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Biological activities of *Lavandula angustifolia* essential oil
(Diplomová práce)

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Summary

1. INTRODUCTION.....	7
2. AIM OF WORK.....	9
3. THEORETICAL PART	10
3.1. Essential oils synthesis.....	10
3.2. Essential oil characterisation	10
3.3. Essential oils isolation and characterisation	12
3.3.1. Hydrodistillation.....	12
3.3.2. Steam and water distillation	13
3.3.3. Steam distillation	13
3.3.4. Organic solvents extraction	13
3.3.5. Pressing.....	14
3.3.6. Simple extraction with oils (fats) and enfleurage.....	14
3.3.7. Analytical techniques	14
3.3.8. Parts of chromatograph.....	14
3.4. Lavender	16
3.4.1. Family Lamiaceae	16
3.4.2. Genus Lavandula	17
3.4.3. Botanical description	17
3.4.4. Lavandula angustifolia Mill.	18
3.4.5. Lavandula angustifolia Essential oil.....	20
3.4.6. Biological activities	21
3.4.6.1. Cytotoxicity.....	21
3.4.6.2. Anti-inflammatory activity.....	21
3.4.6.3. Antioxidant activity.....	23
3.4.6.4. Antifungal activity	24
3.4.6.5. Nematical activity	27
3.4.6.6. Repellency assay	30
4. EXPERIMENTAL PART	33
4.1. Biological material.....	33
4.2. Chemicals.....	34
4.3. Instruments:	36
4.4. Methodology.....	37

4.4.1.	Essential oil isolation and characterisation.....	37
4.4.1.1.	Plant material:	37
4.4.1.2.	Isolation:.....	37
4.4.1.3.	Analysis:.....	37
4.4.2.	Antifungal activity.....	38
4.4.2.1.	Fungal strains	38
4.4.2.2.	Method	39
4.4.3.	Cytotoxicity assay	40
4.4.3.1.	Material	40
4.4.3.2.	Method	40
4.4.4.	Anti-inflammatory activity	42
4.4.4.1.	Material	42
4.4.4.2.	Nitric oxide measurement	42
4.4.5.	Antioxidant activity	43
4.4.5.1.	Method	43
4.4.6.	Nematicidal activity.....	44
4.4.6.1.	Method	44
4.4.7.	Insect repellency	45
4.4.7.1.	Material	45
4.4.7.2.	Method	45
5.	RESULTS	47
5.1.	Essential oil isolation and characterization.....	47
5.2.	Antifungal activity	48
5.3.	Cytotoxicity	49
5.4.	Anti-inflammatory activity	51
5.5.	Antioxidant activity	52
5.6.	Nematicidal activity.....	53
5.7.	Insect repellency	55
6.	DISCUSSION	56
6.1.	Essential oil isolation and characterization.....	56
6.2.	Antifungal activity	57
6.3.	Cytotoxicity	58
6.4.	Anti-inflammatory activity	59

6.5. Antioxidant activity	59
6.6. Nematicidal activity.....	59
6.7. Insect repellency	61
7. CONCLUSION	62
8. REFERENCES.....	63

1. INTRODUCTION

From ancient times, herbs were used by people as food, protection for their homes or filling for their pillows. Through the times they recognized that some plants have special potential. They drank tea from willow bark....and the pain was gone, some plants were good when people had troubles to fall asleep, some repelled the moths or parasites. Herbs started to be used as a cure. It took a long time, but slowly, the knowledge started to spread. It was always a sort of privilege. People who used herbs were called differently in different cultures – shamans, druids, herbalists, witches. Different names for the same people. People, who knew a lot about herbs and used them to cure and protect people. With expansion of the catholic religion, this knowledge started to be banished.

Still medicinal herbs – herbs with medical properties –are the beginning of pharmacy. Pills, extracts, tinctures, liquids were based on herbs and natural products. There was no industry, no science that could create or synthesize some new compound or modify the old one to become better. Everything we knew was gained from natural resources.

A pharmacist was a very important person, he produced medicine and his knowledge and precision was the most important thing to define treating effect of the remedy.

With development of pharmaceutical industry, the role of pharmacist becomes different. He buys the standardized medical drugs and sells them to the patient. From this moment, the individual preparation of remedies decreased and the pharmacist becomes a specialist. Today, the part of individually prepared remedies is minimal in comparison with industrially crafted ones. The heart of his job starts to be the knowledge about the cure, principles and knowing as much as possible. But still the industrially made substances are similar or based on structures we can find in nature.

The medicinal plants can be very useful now. They can be often used to treat many diseases without side effects provided by industrially produced drugs. But we also have to say that using medical herbs in bad dosages or not knowing the effect of the plant can cause harm to the organism. That is why the herbal treatment and dosages should be decided by a medical doctor or pharmacist.

Every country has plants which are traditionally used for their treating, calming or protecting power. But is this really true, or is it just another tradition with no logical argumentation? Many of traditionally used herbs are now tested to prove their activity, to define active substances and to find possibilities how to use them more effectively or in new department.

2. AIM OF WORK

This work - Biological activities of *Lavandula angustifolia* essential oil - is to prove or refuse traditional and other effect of *Lavandula angustifolia* essential oil.

The aim of this thesis is to explore:

- To isolate essential oil from *Lavandula angustifolia*
- To define main compounds of the essential oil
- To test antifungal activity
- To test antioxidant activity
- To test anti-inflammatory activity
- To evaluate cytotoxicity
- To test nematicidal and repellency activity

3. THEORETICAL PART

3.1. Essential oils synthesis

Essential oils are also known as volatile oils, etheric oils or aetherolea from Latin language. They are concentrated natural products and contain a mixture of volatile compounds, usually of monoterpenoids and sesquiterpenoids, benzoides and phenylpropanoids.[1, 2] The mixture is very complex, usually consists of 20 – 60 components from which two or three are the main ones.[3] Terpenes result from condensation of the pentacarbonate unit, 2 - methylbutadiene or isoprene – that is why they are often called isoprenoides. In higher plant synthesising pathways through isopentenylidiphosphate and dimethylallyldiphosphate take place. Terpenes are then synthesized through the mevalonate, non-mevalonate or deoxyxylulose pathways. Mevalonate pathway is located in cytoplasm, the non-mevalonate pathway is taking part in chloroplasts. Phenylpropanoids originates in Shikimate pathway.[2]

3.2. Essential oil characterisation

Internationally, essential oils are defined as products obtained by hydrodistillation, steam distillation or dry distillation or by a suitable mechanical process without heating a flower.[2] Essential oils are secondary metabolites, which means that they do not possess essential role in plant metabolism and they are not present in every plant. Plants which produce essential oil are often called aromatic plants, because of the specific odor. Composition of essential oils is very variable. It is influenced not just by genetic and evolution factors, but also by other conditions.[4] We can mention environmental condition (the climate in which the flower grows, edaphic factors or hydric stress, appearance of pests or other irrigations), geographical variations, physiologic variations (type of organ and its development, age of organ, type of secretory structure, part of pollination cycle).[3] Essential oils are very important for plants and often provide surviving in severe conditions. They cause many effects on human, animal or plant subjects. Herbs often use them to attract insect in pollination or spreading of seeds, or on the other side, to protect themselves against predators (mainly

herbivores or pathogens).[2] Very important is protection against water loss. By evaporating of the oil, the surface of the leaves becomes shiny and reflects light, which prevents evaporation. This may be the reason why xerophytic plants are so rich in essential oils.[5]

Now, volatile oils are used in three main domains: as odorants in perfumes, detergents, soaps and other products; to odor the baked goods, soft drinks and other food [6]; as pharmaceuticals and dental products.[7, 4] In pharmaceuticals they irritate skin and causes warmth, they can be found in ointments against rheumatism. They are also slightly irritating gastric mucosa so they can be used as stomachic (to increase appetite), expectorant (for their effect on the bronchi and facilitation of expectoration) or spasmolytic drugs (relaxing of smooth muscle spasms in digestive tract). It can also be used against intestinal flatulence or oedema (mild diuretic effect).[8] Essential oils often possess antibacterial, insecticidal and antifungal properties.[9] It is believed that lavender has strengthening effect on nervous system and helps to temper physical diseases.[10] Their strong biological activities may help to control human and plant pathogens through an economical and environmentally friendly way.

