penetration and migration to *differentiated vascular tissue, J2 becomes sedentary and juvenile stage ends with sexual differentiation during J4 stage. The female is capable of producing hundreds of eggs. Females can be found inside cortex or root epidermis (**Abrantes et al., 2007**).*

5.3 Previous studies on nematicidal activity of EOs against *Meloidogyne* species

Many EOs have been reported to be nematicidal. However, their effect when incorporated into the soil has not been well studied. Although many plants have been used as soil amendments to control nematodes in small-scale experiments, only a few reports have identified the nematicidal compounds in such plants and only a small number of plants known to contain such compounds have been used as soil amendments for nematode control in commercial fields (**Oka, 2010**).

Oka *et al*. (**2000**) evaluated the nematicidal activity of 27 spices and aromatic plants *in vitro* and in pot experiments against the root-knot nematode *Meloidogyne javanica*, including *Lavandula angustifolia* (extraction part was foliage). Oka *et al*. refer that previous studies had already shown that *Pelargonium graveolens*, *Ocimum sancutum*, *Ocimum basilicum*, *Mentha piperita*, and *Mentha spicata* and their components (citronellol, eugenol, geraniol, and linalool) had nematicidal activity against *Meloidogyne* spp. Nematicidal activity was obtained with the following EOs and main components: *Artemisia judaica* (*Artemisia* ketone), *Carum carvi* ((+)-carvone and

limonene), *Coridothymus capitatus* (carvacrol), *Cymbopogon citratus* (geranial and neral), *Foeniculum vulgare (t-*anethole and limonene), *Mentha rotundifolia* (isomers of 1,2-epoxymenthyl acetate and piperitone), *Mentha spicata* ((−)-carvone and limonene), *Micromeria fruticosa* (pulegone), *Origanum syriacum* (C-type) (carvacrol), *Origanum syriacum* (CT-type) (carvacrol and thymol)*, Origanum vulgare* (C-type) (carvacrol), *Origanum vulgare* (T-type) (thymol) and *Thymus vulgaris* (thymol and carvacrol) [4]. The most effective EOs at J2 immobilization and hatching inhibition were *Carum carvi*, *Foeniculum vulgare, Mentha rotundifolia*, and *M. spicata* (**Oka *et al*., 2000**).

Torres *et al*. (**2008**) evaluated nematicidal activities of the leaf EO of *Croton regelianus* (Euphorbiaceae). The bioassay results proved both the EO of *C. regelianus* and main component ascaridole (a monoterpene) to be moderately active against the *M. incognita* (**Torres *et al*., 2008**).

Onifade *et al*. (**2008**) investigated the potentials of *Haplophyllum tuberculatum* (Rutaceae) and *Plectranthus cylindraceus* (Lamiaceae) oils to control *Meloidogyne javanica in vitro* and in soil. *In vitro*, both oils were toxic to *M. javanica*. At 12.5 mg/ml, the two oils were comparable in their inhibition of hatching and lethality to *M. javanica* juveniles, but less effective than carbofuran. However, carbofuran seemed to be equivalent to 1:1 mixtures of the two oils at 12.5 mg/ml and higher concentrations in inhibiting hatching, and killing the juveniles. When used at 25 mg/ml, *H. tuberculatum* oil was as effective as carbofuran at killing the juveniles of *M. javanica*. On soil bioassays, treated tomato plants had fewer galls on the roots than untreated but nematode-infested pots; nevertheless, carbofuran was more effective at reducing root galling at all concentrations (**Onifade *et al*., 2008**).

Meyer *et al*. (**2008**) tested clove oil from *Syzygium aromaticum* (Myrtaceae) – a soy lecithin/detergent formulation of clove bud oil - against *Meloidogyne incognita* and the results demonstrated the tested formulation to be active against *M. incognita* eggs and J2 in microwell assays, and against J2 in soil tests (**Meyer *et al*., 2008**).

Ntalli *et al*. (**2010**) tested nematicidal activity, with motility bioassays, of the EOs from 8 Greek Lamiaceae aromatic plants and 13 terpene components against J2 *M. incognita*. The EOs were isolated from: *Melissa officinalis, Sideritis clandestina, Origanum dictamnus*, *Ocimum basilicum*, *Mentha pulegium*, *Origanum vulgare*, *Vitex*

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[4] C-type = carvacrol type: a plant chemotype in which carvacrol is the major component of its essential oil. CT-type = carvacrol-thymol type: a plant chemotype in which carvacrol is present in the essential oil in addition to thymol. T-type = thymol type: a plant chemotype in which thymol is the major component of its essential oil.

agnus-castus, and *Salvia officinalis*. The highest nematicidal activity was obtained with *O. vulgare*, *O. dictamnus*, *M. pulegium* and *M. officinalis* EOs. The highest nematicidal terpenes were found to decrease in the order L-carvone, pulegone, trans-anethole, geraniol, eugenol, carvacrol, thymol, and terpinen-4-ol (**Ntalli *et al*., 2010**).

Sosa *et al*. (**2012**) conducted a study on insecticidal and nematicidal bioactivities of EOs from Argentinean *Eupatorium* and *Baccharis* spp. The motility bioassays proved *E. viscidum* to have a strong nematicidal effect against *M. javanica* J2 (**Sosa *et al*., 2012**).

The aim of the present study was to evaluate different biological activities of the essential oils of *Lavandula angustifolia* subsp. *angustifolia* from an established population in Castro D'Aire, Portugal:

- the antifungal activity against common invasive fungal infections on humans caused by *Candida*, *Cryptococcus*, dermatophyte and *Aspergillus* strains, within essential oils investigation on plant-derived antimycotics, keeping step with still work-in-progress scientific validation of lavender's traditional uses, and

- the nematicidal activity against root-knot nematode *Meloidogyne javanica*, within essential oils investigation on biopesticides.

II. MATERIALS AND METHODS

1. PLANT MATERIAL FOR HYDRODISTILLATION

Lavandula angustifolia subsp. *angustifolia* plants were provided by "Ervital" from an established population in Castro D'Aire, Portugal.

Voucher specimens were deposited in the Herbarium of the Department of Botany of the University of Coimbra, COI.

The plants were air-dried for a few days.

2. ESSENTIAL OIL ISOLATION AND ANALYSIS

EOs isolation and analysis took place at the Laboratory of *Pharmacognosy, Faculty of Pharmacy of University of Coimbra*.

The essential oils were obtained from leaves and flowers by hydrodistillation for 3 hours using a modified Clevenger-type apparatus, according to the Portuguese Pharmacopoeia method (**2005**). Distilled water was added to plant material in a 1:10 (v/v) proportion. After decantation, the EOs were stored at $0 - 4 \ ^{o}C$, in dark glass vials. The oil yield was determined according to the referred Pharmacopoeia.

Oil analyses were carried out by both GC and GC/MS using fused silica capillary columns with two different stationary phases (SPB-1 and SupelcoWax-10), in conformity with Cavaleiro *et al.* (**2004**).

3. ANTIFUNGAL ACTIVITY METHODS

Antifungal activity bioassays of the LAEO took place at the Laboratory of *Pharmacognosy, Faculty of Pharmacy of University of Coimbra*.

Antifungal activity of the EO was evaluated against yeast and filamentous fungi strains.

Yeasts: two clinical strains of *Candida* from isolated recurrent cases of vulvovaginitis candidiasis (*C. krusei* H9 and *C. guillermondii* MAT23) and three strains from American Type Culture Collection (*C. albicans* ATCC 10231, *C. tropicalis* ATCC 13803 and *C. parapsilopsis* ATCC 90018), and one strain from Colección Española de Cultivos Tipo (*Cryptococcus neoformans* CECT 1078).

Filamentous fungi: one clinical strain of *Aspergillus* isolated from bronchial secretions (*A. flavus* F44) and two strains from American Type Culture Collection (*A. niger* ATCC 16404 and *A. fumigatus* ATCC 46645); three clinical strains of dermatophytes isolated from nails and skin (*Epidermophyton floccosum* FF9, *Trichophyton mentagrophytes* FF7 and *Microsporum canis* FF1) and four strains from American Type Culture Collection (*Trichophyton mentagrophytes* var. *Interdigitale* CECT 2958, *Trichophyton verrucosum* CECT 2992, *Trichophyton rubrum* CECT 2794 and *Microsporum gypseum* CECT 2905).