There are some botanical families which are very common in oil producing species, mainly the families *Lamiaceae*, *Asteraceae*, *Apiaceae*.

Essential oils occur in all plant organs (buds, flowers, fruits, leaves, root, seeds, stem, twigs). They are produced and accumulated in different secretory structures. We can distinguish internal structures: secretory cells, secretory cavities and secretory ducts and external structures: osmophores, epidermal cells and glandular trichomes.[2] In *Lamiaceae* family, the essential oils are stored in glandular trichomes on the epidermis of the plant.[11]

Trichomes are outward elongated epidermal cells. We can find them on the surface of many plants. Their function is very important. They can make it difficult for insects to land on the plant, walk on it or chew it.[12] They can also shade the leaf and protect it from evaporation. Trichomes exist in different shapes or sizes, unicellular or

multicellular. In some species, they can contain specialized cells which can produce specific complexes such as antiherbivore compounds or digestive enzymes, trichomes of carnivorous plants can produce poisonous compounds for stinging nettle.

Glandular trichomes have a stalk and head region. The stalk can be unicellular or multicellular and it can have several rows of cells. The head can also be unicellular or multicellular. To create a trichome, first the epidermal cell wall has to outgrow. The cytoplasm distribution is unequal, it cumulates on the outer surface of the cell. As the cell division follows, the head and vacuolated supporting cell are created. After this, the division of head cells begins and the cytoplasm is now more or less uniformly distributed. The head cells of glandular trichomes are covered with cuticle. Beneath the permeable cuticle, the secrets are accumulated.[13] Family *Apiaceae* creates essentials oil canals, family *Gingiberraceae* and *Piperaceae* create the special cells only for essentials oil. They can be stored in specific plant organ, so we can distinguish essential oil of blossoms, leaves and so on. Sometimes the volatile oil can permeate through the whole plant – which is the case of conifers. Usually the plants which contain large amount of alkaloids do not contain essential oil (or in a very little amount).[14]

3.3. Essential oils isolation and characterisation

Essential oils can be obtained from the plant by few methods: distillation, organic solvent extraction, pressing and simple extraction with oils or fats or enfleurage. If we use pressing (only for citrus fruits), the gained product is not pure, but contains also water or pectines and it is very difficult to separate them.

3.3.1. Hydrodistillation

The main method used in laboratory is hydrodistillation. The principle of this method is to boil fresh or dried material in water. The plant is in contact with water during the whole process. During boiling of the mixture, the volatile compounds are vaporized and then condensed in condenser. The following separation is guaranteed by immiscibility of essential oils with water. Hydrodistillation has also disadvantages. The

process is long and some components can react with water because of the heat and long time of boiling, also, it can be used mainly for stable essential oils.[14]

The process requires special apparatus: Clevenger apparatus. According to the Portuguese Pharmacopoeia [15, 16] it comprises of round bottom flask with a short, ground – glass neck having an internal diameter about 29 mm at the wide end, where the mixture of plants and the water boils, a condenser assembly that closely fits to the flask, appropriate heating device which allows fine control of temperature and vertical support with a horizontal ring covered with insulating material.

3.3.2. Steam and water distillation

During using this method, the plant material is not in direct contact with water. The plant is placed on a grid over the boiling water. The water is heated by open fire and saturated steam rises through the plant material. The steam extracts the volatile compounds from the plants. Steam and water distillation is more effective, taking a shorter time.[17]

3.3.3. Steam distillation

Similarly as with the steam and water distillation, the plant material is held on the grid. The difference is that the steam is supplied from an outside source. Volatile compounds are dragged by steam and create oil layer in distillation bulb.[14] Today, we can use modern pressure boiler and thus we can use higher pressure and higher temperature, too. This is a modern method, which is quick and allows more complete isolation of essential oil. The steam is highly controlled, so the essential oil does not suffer thermal decomposition and the method is suitable for commercial usage.[17]

3.3.4. Organic solvents extraction

Organic solvent extraction can be used for extraction from plants with small amounts of volatile components, especially for those which are easily dissolvable in water. Solvents like ethanol, hexane, benzene, toluene or petrol are often used.[18]

3.3.5. Pressing

Pressing can be used at plants with high essential oil content, where essential oils are stored in exterior part of the plant. This method is used mainly with citrus fruits.[14]

3.3.6. Simple extraction with oils (fats) and enfleurage

There is simple oils/fats extraction and enfleurage. Enfleurage is special process in which leaves of plants are placed between glass plates covered with thin layer of fatty substance. Essential oil then gradually passes into the fat. Leaves are periodically changed till the fat saturation. Essential oils have to be separated from fat by extraction with alcohol or by supercritical fluid extraction.[18]

3.3.7. Analytical techniques

The generalized and most common technique used to evaluate the composition of essential oil is gas chromatography (GC). GC separates the parts of mixture. This technique was first used in the essential oil characteristic in 1956 by Liberti & Conti.[19] Gas chromatograph consists of a mobile phase and a stationary phase. The mobile phase is composed by a gas, usually nitrogen, helium, hydrogen or argon. The gas has to be chemically inert. On the other hand, the stationary phase is liquid in an inert support. The separation is due to different distribution of each substance between stationary and mobile phase. Each compound then elutes in different time. Very important is also the final detection of components. Between compatible detectors belong flame-ionization detector, Fourier transform infrared detector and the mass spectrometer (MS).[14]

3.3.8. Parts of chromatograph

The first thing is the supply of the gas, usually in a gas bomb with a flow controller and a sample injection port. The sample has to have the right volume, the most effective form of applying the sample is injecting by microsyringe through a rubber septum into a flash evaporator on the top of the column. There are different types of columns. Packed columns contain finely divided inert solid support material, coated

with stationary phase. They are 1 - 10 m long and 2 - 4 mm wide in diameter. Capillary columns are very thin in internal diameter and we can distinguish two types: a wall-coated open tubular column and a support-coated open tubular column. The wall-coated ones have a wall coated with liquid stationary phase, in the support-coated columns, the walls are covered with support material on which the stationary phase is absorbed. The most efficient type of column is the wall-coated open tubular column.

The temperature in the spectrometer has to be precise so there should be the possibility to control it.

Detectors can be variable. According to the type of detector (selective or non-selective one), there is a different selectivity. The selective one responds to the range of substances with some common physical or chemical condition, the non-selective one responds to every signal except for carrying gas. Specific detector responds to only one selective signal. We can also divide detectors into the concentration dependant detector and the mass flow dependant detectors which usually destroy the sample. Mass flow detectors are flame-ionization detector, nitrogen-phosphorous detector, flame photometric detector and hall electrolytic conductivity detector. Between the concentration dependent ones belong thermal conductivity detector, electron capture detector and photo-ionization detector.

The mass spectrometer connected to the gas chromatographer identifies the compounds. The molecules are hit by electrons. This turns them to the ions – positively charged ones. Positive particles can pass through the filter to the electromagnetic field. In the field, the ions are scanned according to the similar mass number. This information is send to the computer which makes a graph.[14, 20, 21]

3.4. Lavender

3.4.1. Family Lamiaceae

The family *Lamiaceae* was established by De Jussieu in 1789, but under a different name – order *Labiatae*. [22] This name refers to the typical shape of petals fused into an upper and lower lip. The new name was derived from genus *Lamium*. The family is divided into several subfamilies from which the richest in genera is the subfamily *Nepetoideae*. The *Lamiaceae* family is genetically very close to the family *Verbenaceae*.