The fungal isolates were identified by standard microbiology methods and *stored* on *Sabouraud* broth with *glycerol* at − 70°C. Prior to antifungal susceptibility testing, each isolate was inoculated on Sabouraud agar to ensure optimal growth characteristics and purity.

Broth macrodilution methods based on the Clinical and Laboratory Standards Institute (CLSI) reference protocols M27-A3 (**CLSI, 2008a**) and M38-A2 (**CLSI, 2008b**), for yeasts and filamentous fungi, respectively, were used to determine MIC's of the EOs and their major constituents.

The minimal inhibitory concentration is defined as the lowest EOs concentration in the broth resulting in the lack of visible microorganism growth changes (**Lalemba & Kunicka, 2003**).

A macrodilution rather than a microdilution design was used to allow the use of glass test tubes, thus avoiding the interaction of the EO with the plastic polymer material of the 96-well microtitre plates (**Zuzarte *et al.*, 2011**).

For each assay, serial twofold dilutions of the oil were prepared in dimethyl sulfoxide (DMSO), with concentrations ranging from 0.16 to 20 µL mL^{-1}. Final DMSO concentrations never exceeded 2% (v/v). All tests were performed in RPMI-1640-MOPS medium and pH was adjusted at 7.0 with NAOH. For each tested fungal strain, the growing conditions as well as the sterility of the medium were checked in two control tubes. Recent cultures of each strain were used to prepare the inoculums at approximately 0.5 McFarland units for yeasts, 1.0 McFarland units for dermatophytes, and $0{,}4 - 5 \times 10^4$ spores per mL for *Aspergillus* strains, in order to obtain final cell suspensions in RPMI medium of $1\text{-}2\times10^3$ cells per mL for yeasts, and $1\text{-}2\times10^4$ cells per mL for filamentous fungi. The test tubes were incubated aerobically at 35 $^{\circ}$C for 48h / 72h (*Candida* spp. and *Aspergillus* spp. / *Cryptococcus neoformans*) and at 30 $^{\circ}$C for 7 days (dermatophytes) and MICs were determined. In addition, two reference antifungal compounds, amphotericin B (Fluka) and fluconazole (Pfizer) were used to control the sensitivity of the tested microorganisms.

In order to estimate the lethal activity of an EO, the microorganisms are transferred from the liquid (or agar) broth where no growth is observed into a new broth medium and incubated. The lowest EO concentration resulting in total growth inhibition is recognised as the minimal lethal concentration (MLC) (**Lalemba & Kunicka, 2003**). To evaluate MLC, aliquots (20 µL) of broth were taken from each negative tube after MIC reading, and cultured in Sabouraud dextrose agar plates. Plates were then incubated at 35ºC for 48h (*Candida* spp. and *Aspergillus* spp.) and 72h for *Cryptococcus neoformans,* and 30ºC for 7 days (dermatophytes).

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.

Aseptic technique included a *Bunsen burner* and a *laminar flow cabinet.*

4. NEMATICIDAL ACTIVITY METHODS

Nematicidal activity of the EO was evaluated against *Meloidogyne javanica* population (P018) eggs with second-stage juveniles (J2).

Nematode population: *Meloidogyne javanica* population (P018) was maintained on previously infected tomato plants, *Solanum lycopersicum*, cv. Easypeel, in pots containing sterilized sandy loam soil and sand (1:1), in a growth chamber, at 25±2°C, with a 12 h photoperiod and 70–75% relative humidity, at the Nematology Laboratory at University of Coimbra. Soil inoculation was performed with 10 egg masses per pot and sub-cultures were made every 2 months.

Hatching bioassay

Egg masses were picked from S. *lycopersicon* roots. At 60 days after soil inoculation, plants were harvested and the root systems were washed carefully.