The *Lamiaceae* family is a very important one with a great economic value. Its plants are widely used in traditional medicine and also horticulture. The *Lamiaceae* family comprises of over 240 genera and 6500 species all around the world except for the coldest polar region. They are very well represented in tropical and subtropical region and very adaptable to different habitats. [23]

Plants from this family can be trees, shrubs or herbs with typically square stems and leaves standing opposite with no stipules. Typical for this family is a plant body often covered with hairs which can be unicellular or multicellular and in different forms. Many of the species produce essential oils and therefore are very aromatic. Flowers are hermaphrodite, arranged in thyrsoid inflorescences. Cymens are extremely complex or even reduced and the flower can be strongly condensed - they can even form heads. Calyx has five tubular or tunnel-shaped lobes, often two-lipped, but it can be extremely diverse. Corolla is sympetalous, often zygomorphic and tubular. Stamen is usually didynamous with longer anterior pair, anthers are basifixed and the structure and shape of connective can be diverse. Style is gynoblastic, arising between deeply four lobed ovaries which are superior and composed of two carpels, each divided into two loculi, each containing a single ovule. Nectarious disk is often below the ovaries. Fruits consist of four single seeded mericarps, called nutlets. [5]

3.4.2. Genus *Lavandula*

Today, many products from lavender are commercially used. We can mention essential oils, fresh and dried flowers and landscape plants. The genus has 39 species and four of them have the main importance – *Lavandula angustifolia*, *Lavandula latifolia*, *Lavandula x. intermedia*, *Lavandula stoechas*. [9, 4] Lavender was used since the ancient Roman times for personal care – such as bathing and washing clothes. Also the name itself – lavender – refers to the Latin word *lavare* which means *to wash*. Later it started to be part of perfumes – eau de Cologne, Russian cologne and also some floral cologne. From 1920s, lavender has been very popular to fragrance, for example soaps, talcum powder or bath salts. During the years, lavender essential oil became the most used one in perfumery industry. Now we can also find lavender in food industry (beverages, baking products) and for association with cleanliness, we also put the dried herb in drawers with clothes to keep them fresh and to repel the moths. These are the reasons why the wild plant started to be cultivated and then spread almost all around the world. [5]

3.4.3. Botanical description

Originally, lavender is native to the mountains of the Mediterranean region, [24] it grows in sunny and stony habitats and it doesn't need a lot of water. Soil pH should be neutral to alkaline (the best is between pH 7,0 – 9,0).

Morphology of lavender is very variable, but we can still trace some common characteristics. Problematic is also the fact, that in *lavandula* genus, there is a lot of different species, different cultivars and new ones are still cultivated.

The habit of the plant can be woody shrub, or woody-perennial to short-lived herbs. Usually new stems are herbal and old ones become woody and also have some type of bark.

Typical for genus *lavandula* is indumentum. It covers most parts of the plant – mainly leaves, stem, calyces and branches. Indumentum contains two types of

trichomes – glandular and non-glandular. The glandular ones have usually round shape, the non-glandular has many different shapes and both types are important for determination of different species. It is the indumentum that makes lavender flower look grey, green-grey or silvery-grey.[5]

Leaves are also very variable with great taxonomic significance. The basic ones are narrowly elliptic, but they can also be dissected, sessile or petiolate.

Inflorescence is characteristically borne on distinct peduncles and can be branched or not. Also the margin of the peduncle can be colored in purple, depending on the type of the soil. The cymes are multi-flowered or single-flowered and the type of flowering is centrifugal (multi-flowered) or acropetalous (single-flowered).

Calyx is tubular and consists of five fused sepals. As usual for family *Lamiaceae*, it is two lipped. The upper lip has three lobes and the lower lip two lobes. Calyx lobes can be all the same, or they can differ in the upper and lower part, or just one lobe can be slightly different, it is persistent and retains after flowering to protect developing nutlets.

The corolla consists of five fused petals with a distinct calyx tube and five lobes forming a two lipped flower. The upper lip consists of two lobes, usually erected and they can be two times bigger than the lower lip ones. The color of petals is in shades of violet and blue, rarely deep purple. Today, many cultivars had been produced with different colors as white or pink.[5]

3.4.4. *Lavandula angustifolia* Mill.

Lavandula angustifolia is a low woody shrub 40 – 80 cm tall. Leaves are linear to narrowly ovate, 3 – 4 x 0,3 – 0,5 cm with smaller ones in axils. Peduncles are unbranched, erected, (7 -) 10 – (20 -) 30 cm long. Spikes are compact (2 – 5 cm long) or interrupted (6 – 10 cm long). It is very usual for the plant to have remote verticillasters. Bracts are ovate or broadly ovoid, apex is acuminate or apiculate, membranous, approximately half as long as calyces with prominent reticular veins. Bracteoles are

small (1 mm), linear and scarious. Calyx is tubular with short round lobes and dense wooly indumentum of long branched hairs and sessile glands. The color of hairs is pale gray or partially violet-blue. Corolla is 1 – 1,2 cm long with upper lobes typically twice as big as lower ones. Colors are in shades of violet or blue. The lavender flowers from late June to mid July, but it depends on altitude. Lavender can grow in open, arid places on calcareous soil between low growing vegetation.

As other lavenders, it is widely used in perfumery, medicine and as a garden decorative plant. We can find it as part of colognes, toilet waters, salts, soaps or lotions. However, due to oil quality, the products are the more expensive ones than from other lavenders. It is believed that lavender has strong antiseptic and anti-inflammatory effects, so it is very often used to treat many types of skin diseases, such as acne, eczema, ulcers, sores, burns and small infections.[9, 25] It is also believed, that lavender essential oil can prevent creating of scars. It can be used in aromatherapy, where it has sedative and calming properties or against anxiety, when one cannot fall asleep or suffers from stress. It can be used also as analgesic and anti-spasmodic especially when the condition has stress origin. Traditionally, the plant is used against cold, flu and fever. Lavender has also insecticidal properties, being used in wardrobes to protect clothes from moths and flies.[9, 26]

Picture 1: *Lavandula angustifolia* [27]



3.4.5. *Lavandula angustifolia* Essential oil

Lavender plants are widely grown in fields for the essential oil production. The main industrial producers of lavender essential oil are France, Bulgaria, China, Spain, Russia, Ukraine, Australia, Argentina and England. High quality lavender oil is obtained by steam distillation of the inflorescence and for obtaining one kilogram of the oil, about 120 – 150 kg of lavender inflorescence is needed.[28] For the industrial purposes, the oil has to be pure with no residuals like artificially added substances, pesticides residues or toxic elements. Metal elements can cause worse stability during the storage or increase toxicity of the oil. Main compounds are linalool, cineol, α -terpineol, borneol, geraniol. Minor compounds, which can be found in volatile oil, are kumarin, resin and tannin.

The lavender essential oil has transparent to pale yellow color. Its characteristic odor is sweet, fresh and floral with balsamic-woody undertone.[5]

3.4.6. Biological activities

3.4.6.1. Cytotoxicity

Testing the cytotoxicity is very important assay for every new molecule which has potential to become medical cure. It is needed to know how toxic it is for human cells, so we can decide whether it is possible to introduce a molecule in the organism and through which way.

The assays to test *in vitro* cytotoxicity were created to quickly evaluate the toxicity of the tested compound and are widely used in drug discovery research. These assays lower the need of animal testing and lower the time and expenses for the research – it allows remove the toxic compounds early in discovery process. In latest years the methods were optimized for use in microplates to allow testing of large amount of specimens at one time. Also the colorimetric based assays can be measured directly in the plate by using automatic plate reader or ELISA plate reader. Among the most used ones belong LDH release, MTT metabolism and neutral red uptake and ATP content assays.[29] Essential oils are being tested mostly for carcinoma cell cytotoxicity. *Casearia sylvestris*,[30] *Zanthoxylum rhoifolium*,[31] *Lindera strychnifolia* [32] or *Amonum tsao-ko*[33] belong between essential oils with great potential. They showed significant results in fighting hepar carcinoma, lung carcinoma or cervical carcinoma. These oils will be further tested for medical use.

3.4.6.2. Anti-inflammatory activity

Inflammation is a very important process characterized by a normal response of the body to injury or infection. It also helps the body to remove damaged or dead cells.[2] If the inflammation becomes chronic, the body can be severely damaged. Several diseases were identified as a state caused by chronic inflammation – rheumatoid arthritis, diabetes, neurodegenerative diseases...Chronic inflammation is one of the main causes of mortality in western countries. These days, new drugs to combat the inflammation are needed. The current ones have limitations, such as side or adverse effects, tolerance, loss of effectiveness, difficult way of delivery. At the field of plant

medicine, there is a great potential to obtain new molecules that will be less toxic, with good administration and better results.[34] Essential oils from *Cinnamomum osmophloemum*[35] and *Distichoselinum tenuifolium*[36] have been already tested to prove a great potential and will be further tested.