LAEO was dissolved in sterile distilled water with 0.5% Triton X-100 with the following EO final concentrations: 2.5 µL/mL, 5.0 µL/mL, and 10.0 µL/mL. Each treatment should have consisted of 5 replicates of exactly 15 eggs in 1 mL of each EO concentration, but due to 'inexperience' some replicates had 30 eggs. The experiments were conducted in glass-staining blocks maintained in a moist chamber, in the dark, at 25°C. Eggs were handpicked using a bristle (based on morphological criterion: eggs with clearly visible juveniles). Observations of *egg*-hatching were made using a stereomicroscope in a 24 hour-period during 15 days.

Data on hatching were converted to percentage cumulative hatching inhibition, corrected by Abbott's formula (**Abbott, 1925**)[5], and subjected to probit analysis (**Finney, 1971**) and, finally, calculated the essential oil concentration inhibiting hatching by 50% (IC_{50}) after 360 hours exposure. The statistical software used was SPSS for Windows (version 19.0).

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[5] Cumulative percentage inhibition, for each exposure period, is calculated according to the formula I = 100 – E, where: I = cumulative hatching inhibition (%); E = cumulative hatching (%). These values are then corrected by Abbott's formula (**1925**): $I_C = (I_E-I_T) \times 100/(100-I_T)$, where I_C = corrected cumulative hatching inhibition (%); I_E = cumulative hatching inhibition (%) on the essential oil; I_T = cumulative hatching inhibition (%) in control.

III. RESULTS AND DISCUSSION

ESSENTIAL OIL YIELD AND ANALYSIS

The EO was obtained in yields of 2.0 % (v/w). The oil was characterized by high contents of linalool (24.7%) and linalyl acetate (25.3%), which is in accordance with the international monographs.

ANTIFUNGAL ACTIVITY

The results of the antifungal tests are summarized in Table 2.

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

Strains	*L. angustifolia* subsp. *angustifolia* essential oil		Fluconazole		Amphotericine B	
	MIC (a)	MLC (a)	MIC (b)	MLC (b)	MIC(b)	MLC (b)
Candida albicans ATCC 10231	1.25	1.25	1	>128	N.T	N.T
Candida tropicalis ATCC 13803	1.25-2.5	1.25-2.5	4	>128	N.T	N.T
Candida krusei H9	1.25-2.5	2.5	64	64-128	N.T	N.T
Candida guillermondii MAT23	1.25	1.25	8	8	N.T	N.T
Candida parapsilopsis ATCC 90018	1.25-2.5	1.25	< 1	< 1	N.T	N.T
Cryptococcus neoformans CECT 1078	0.32-0.64	0.64	16	128	N.T	N.T
Trichophyton mentagrophytes FF7	0.64	0.64	16-32	32-64	N.T	N.T
Trichophyton mentagrophytes var. *interdigitale* CECT 2958	0.64	1.25	128	≥128	N.T	N.T
Trichophyton verrucosum CECT 2992	1.25	1.25	>128	>128	N.T	N.T
Microsporum canis FF1	0.64	0.64	128	128	N.T	N.T
Trichophyton rubrum CECT 2794	0.32	0.32-0.64	16	64	N.T	N.T
Microsporum gypseum CECT 2905	0.64-1.25	1.25	128	>128	N.T	N.T
Epidermophyton floccosum FF9	0.64	0.64	16	16	N.T	N.T
Aspergillus niger ATCC 16404	1.25	10-20	N.T	N.T	1-2	4
Aspergillus fumigatus ATCC 46645	1.25	2.5-5	N.T	N.T	2	4
Aspergillus flavus F44	2.5	5	N.T	N.T	2	8

(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.

The highest antifungal activity was observed against dermatophyte strains and *Cryptococcus neoformans*, with MIC and MLC values ranging from 0.32 to 0.64 μL mL^{-1}, except for *Microsporum gypseum* CECT 2905 and *Trichophyton verrucosum* CECT 2992, where values ranged from 0.64-1.25 μL mL^{-1}. For *Candida* strains, MIC and MLC values ranged from 1.25 to 2.5μL mL^{-1}. The oil was less effective against *Aspergillus* strains (**Table 2**). We considered 0.64 μL mL^{-1} the upper value for good antifungal activity and values from 1.25 to 2.5μL mL^{-1} as mild activities.

For *Trichophyton mentagrophytes* FF7, *Trichophyton verrucosum* CECT 2992, *Microsporum canis* FF1, *Epidermophyton floccosum* FF9, *Candida albicans* ATCC 10231 and *Candida guillermondii* MAT23, the MIC was equivalent to the MLC, indicating fungicidal activity of LAEO. Our results were very similar to the ones of Zuzarte *et al*. against same fungal strains.

Though we have obtained good values for *C. neoformans*, previous studies are more consensual towards dermatophytes. The higher susceptibility of dermatophytes to some EOs was already referred. Tullio *et al*. (**2006**), comparing broth microdilution and vapour contact methods for antifungal activity of EOs, reported that this susceptibility of dermatophytes does not seem to depend on the method used and summarize that more data will be necessary either to confirm this good *in vitro* efficacy (**Tullio *et al*., 2006**).

Tavares *et al*. (**2010**) and Gonçalves *et al*. (**2010**, **2011**) also obtained MIC and MLC values ranging from 0.32 to 0.64 μL mL^{-}1 for *C. neoformans* CECT 1078.

Terpene alcohols such as linalool have already been proved to exhibit strong antimicrobial activity (**Skaltsa *et al*., 2003**; **Lopes-Lutz *et al*., 2008**). In Salgueiro *et al*. (**2006**), major constituents of each chemotype of *Thymus capitellatus* (1,8-cineole, borneol, linalyl acetate and linalool) were assayed against dermatophytes, which included some of the dermatophyte clinical strains tested in the present study - *Microsporum canis* FF1, *Trichophyton mentagrophytes* FF7 and *Epidermophyton floccosum* FF9. Salgueiro *et al*. concluded that the chemotype of *T. capitellatus* with the highest antifungal activity could be associated with the contribution of linalyl acetate. In Zuzarte *et al*. (**2011**), major constituents of *Lavandula viridis* oil (1,8-cineole, camphor, α-pinene and linalool) were also assayed individually against tested microorganisms and antifungal activity was associated the presence of α-pinene in the oil, while linalool proved moderate activity. To evaluate what component(s) are

responsible for the obtained antifungal activity of *Lavandula angustifolia*, after mandatory GC-MS analysis, further assays with linalool and linalyl acetate are the next step. Usually, major compounds are the ones responsible for the antifungal activity of the EOs, however, some studies show that minor components may have a crucial role and their activities can be revealing by themselves of for inferring a synergism between components. Moon *et al*., as earlier said, reported a lack of correlation between major components and antifungal activity in LAEO (**Koroch & Juliani, 2007**; **Moon *et al*., 2007**).

Prashar *et al*. (**2004**) investigated the cytotoxicity of lavender oil and its major components to human skin cells. They reported an *in vitro* cytotoxic activity of lavender oil and its main components linalyl acetate and linalool on human skin cells (endothelial cells and fibroblasts) at a concentration of 0.25% (v/v) in the cell types tested. Combining these data with our results for dermatophytes, we can say that the range of values obtained for antifungal activity are well below citotoxicity levels for skin cells and LAEO may be a good candidate for alternative antifungal agents in treatment of dermatophytoses. Even on systemic uses, linalool has been reported to be safe and without toxicity. Burdock & Carabin (**2009**) refer that the majority of linalool and its metabolites are excreted in the urine and, smaller amounts excreted in expired air and feces. Furthermore, the most common fragrance ingredients in cosmetics and other scented products have shown that linalool is the most frequently incorporated fragrance found 97% of deodorants analysed were shown to contain linalool (**Prashar *et al*., 2004**; **Burdock & Carabin, 2009**).

As with other EOs, more data will be necessary, such as assays to evaluate its mechanism of action and clinical trials are required also to evaluate the practical relevance of *in vitro* research.