During the inflammation response of the body, the endothelial lining cells become more permeable to influx leukocytes in the intersticium, oxidative burst and release of interleukins and tumor necrosis factor- α (TNF- α). The activity of oxygenases, nitric oxide synthases and peroxidases is induced and so is the arachidonic acid metabolic pathway.[2]

Arachidonic acid is a polyunsaturated fatty acid released from lipid membranes by phospholipase A2. It is metabolized by cyclooxygenase and lipoxygenase creating prostaglandins or leucotriens which are important inflammatory mediators. The cyclooxygenase has two main isoforms (COX-1 and COX-2) and one variation (COX-3). COX-1 is a constitutive enzyme of many tissues and its activity does not change with inflammation. On the other hand, COX-2 is an inducible enzyme induced only in inflammatory cells. After the stimulus from COX-2 macrophages, which play major role in inflammation, the production of prostaglandins in large amounts occurs. Prostaglandin E2 amplifies the pain mechanism and enlarges the vascular permeability. Macrophages then produces large amount of nitric oxide synthase (we know three isoforms of NOS: inducible, endothelial and neuronal). Inducible nitric oxide synthase is responsible for production of nitrite oxide from L-arginine and molecular oxygen. This process contributes to pathogenesis of inflammatory disease and the volume of released products is strictly triggered by series of signaling pathways including nuclear factor- κ B transcription factor and mitogen-activated protein kinases. MAPKs are signaling molecules which play the major role in cell growth regulation, apoptosis, differentiation or response to the cytokines and stress. MAPKs have three subclasses, which are activated by LPS and participate in COX-2 and iNOS expression in macrophages - P38 MAPK, c-jun NH2-terminal kinase (ERK) and extracellular signal – regulated kinase (ERK). Inhibition of expression of COX-2 is through NF- κ B,

CCAAT/enhancer binding protein (C/EBP), activator protein 1 and CRE-binding protein. iNOS expression can also be modulated by phosphatidylinositol-3-kinase/Akt pathway.[34, 2]

3.4.6.3. Antioxidant activity

Antioxidant activity of different substances is very important for healthy cell tissues. Antioxidants have health-enhancing effect on human organism, protecting cell tissues from oxidant damage.[1] Today, there are some pressures to find new drugs to prevent deterioration of food and lower oxidative damage of living cells.

The oxygen has a potential to become a toxic element. This can happen through many metabolic pathways. Oxygen can be transformed into reactive forms, such as superoxide, hydrogen peroxide, singlet oxygen or hydroxyl radicals which can cause damage to the tissues. We live in environment, which contributes to the formation of free radicals – cigarette smoke, burning of fossil fuels, ozone, nitrogen oxide, sulphur dioxide, UV radiation. Hydrogen peroxide can cross biological membranes, hydroxyl radical can react with most of molecules we can find in living organisms. Oxidation causes unsaturated oils degradation – so the lipids, proteins, carbohydrates or DNA represents substrates for the active oxygen. Lipid peroxidation causes changes in membrane structures and this can stimulate apoptosis and finally cause death. Free radicals can also cause mutations, growth of malignant cell types, they can play an important role in chronic inflammatory diseases. Oxidation can be evaluated through different methods which are different to compare and each of them indicates different potential use of substance. *Amonum tsao-ko*,[33] *Croton urushurana*[37] showed weak antioxidant activity. *Lycopus lucidus* proved to have moderate antioxidant action.[38] On the contrary, *Lippia grandis*,[39] *Conobea scoparioides*,[40] *Melilothus officinalis*, *Artemisia dracuncululus* and *Foeniculum vulgare*[41] and many types from the genus *Thymus*[42] showed significant antioxidant activity.

3.4.6.4. *Antifungal activity*

Over the last few decades, there has been an increase in the number of serious human infections in immunocompromised patients caused by fungi. The range of severity of these infections is a consequence of the host reaction to the metabolic and environmental factors. Nowadays, the increasing impact of these infections, the limitations encountered in their treatment (e.g. resistance, side-effects and high toxicity) and the rising overprescription and overuse of conventional antifungal drugs all stimulate searching for alternative natural drugs. We tested several human pathogenic fungal strains. Up to now, mostly plant pathogenic fungi were tested to prevent infection of crop plants – *Illicium verum* essential oil[43] and, *Ocimum basilicum* essential oil is useful against *Botrytis fabae*,[44] essential oil of *Hyssopus officinalis* was tested against *Pyrenophora avenae* and *Pyricularia oryzae*. [45] Australian *Lavandula spp.* were successfully tested against *Aspergillus nidulans*, *Trichophyton mentagrophytes*, *Leptosphaeria maculans* and *Sclerotinia sclerotiorum*. [46]

Candida spp.

Candida is the most common cause of mycoses worldwide. *Candida* species are normal colonizers of human skin, mouth, vagina, or stool. When the fungus overgrowth the disease state appears and we call it candidiasis. Occasionally, the disease can be acquired from an exogenous source, such as person to person transmission. Only 6 of 154 known species are known to cause human diseases – *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, *C. lusitaniae*.

Yeasts are small, thin walled and reproduce by budding. Colonies of yeasts are cream to yellowish color, grow rapidly and mature in three days.

Candidiasis can be located on skin and mucosas or they can become systemic. Located infections can be most commonly found in mouth, vagina or nails. Mucosal candidiasis can be treated more easily than systemic ones.

Systemic candidiasis goes past the skin and is very difficult to cure. However they appear only on a person with a weak immune system - then *candida* can infect almost any organ in the body.

The fact that *candida* can cause oropharyngeal candidiasis in patients with HIV-AIDS have made candidiasis a leading fungal infection in this immunosuppressed population.[47, 48]

Aspergillus spp.

Aspergillus is filamentous fungus commonly isolated from soil, plant debris or indoor air. *Aspergillus* strain contains over 185 species and around 20 have been proven to be cause of human disease infection.

Aspergillus species can cause three states of infection in human body: opportunistic infection, allergic state and toxicoses. Opportunistic infection appears mostly in immunocompromised people and it can vary from localised to hard systemic infection called aspergillosis. It can also act as an allergen and cause an allergic bronchopulmonary aspergillosis, particularly in people with atopic eczema. Many of the species, e.g. *Aspergillus flavus*, can produce aflatoxins which are harmful and by chronic ingestion they can cause hepatocellular carcinoma.

Aspergillus grows rapidly and forms colonies down and powdery in texture.[49, 50]

Dermatophytes

Dermatophytes are a group of three genera causing hair, skin and nail diseases: *Trichophyton*, *Epidermophyton* and *Microsporum*.

Trichophyton spp.

Trichophyton spp. inhabit soil, humans or animals. Most of the species have teleomorphic forms which are classified in genus *Arthroderma*.

The growth rate of *Trichophyton* colonies is slow to moderately rapid. The texture is waxy, glabrous to cottony. From the front, the color is white to bright yellowish-beige or red violet. Reverse is pale, yellowish, brown, or reddish-brown.[51, 52]

Epidermophyton spp.

Epidermophyton is filamentous fungi distributed world wide. The only pathogenic species is *Epidermophyton floccosum*. It can affect otherwise healthy individuals. *Epidermophyton* infects the cornified parts of human skin but has no ability to penetrate beneath them. Common diseases caused by *Epidermophyton* are tinea pedis, tinea corporis, tinea cruris or onychomycosis.

The colonies of *E. floccosum* grow moderately rapid and mature within 10 days. The colonies vary from brownish-yellow to olive-gray or khaki from the front and orange to brown with an occasional yellow border from the reverse side. The texture is flat and grainy initially and becomes radially grooved and velvety by aging. The colonies quickly become downy and sterile.[53, 54]

Microsporum spp.

Genus *Microsporum* contains 17 species of filamentous keratinophilic fungi, two of them are anthropophilic – *M. Audouinii* and *M. Ferrugineum*. *Microsporum* is the asexual state of the fungus and teleomorph phase is organised in the genus *Arthroderma* as in *Trichophyton*.