NEMATICIDAL ACTIVITY

The *Lavandula angustifolia* essential oil concentration inhibiting hatching by 50% (IC_{50}) after 360 hours exposure was 5.39 µL/mL. The probit analysis shows a dose-response effect.
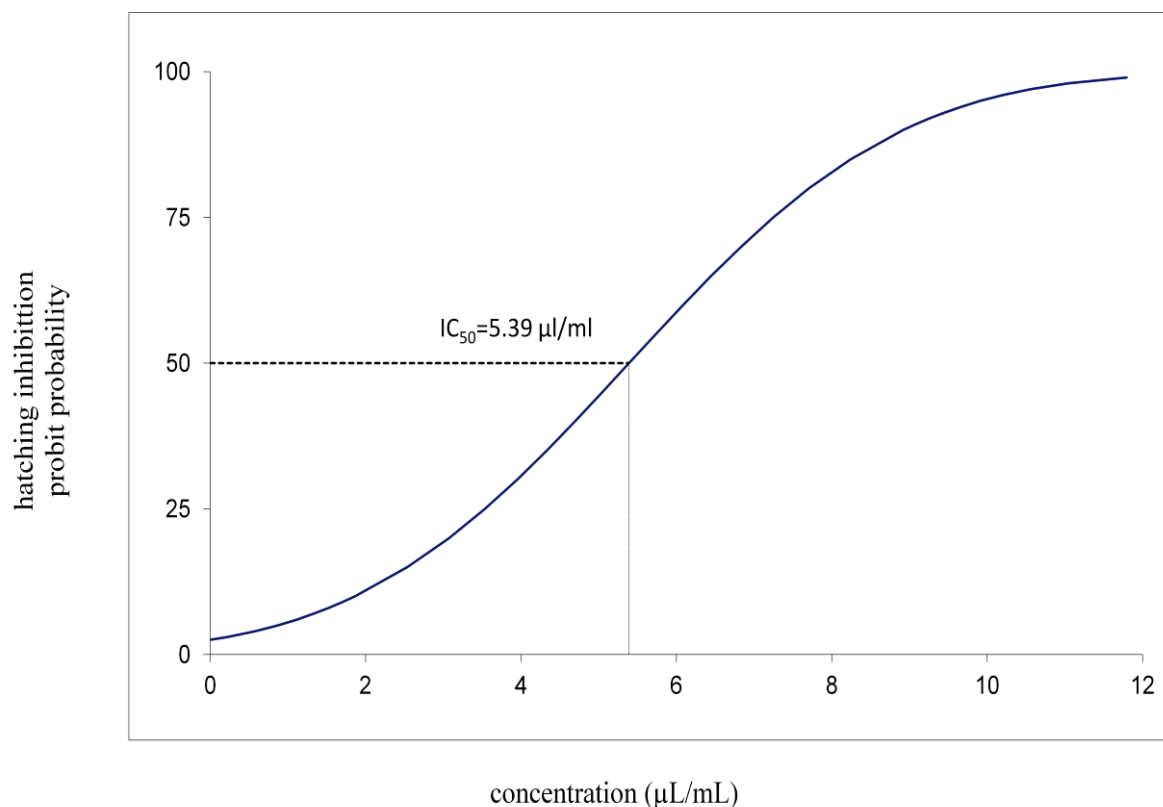

Figure 5 . Probit plot of the effect of exposure for 360 hours to *Lavandula angustifolia* essential oil on *Meloidogyne javanica* egg-hatching ($P < 0.05$).

Non-treated results of the hatching bioassay are presented in Annex.

We did not find any published or other available previous studies on nematicidal activity of EOs from genus *Lavandula* against *Meloidogyne* species.

Chatterjee *et al*. (**1982**) reported linalool, the major constituent of *Ocimum basilicum* (Lamiaceae) as having nematicidal activity on "*Meloidogyne incognita* larvae". Oka *et al*. (**2000**) did not find *Lavandula angustifolia* essential oil to have nematicidal activity against *Meloidogyne javanica*, but the extraction part used was foliage. According to Guitton *et al*. (**2010**) accumulation of linalool content in *L.*

angustifolia is found in 10 times lower in leaves than in inflorescences, as terpene secretion mainly takes place in peltate glandular trichomes in the depressed sinuses of the adaxial calyx epidermis, a result based on volatile organic content analyzed by GC-MS and histochemical analyses by scanning electron microscopy.

A preliminary mortality bioassay was conducted. In the same range of EO concentrations used in hatching bioassay, we obtained mortality values of 100% (data not shown). A mobility bioassay is recommended.