Microsporum colonies are glabrous, downy, wooly or powdery. The growth on Sabouraud dextrose agar at 25 °C may be slow or rapid and the diameter of the colony varies between 1 – 9 cm, after 7 days of incubation. The color of the colony varies and depends on the species. It may be white to beige or yellow to cinnamon. From the reverse side, it can be yellow to red-brown.[55, 56]

3.4.6.5. *Nematical activity*

Plant parasitic nematodes belong to the important group of pathogens transmitted by earth (soilborne pathogens). These pathogens cause huge damage on the crop and have to be controlled – chemically or by natural nematicides. However the effect of their agents is usually only short-term and very toxic. New potential nematicidal drugs with safer toxicity and ecological profile may be found in natural resources.[57, 58] Plant essential oils may provide alternative to currently used control agents because they consist of many bioactive molecules and are commonly used as fragrances and flavoring agents for food and beverages.[59]

Some of nematodes parasite on plants and can play an important role in the predisposition of the host plant to the invasion by secondary pathogens. Plants attacked by nematodes often show retarded growth and development and also lower quality and fewer products to harvest.[60] Essential oils can be part of natural repellents, many of them already showed their potential – *Cymbopogon citratus*, *Cinnamomum verum*, *Allium sativum*, *Leptospermum petersonii*, *Eugenia caryophyllata*, *Asiasarum sieboldi*, *Mentha spicata*, *Boswellia arterii* and *Pimenta racemosa*,[61] *Liquidambar orientalis*, *Valeriana wallichii*,[58] *Gaultheria fragrantissima* and *Zanthoxylum alatum*. [62]

Pine wilt disease(PWD)

Pine wilt disease is characterized by a reduction in the oleoresin flux of tree and browning/reddening of the needles. This is a result of collapse of photosynthesis and water blocking mechanisms. These symptoms are comprised of three conditions - the nematode, the insect vector and the susceptible host. The combination of these and environmental factors are the main factors in development of the disease.[63]

The weevil was recognized in 1891 in India for the first time [64] and in 1971, *Bursaphelenchus lignicolus*, presently known as *Bursaphelenchus xylophilus* (the pinewood nematode - PWN), was confirmed as the pathogenic agent of PWD.[63]

The genus *Bursaphelenchus* comprises of mycophagous nematodes, mainly distributed in the northern hemisphere. Among approximately one hundred species within this genus, only two are plant parasitic - *B. xylophilus* and *B. cocophilus* (causes “red ring disease”). *B. xylophilus* has a life cycle which comprises from phytophagous and mycophagous phase of development. The vectors of the PWN are longhorn beetles belonging to the genus *Monochamus Dejean* (order *Coleoptera*, family *Cerambycidae*). In Portugal, the only known vector is *Monochamus galloprovincialis* Olivier.[63]

The host plants for PWN are mainly conifers of the genus *Pinus* such as *P. bunjeana*, *P. densiflora*, *P. luchuensis*, *P. massoniana* and *P. thunbergii* for Far Eastern countries and *P. nigra*, *P. sylvestris* and *P. pinaster*. *Pinus pinaster* is the only susceptible species in Portugal.[63]

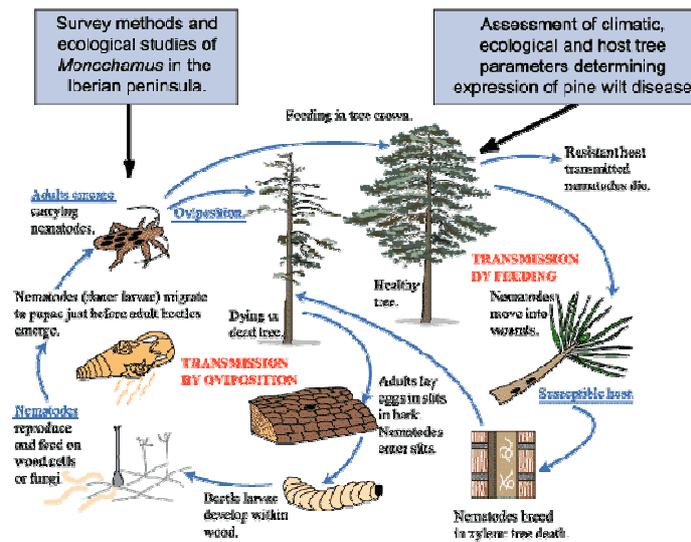
There are theories about interaction between host, beetle and nematode suspecting releasing toxic proteins or infecting the tree by parasitic bacteria (*Pseudomonas fluorescens* and *Pantoea agglomerans*). Although we could postulate a potential involvement of bacteria in PWD, this subject is still controversial and further studies are needed to understand the effective role of bacteria on this complex disease.

Many *Bursaphelenchus* species, including the PWN, have been routinely intercepted in packaging and wood products in several countries, stressing the importance of trade globalization for the potential entry of this disease into pathogen free region. Once the PWN becomes established in a new region, the evolution of the PWD is guided by a widely studied framework involving two processes: 1) transport of contaminated wood by human activities, and 2) biological development of the insect vector.

Since wood industry is the main cause of the spread of the disease, control must be concentrated on the activities which possess risk of entry and dissemination of the pathogen. Wood trade between countries is nowadays highly monitored and all infested wood should be carefully treated before shipment or transformation.

Authorities search for new ways of controlling the insect vector: preventing movement of contaminated wood, cutting down symptomatic trees and monitoring the healthy ones.[62] European Union (EU) has taken actions to ensure control of the PWN beyond its present geographic area and, if possible, to eradicate it from the EU territory.[63]

Picture 2: Life cycle of *Bursaphelenchus xylophilus* [65]



The insect vector transports the PWN in its elytra (wing cases) and tracheae (breathing tubes). During insect maturation feeding on healthy pine trees, the nematode is transmitted and spreads through its vascular system and resin canals.[66, 63] The nematodes feed on epithelial cells and living parenchyma – this is called phytophagous phase.

The whole life cycle comprises of four stages of propagative juveniles, which finally moult to an adult stage.

The first juvenile stage (J1) is completed inside the egg resulting in hatching as second-stage juveniles (J2) and continues with three moults to become adults. Under favorable conditions, (suitable temperatures i.e approx. 20 °C), the nematodes rapidly reproduce and complete their life cycle from egg to adult in 6 days. Each female gets to lay between 80 and 150 eggs in 28 days (oviposition period). Large amount of

nematodes then block water flow in the xylem and this contributes to the death of the plant.[63]

B. xylophilus develops through two different forms, as reproductive or dispersal life cycle and the first two juveniles stages (J1 and J2) are the same for both types.

The nematodes which live under optimal conditions develop through the reproductive pathway (described above).

When environmental conditions are not ideal with too high or low moisture or lack of food, the nematodes switch to dispersal path of development. Prior to insect vector emergence, the nematodes (J3) surround the pupal chambers (March-April) and moult into J4 - a non-feeding dispersive stage known as *dauer* juveniles. They are attracted into the insects' pupal chamber, where they enter the vectors body through natural openings (e.g. spiracles). Insect then transmits the juveniles during feeding on host trees. J4 juveniles leave the insect body and enter the host through feeding wounds.[63]

Species of the cerambycid beetle genus *Monochamus* are the main vectors of PWN, in which *M. alternatus* is the major vector for Asian countries and *M. galloprovincialis* for Portugal.[67, 63]

3.4.6.6. Repellency assay

Most of the plants contain special compounds which help to protect themselves against herbivores. Although the primal purpose of these compounds is against phytophagous insects, many are proven to be effective against flying *Diptera*, too. This fact can be evolutionary relict from plant feeding ancestors of *Diptera*.[68]

This repellency effect of plant material has been used for thousands of years. People often hanged dried or fresh plant in their houses, planted the herb nearby or burned it in fire place. Many of them are also used as spices. These methods are still used among rural poor tropic tribes or communities, because it is their only available repellent method.[68]

There is the possibility to find some important substances with very good results, which will not be dangerous neither for people nor for our planet.[68] Between essential oils mostly used as insecticides belong the ones from genus *Cymbopogon*, *Ocimum forskolei* or *Tanacetum cinerariifolium* or *Tanacetum coccineum*. [68] *Boxus chinensis* already demonstrated good results against *Rhynchophorus ferrugineus*. [69] The low toxicity and duration of effect is very important for natural repellent substances. They are environmentally friendly. [1]

Rhynchophorus ferrugineus

This beetle, also known as *Calandra ferruginea*, *Curculio ferrugineus* or *Rhynchophilus signaticollis*, belongs to the family *Coleoptera*, genus *Curculionidae*. The adult beetles are quite robust, 35 x 10 mm, with long curved rostrum and dark spots on the upper part of thorax.