IV. CLOSING REMARKS AND FUTURE PERSPECTIVES

Lavandin, *L.* x *intermedia*, and fine lavender, *Lavandula angustifolia*, have high industrial and commercial value, used in food industry, perfumery and pharmaceutical preparations. Beyond scientific validation of traditional uses of lavender, investigation on these essential oils aims to develop reliable protocols for industrial purposes.

It must be emphasized that, although *Lavandula angustifolia* oil is the most commercialized oil of all lavenders, on scientific validation of its properties in herbal medicine it is of the utmost importance to study *Lavandula angustifolia* distilled oil obtained from raw plant material instead of commercial oil because racemic mistures are frequent.

Within antifungal activities of the *Lavandula angustifolia* essential oil used in this study, although the range of values obtained for antifungal activity are well below cytotoxicity on human skin cells reported by Prashar *et al*., (**2004**) it would be desirable to undertake corroboration tests on its citotoxicity.

Within nematicidal activities of the *Lavandula angustifolia* essential oil used in this study, the next steps would be mobility and mortality bioassays, followed by an infectivity bioassay against *Meloidogyne javanica*.

As referred earlier, natural and synthetic analogs based on natural compounds in plants have been developed for use in pest control, but mainly as insecticides or insect-repellents. According to recently revised European Legislations, and taking into account a possible water crisis in the next 10 to 20 years, finding natural pesticides for crops that protects groundwater may become a matter of urgency for horticultural and agronomic industries.

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Index of figures and tables

Table 3e (Annex). Hatching bioassay with *Meloidogyne javanica* (P018) using different concentrations of *Lavandula angustifolia* essential oil: 10.0 µL/mL of *Lavandula angustifolia* essential oil

Table 3a. Hatching bioassay with *Meloidogyne javanica* (P018) using different concentrations of *Lavandula angustifolia* essential oil: CONTROL 1 – H2Ode (sterile distilled water).

	REPLICATE 1		REPLICATE 2		REPLICATE 3		REPLICATE 4		REPLICATE 5	
	Eggs	J2	Eggs	J2	Eggs	J2	Eggs	J2	Eggs	J2
0 H	18	0	15	0	19	0	17	0	20	0
24 H	13	5	15	0	19	0	9	8	16	4
48 H	13	5	11	4	16	3	7	10	15	5
72 H	11	7	9	6	9	10	7	10	15	5
96 H	10	8	9	6	5	14	4	13	15	5
120 H	7	11	8	7	5	14	3	14	15	5
144 H	7	11	8	7	4	15	3	14	15	5
168 H	7	11	7	8	5	14	3	14	15	5
216 H	7	11	5	10	5	14	3	14	15	5
240 H	6	12	5	10	5	14	3	14	15	5
264 H	5	13	5	10	5	14	3	14	15	5
288 H	5	13	5	10	5	14	3	14	15	5
312 H	5	13	5	10	5	14	3	14	15	5
336 H	5	13	5	10	5	14	3	14	15	5
360 H	5	13	5	10	5	14	3	14	15	4

Table 3b. Hatching bioassay with *Meloidogyne javanica* (P018) using different concentrations of *Lavandula angustifolia* essential oil: CONTROL 2 – [H2Ode + Triton X (5000 ppm)].

	REPLICATE 1		REPLICATE 2		REPLICATE 3		REPLICATE 4		REPLICATE 5	
	Eggs	J2	Eggs	J2	Eggs	J2	Eggs	J2	Eggs	J2
0 H	17	1	17	0	15	0	16	0	17	0
24 H	16	2	17	0	13	2	16	0	17	0
48 H	14	4	13	4	13	2	16	0	16	1
72 H	14	4	12	5	12	3	16	0	16	1
96 H	14	4	7	10	10	5	15	1	16	1
120 H	13	5	1	16	9	6	13	3	16	1
144 H	13	5	1	16	9	6	10	6	13	4
168 H	11	7	0	17	9	6	7	9	11	6
216 H	8	10	0	17	7	8	3	13	7	10
240 H	4	14	0	17	7	8	2	14	5	12
264 H	3	15	0	17	3	12	1	15	3	14
288 H	3	15	0	17	3	12	0	16	0	17
312 H	3	15	0	17	3	12	0	16	0	17
336 H	3	15	0	17	3	12	0	16	0	17
360 H	3	14	0	17	3	12	0	16	0	17

Table 3c. Hatching bioassay with *Meloidogyne javanica* (P018) using different concentrations of *Lavandula angustifolia* essential oil: 2.5 µL/mL of *Lavandula angustifolia* essential oil.

	REPLICATE 1		REPLICATE 2		REPLICATE 3		REPLICATE 4		REPLICATE 5	
	Eggs	J2	Eggs	J2	Eggs	J2	Eggs	J2	Eggs	J2
0 H	15	0	17	0	17	0	18	0	20	0
24 H	15	0	17	0	16	1	18	0	20	0
48 H	15	0	10	7	11	6	10	8	18	2
72 H	10	5	7	10	9	8	10	8	17	3
96 H	6	9	6	11	10	10	5	12	15	4
120 H	6	9	5	12	7	10	5	12	14	5
144 H	5	10	5	12	5	12	5	12	14	5
168 H	3	12	2	15	5	12	5	12	13	6
216 H	2	13	0	17	4	13	3	14	11	8
240 H	2	13	0	17	3	14	3	14	6	12
264 H	2	13	0	17	3	14	3	14	6	12
288 H	2	13	0	17	3	14	3	14	6	12
312 H	2	13	0	17	3	14	3	14	6	12
336 H	2	13	0	17	3	14	3	14	6	12
360 H	2	13	0	17	3	13	3	14	6	13

Table 3d. Hatching bioassay with *Meloidogyne javanica* (P018) using different concentrations of *Lavandula angustifolia* essential oil: 5.0 µL/mL of *Lavandula angustifolia* essential oil.

	REPLICATE 1		REPLICATE 2		REPLICATE 3		REPLICATE 4		REPLICATE 5	
	Eggs	J2	Eggs	J2	Eggs	J2	Eggs	J2	Eggs	J2
0 H	17	0	17	0	15	0	15	0	19	0
24 H	17	0	14	1	13	2	14	1	19	0
48 H	14	3	8	7	13	2	12	3	19	0
72 H	14	3	8	7	13	2	12	3	17	2
96 H	14	3	7	8	9	6	8	7	15	4
120 H	14	5	7	8	5	10	7	8	12	7
144 H	12	5	7	8	5	10	5	10	10	9
168 H	9	8	7	8	5	10	3	12	7	12
216 H	6	11	7	8	4	11	3	12	7	12
240 H	6	11	7	8	4	11	3	12	7	12
264 H	6	11	7	8	4	11	3	12	7	12
288 H	6	11	7	8	4	11	3	12	7	12
312 H	4	13	7	8	4	11	3	12	7	12
336 H	4	13	5	10	4	11	3	12	7	12
360 H	4	13	5	10	4	11	3	12	7	12

Table 3e. Hatching bioassay with *Meloidogyne javanica* (P018) using different concentrations of *Lavandula angustifolia* essential oil: 10.0 µL/mL of *Lavandula angustifolia* essential oil.

	REPLICATE 1		REPLICATE 2		REPLICATE 3		REPLICATE 4		REPLICATE 5	
	Eggs	J2	Eggs	J2	Eggs	J2	Eggs	J2	Eggs	J2
0 H	17	0	16	0	30	0	23	0	16	
24 H	17	0	16	0	30	0	23	0	16	0
48 H	17	0	16	0	30	0	23	0	16	0
72 H	17	0	16	0	30	0	23	0	16	0
96 H	17	0	16	0	30	0	23	0	16	0
120 H	17	0	16	0	30	0	23	0	16	0
144 H	17	0	16	0	30	0	23	0	16	0
168 H	17	0	16	0	30	0	23	0	16	0
216 H	17	0	16	0	30	0	23	0	16	0
240 H	17	0	16	0	30	0	23	0	16	0
264 H	17	0	16	0	30	0	23	0	16	0
288 H	17	0	16	0	30	0	23	0	16	0
312 H	17	0	16	0	30	0	23	0	16	0
336 H	17	0	16	0	30	0	23	0	16	0
360 H	17	0	16	0	30	0	23	0	16	0