Adults are active during both day and night, but flying and crawling is taking part strictly during the day. Mating can happen any time of the day. They are able to fly for 900 m to find a new area. One female beetle can lay 204 eggs in average and adults live in average 2 - 3 months. A female dies in ten days after laying the eggs.

Red palm weevil (*Rhynchophorus ferrugineus*) affects palm trees from genus *Arecaceae* (*Areca catechu*, *Arenga pinnata*, *Borassus flabelifer*, *Calamus merillii*, *Caryota maxima*, *Caryota cumingii*, *Cocos nucifera*, *Corypha gebanga*, *Corypha elata*, *Elaeis guineensis*, *Livistona decipiens*, *Metroxylon sagu*, *Oreodoxa regia*, *Phoenix canariensis*, *Phoenix dactylifera*, *Phoenix sylvestris*, *Sabal umbralicufera*, *Trachycarpus fortunei*, *Washingtonia spp.*) It can also attack *Agave americana* and *Saccharum officinarum*. The plants in danger have to have at least 5 cm at the basis of the plant. The whole life cycle of the beetle takes part in the trunk of the palm (with larvae as the most damaging stage), which makes very difficult to recognize infected trees. Only two or three generation of beetles can cause death of infested tree. [70, 64, 71, 72]

It is very difficult to detect the *rhynchophorus* in early stadium, when the palm tree is not so badly damaged. When the symptoms appear (holes in the crown or trunk, crunching noises, withered bud/crown), it is usually too late and the only possibility is to cut the tree down and burn it to prevent spreading of infection.[73, 71]

4. EXPERIMENTAL PART

4.1. Biological material

- *Lavandula angustifolia* Mill. (Ervital, Mezio, Portugal)
- *Candida crusei* H9 (isolated from recurrent cases of vulvovaginal candidosis, IBILI, University of Coimbra, Coimbra, Portugal)
- *Candida guilliermondii* MAT23 (isolated from recurrent cases of vulvovaginal candidosis, IBILI, University of Coimbra, Coimbra, Portugal)
- *Candida albicans* ATCC 10231 (ATCC, Manassas, Virginia, USA)
- *Candida tropicalis* ATCC 13803 (ATCC, Manassas, Virginia, USA)
- *Candida parapsilopsis* ATCC 90018 (ATCC, Manassas, Virginia, USA)
- *Cryptococcus neoformans* CECT 1078 (CECT, University of Valencia, Paterna, Spain)
- *Aspergillus flavus* F44 (isolated from bronchial secretion, IBILI, University of Coimbra, Coimbra, Portugal)
- *Aspergillus niger* ATCC 16404 (ATCC, Manassas, Virginia, USA)
- *Aspergillus fumigatus* 46645 (ATCC, Manassas, Virginia, USA)
- *Epidermophyton floccosum* FF9 (isolated from nails and skin, IBILI, University of Coimbra, Coimbra, Portugal)
- *Trichophyton mentagrophytes* FF7 (isolated from nails and skin, IBILI, University of Coimbra, Coimbra, Portugal)
- *Microsporum canis* FF1 (isolated from nails and skin, IBILI, University of Coimbra, Coimbra, Portugal)
- *Trichophyton rubrum* CECT 2794 (CECT, University of Valencia, Paterna (Valencia), Spain)
- *Trichophyton verrucosum* CECT 2992 (CECT, University of Valencia, Paterna, Spain)
- *Trichophyton mentagrophytes* var. *interdigitale* CECT 2958 (CECT, University of Valencia, Paterna, Spain)

- *Microsporium gypseum* CECT 2908 (CECT, University of Valencia, Paterna, Spain)
- Macrophages RAW 264.7, ATCC number: TIB-71 (supplied by Dr. Otilia Vieira, Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal)
- Lung carcinoma cells A549, ATCC CCL-185, (ATCC, Manassas, Virginia, USA)
- *Bursaphelenchus xylophilus* (supplied by Department of Life Sciences of the Faculty of Science and Technology of the University of Coimbra, Coimbra, Portugal)
- *Rhynchophorus ferrugineus* (beetles were gained during cutting down infected tree by the municipal authority, Coimbra, Portugal)

4.2. Chemicals

- DMEM medium (supplemented with glucose (25 mM), 3,70 g.L-1 sodium bicarbonate, 10 % (v/v) fetal calf serum (FCS), 100 µg/L streptomycin, 70 µg/L penicillin and adjusted to pH 7.2.) (Sigma Chemical Co., Saint Louis, MO, USA)
- Iscoove's modified Dulbecco's medium (with L-glutamine (4mM), Hepes (25 mM) and supplemented with 10 % (v/v) FCS, 3,02 g/L sodium bicarbonate, 100 µ/L streptomycin, 100 U/mL penicillin, adjusted to pH 7.2.), (Sigma Chemical Co., Saint Louis, MO, USA)
- Polydimethylsiloxane (Sigma Chemical Co., Saint Louis, MO, USA)
- Polyethyleneglycol (Sigma Chemical Co., Saint Louis, MO, USA)
- Dimethyl sulfoxide (DMSO) (Sigma Chemical Co., Saint Louis, MO, USA)
- Amphotericin B (Fluka - Sigma Chemical Co., Saint Louis, MO, USA)
- Fluconazole (Pfizer, NY, UK)

- RPMI - 1640 medium (containing L-glutamine, phenol red pH indicator and without bicarbonate), (Sigma Chemical Co., Saint Louis, MO, USA)
- Trypsine-EDTA solution, (Sigma Chemical Co., Saint Louis, MO, USA)
- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co., Saint Louis, MO, USA)
- LPS obtained from E. coli (serotype 026:B6) (Sigma Chemical Co., Saint Louis, MO, USA)
- Acidified isopropanol (0.04 N HCl in isopropanol) (Sigma Chemical Co., Saint Louis, MO, USA)
- Griess reagent [0,1 % (w/v) N-(1-naphtyl)-ethylendiamine dihydrochloride and 1% (w/v) sulphanilamide containing 5 % (w/v) H₃PO₄] (Sigma Chemical Co., Saint Louis, MO, USA)
- TBA , 2 – thiobarbituric acid (Sigma Chemical Co., Saint Louis, MO, USA)
- ABAP, 2,2' – azobis (2-methylpropionamidine) dihydrochloride (Sigma Chemical Co., Saint Louis, MO, USA)
- KCl (1,15 % (w/v) (Sigma Chemical Co., Saint Louis, MO, USA)
- Egg yolk (supplied by Célia Cabral)
- Methanol (Merck, Darmstadt, Germany)
- BHA – butylated hydroxyanisole (Sigma Chemical Co., Saint Louis, MO, USA)
- BHT - butylated hydroxytoluene (Sigma Chemical Co., Saint Louis, MO, USA)
- Acetic acid (Sigma Chemical Co., Saint Louis, MO, USA)
- SDS (sodium dodecil sulphate) (Sigma Chemical Co., Saint Louis, MO, USA)
- 1 - Butanol (Merck, Darmstadt, Germany)
- Triton-X 100 (5000 ppm) (Sigma Chemical Co., Saint Louis, MO, USA)

4.3. Instruments:

- Gas chromatograph Hewlett-Packard 6890 (Agilent Technologies, Palo Alto, CA, USA), HP GC ChemStation Rev. A.05.04 data handling system, single injector and two flame ionization detection (FID) systems, graphpack divider (Agilent Technologies, Palo Alto, CA, USA, part no. 5021-7148), Supelco (Supelco, Bellefonte, PA, USA) silica columns
- Mass spectrometry analyses were carried out in a Hewlett-Packard 6890 gas chromatograph with Hewlett-Packard mass-selective detector 5973 (Agilent technologies, Palo Alto, CA, USA) operated by HP enhanced ChemStation software, version A.03.00.
- Inverted Microscope - Axiovert 135 (Carl Zeiss Microscopy, LLC, NY, USA)
- Laminar Box- NuAire Biological safety Cabinets Class II Type B2 (NuAire, Caerphilly, UK)
- Centrifuge - Eppendorf centrifuge 5415r (Eppendorf, Hamburg, Germany)
- ELISA automatic microplate reader (SLT Labinstruments GmbH, Salzburg, Austria)
- Ultrasonic bath - Bandelin sonorex digitec (BANDELIN electronic GmbH & Co. KG, Berlin, Germany)
- Test tube shaker - Vortex Reax top/Reax kontrol, Heidolph (Heidolph instruments, Schwabach, Germany)
- GFL-1083 water bath (GFL – Gesellschaft für Labortechnik, Burgwedel, Germany)
- Centrifuge - Sigma laborzentrifugen 3k10 (SIGMA Laborzentrifugen, Osterode am Harz, Germany)
- Spectrophotometer - Cintra 101 GBC, software Cintral General Applications (GBC Scientific Equipment, Braeside, Australia)
- Balance- Jenway 1000 (Bibby Scientific Limited, Staffordshire, UK)
- Stereomicroscope - Leica Zoom 2000 (Leica Microsystems, Wetzlar, Germany)

4.4. Methodology

4.4.1. Essential oil isolation and characterisation

4.4.1.1. *Plant material:*

Lavandula angustifolia Mil. is not naturally occurring plant in Portugal, so it has to be artificially cultivated in fields. Flowers were provided by Ervital, a Portuguese plant producer. These plants were cultivated in Castro de Aire region. Fresh plants were harvested and then dried in the laboratory for two days, in an open space in the shadow.

4.4.1.2. *Isolation:*

Essential oil was obtained from flowering aerial parts of the plant by hydrodistillation for three hours. The distillation was performed according to European Pharmacopoeia [74] using Clevenger-type apparatus. The oil was obtained in yields of 2 % (v/w) and was stored in refrigerator to conserve it.

4.4.1.3. *Analysis:*

Analytical gas chromatography was carried out in Hewlett-Packard 6890 (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph and HP GC ChemStation Rev. A.05.04 data handling system, equipped with a single injector and two flame ionization detection (FID) systems. A graphpack divider (Agilent Technologies part no. 5021-7148) was used for simultaneous sampling to two Supelco (Supelco, Bellefonte, PA, USA) fused silica capillary columns. Two different stationary phases were used: SPB-1 (polydimethylsiloxane 30 m x 0,20 mm i.d. with film thickness 0,20 µm) and SulpecoWax-10 (polyethyleneglycol 30 m x 0,20 mm i.d. with film thickness 0,20 µm). The oven program temperature was programmed to increase from 70 °C to 220 °C at 3 °C/min increments, 220 °C – 15 min) with injection temperature 250 °C. The carrier gas was helium adjusted to linear velocity of 30 cm/s and splitting ratio 1:40. The temperature of detectors was set for 250 °C.

Mass spectrometry analyses were carried out in a Hewlett-Packard 6890 gas chromatograph fitted with a HP-1 fused silica column (polydimethylsiloxane 30 m x

0,25 mm i.d. film thickness 0,25 μm), interfaced with Hewlett-Packard mass-selective detector 5973 (Agilent technologies) operated by HP enhanced ChemStation software, version A.03.00. The same parameters as described above for gas chromatography were used. Interface temperature was set on 250 °C, MS source temperature was 230 °C, MS quadrupole temperature was 150 °C and ionization energy 70 eV, ionization current 60 μA and scan range 35 - 350 units. Scan/s: 4,51.

Identification of components was gained from retention times of SPB-1 and SupelcoWax-10 columns and their mass spectra. Retention times were compared with samples saved in laboratory database with more than 400 volatile natural compounds). Relative amount of components was calculated according to GC areas without FID response factor correction.

4.4.2. Antifungal activity

4.4.2.1. Fungal strains

The antifungal activity of *Lavandula angustifolia* essential oil was tested on *Candida*, *Dermatophyte* and *Aspergillus* strains. The strains were obtained by several methods. *Candida crusei* H9 and *Candida guilliermondii* MAT23 were isolated from recurrent cases of vulvovaginal candidosis, *Candida albicans* ATCC 10231, *Candida tropicalis* ATCC 13803 and *Candida parapsilopsis* ATCC 90018 were bought from American type Culture Collection. *Cryptococcus neoformans* CECT 1078 was gained from Colección Espanola de Cultivos Tipo. Filamentous fungi *Aspergillus flavus* F44 was isolated from bronchial secretion, *Aspergillus niger* ATCC 16404 and *Aspergillus fumigatus* 46645 were supplied by American Culture Type Collection. Dermatophytes *Epidermophyton floccosum* FF9, *Trichophyton mentagrophytes* FF7 and *Microsporum canis* FF1 were isolated from nails and skin and last dermatophytes *Trichophyton rubrum* CECT 2794, *Trichophyton verrucosum* CECT 2992, *Trichophyton mentagrophytes* var. *interdigitale* CECT 2958, *Microsporum gypseum* CECT 2908 were obtained by Colección Espanola de Cultivos Tipo.

Fungi were identified by standard microbiological methods. All strains were stored on Sabourad agar with 20 % of glycerol at -70 °C. Before the actual testing each isolate was inoculated on Sabourad agar to make sure that the strain is not contaminated and has standard grow characteristic.

4.4.2.2. Method

Antifungal activity was tested by macrodilution broth method (macrodilution was chosen for the possibility to test oil in glass tubes and so avoid reaction of oil with the plastic ones) to detect minimal inhibitory concentrations (MIC) and minimal lethal concentrations (MLC) of essential oil. The whole experiment was performed according to Clinical and Laboratory Standards Institute (CLSI) reference protocols M27-A3[75] and M38-A2.[76]

Dilutions of the oil were prepared by serial dilution (the same amount of essential oil and DMSO were mixed, half of the mixture was added to the specific amount of medium, mixed and half of this new mixture was again transefered to the new medium and so on), concentration ranging from 0,08 to 20 µL/mL. Final concentration of DMSO did not exceed 2 % (v/v).

The inoculum suspensions were prepared from fungal strains diluting in PRMI 1640 broth in appropriate density of $(1-2) \times 10^3$ cells/ml for yeasts or $(1-2) \times 10^4$ cells/ml for filamentous fungi and placed in 12 x 75 mm glass test tubes. The cell density was then confirmed by counting on Sabourad agar.

Different essential oil concentrations were added to the test tubes, which were subsequently aerobically incubated at 35 °C for 48 hours for *Candida* spp. and *Aspergillus* spp, at 35 °C and 72 hours for *Cryptococcus neoformans* and at 30 °C for 7 days for Dermatophytes. The oil-free growth control and DMSO toxicity control tubes were used.

The minimal lethal concentration (MIC) was then evaluated to detect the lowest concentration of the oil which causes the full growth inhibition.

To measure minimal lethal concentration (MLC), 20 μ L aliquots were taken from each negative tube and the first positive tube (as a growth control) from the MIC reading and were cultured in Sabourad dextrose agar. Plates were incubated at 35 °C for 48 hours for *Candida* and *Aspergillus*, at 35 °C and 72 hours for *Cryptococcus neoformans* and for 30 °C for 7 days for dermatophytes. MLC values were determined as the lowest concentration of the oil causing fungal death.

Two reference antifungal compounds, amphotericin B (Fluka) and fluconazole (Pfizer, UK) were used as standard antifungal drugs for quality control. For all conditions the RPMI 1640 medium (containing L-glutamine, phenol red pH indicator and without bicarbonate) was used. The experiment was performed in triplicate.

4.4.3. Cytotoxicity assay

4.4.3.1. Material

Two different types of cell lines were used: lung carcinoma cells A549 and macrophages RAW 264.7. The mouse macrophage cell line, Raw 264.7 (ATCC number: TIB-71) was kindly supplied by Dr. Oflia Vieira (Center for Neuroscience and Cell Biology, University of Coimbra, Portugal) and the Lung carcinoma cells A549 (ATCC number: CCL-185) were bought from American Type Culture Collection and cultured in medium at 37 °C in a humidified atmosphere of 95 % air and 5 % CO₂. Along the experiments, cells were monitored by microscope observation in order to detect any morphological changes.

4.4.3.2. Method

Macrophages were cultured in Costar plastic flask in monolayer. They were treated by Iscoove's modified Dulbecco's medium with L-glutamine (4 mM) and Hepes (25 mM) and supplemented with 10 % (v/v) FCS, 3,02 g/L sodium bicarbonate, 100 μ L streptomycin, 100 U/mL penicillin and adjusted to pH 7.2.

Lung carcinoma cells were also grown in Costar plastic flask in monolayer cultures. In this case with the DMEM medium supplemented with glucose (25 mM),

3,70 g/L sodium bicarbonate, 10 % (v/v) fetal calf serum (FCS), 100 µg/L streptomycin, 70 µg/L penicillin and adjusted to pH 7,2.

Both cell cultures were grown at 37 °C in an atmosphere of 5 % CO₂ in air. The medium was routinely renewed when confluence was reached.

The assessment of cell viability was made through a colorimetric assay, using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). This assay is based on the reduction of yellow tetrazolin salt of MTT by the mitochondrial succinate dehydrogenase to form an insoluble-formazan blue product. Only viable cells with active mitochondria can reduce significant amounts of MTT, being formazan-blue formation measured spectrophotometrically.

The cells were cultured in 48-well microplates in concentration $0,6 \times 10^6$ cells/well for macrophages and $0,05 \times 10^6$ for lung carcinoma cells in a total volume of 600 µL. The cells were left to stabilize in a chamber (in the dark, 37 °C, 5 % CO₂) for 12 hours. After twelve hours, all medium was taken away and replaced with a new one in a total volume of 588 µL (except for control wells, which still had 600 µL). Then 12 µL of different essential oil dilutions were added (different concentrations ranging from 0,08 - 1,25 µL/mL were prepared by serial diluting in DMSO and medium used for treating cells, the same amount of essential oil and DMSO was mixed together, half of the mixture was transferred to the medium, mixed again, half of this new mixture was again transferred to the same amount of medium and so on). The plate was left in the chamber for 1 hour and then 0,6 µL of LPS was added to the wells. Again, the plate was placed in the chamber for 24 hours. After 24 hours, 43 µL of MTT were added to each well. The plate was left to incubate in the chamber for 15 minutes.

After 15 minutes, supernatant was separated to eppendorf tubes and centrifuged (1000 g during 5 min, 4500 rpm) to recover viable cells. To dissolve formazan crystals formed in adherent cells in the microplates, 300 µl of acidified isopropanol (0,04 N HCl in isopropanol) were added to each well and recovered to the respective eppendorf containing the blue formazan pellet formed after centrifugation. The quantification of

formazan was performed using an ELISA automatic microplate reader (SLT, Austria) at 570 nm, with a wavelength of 620 nm.

All the experiments were performed in duplicate, and repeated three times, being the results expressed as mean \pm SEM of the indicated number of experiments. The means were statistically compared using one-way ANOVA, with a Dunnet's multiple comparison test. The statistical tests were applied using GraphPad Prism, version 5.02 (GraphPad Software, San Diego, CA, USA).

4.4.4. Anti-inflammatory activity

4.4.4.1. Material

The mouse macrophage cell line, Raw 264.7 was cultured on DMEM supplemented with 10 % (v/v) non inactivated fetal bovine serum, 3,02 g/L sodium bicarbonate, 100 μ G/mL streptomycin and 100 U/mL penicillin at 37 °C in a humidified atmosphere with 5 % CO₂. Viable cells were counted on a haemocytometer using trypan blue dye and the morphological appearance of the cells was microscopically monitored during the assays.

LPS was obtained from *E. coli* (serotype 026:B6).

4.4.4.2. Nitric oxide measurement

The production of nitric oxide was measured in the culture supernatant using colorimetric reaction with the Griess reagent [0,1 % (w/v) N-(1-naphtyl)-ethylenediamine dihydrochloride and 1 % (w/v) sulphanilamide containing 5 % (w/v) H₃PO₄].

The cells were cultured in 48-well microplates in concentration 0,6 x 10⁶ cells/well for macrophages in total volume of 600 μ L. The cells were left to stabilize in a chamber (in the dark, 37 °C, 5 % CO₂) for 12 hours. After twelve hours, all medium was taken away and replaced with new one – total volume of 588 μ L (except for control wells, which still had 600 μ L). Then 12 μ L of different essential oil dilutions (concentrations ranging from 0,04 - 1,25 μ L/mL were prepared by serial diluting in

DMSO and medium used for treating cells) were added. The plate was left in the chamber for 1 hour and then 0,6 μL of LPS were added to the wells. Again, the plate was placed in the chamber for 24 hours. After the treatments, 170 μL of the supernatant were mixed with 170 μL of Griess reagent. The plate was left for 30 minutes in the dark at the room conditions.

Then the results were read in an automatic plate reader at 550 nm (SLT, Austria). The quantity of nitrites was determined according to a sodium nitrite standard curve. All the experiments were performed in duplicate, and repeated three times, being the results expressed as mean \pm SEM of the indicated number of experiments.

Statistical analysis comparing LPS condition to control was performed using a two-sided unpaired t-test. When comparing the effect of different treatments to LPS-stimulated cells, a one-way ANOVA followed by Dunnett's post-test was used. The statistical tests were applied using GraphPad Prism, version 5.02 (GraphPad Software, San Diego, CA, USA).

4.4.5. Antioxidant activity

4.4.5.1. Method

The method is called TBARS (thiobarbituric acid – reactive substances). The experiment is based on measuring the antioxidant activity of the samples using the modified thiobarbituric acid (TBA), which is the reactive substance here. In the experiment, we were measuring the antioxidant activity of the samples with or without a lipid peroxidation inducer (ABAP). The measured compound is malondialdehyde (MDA) after lipid hydroperoxide decomposition, which makes pink chromophore with thiobarbituric acid (condensation of two molecules of thiobarbituric acid and one molecule of malondialdehyde). The pink chromophore absorbs at 532 nm wavelength. The problem of this method is its low specificity. Many compounds can react with malondialdehyde to form a chromophore (4-hydroxyalkenals, 2,4-alkendials, 2-alkenals, proteins, sugar degradation products, amino acids, nucleic acids with more than 3 carbon atoms, anthocyanins) and cause false positive results.

Egg yolk was used as the source of the lipids. 1,0 g of the fresh egg yolk was diluted with 10,0 mL of 1,15 % (w/v) KCl solution to create an egg yolk solution of final concentration of 10 % (w/v). This yolk solution was then homogenized for 30 seconds and then ultrasonicated for five more minutes. To every experiment tube, 500 µL of the homogenate was placed, than added 100 µL of methanol (for control) or 100 µL of essentials oil dilution (in concentration ranging from 4,5 – 18,0 µL/mL) or standard (BHA – butylated hydroxyanisol, BHT – butylated hydroxytoluen). The tubes were filled up to volume of 1,0 mL with distilled water, followed by 1,5 mL of 20% acetic acid at pH 3,5 and 1,5 mL of 0,8% w/v TBA (2 – thiobarbituric acid) in 1,1% (w/v) SDS (sodium dodecil sulphate). Mixture was shaken at the vortex for five seconds and then given to the water bath (95 °C) for one hour. After the bath, the tubes were cooled down at the room tempreature (20 minutes in water with room temperature in a dark place). After cooling down, 5 mL of n-butanol were added to each tube and centrifugated for 15 min at 4500 rpm. Finally the absorbance of the supernatant was measured at 532 nm on Cintra 101 GBC spectrophotometer in the software Cintral General Applications. The final percentage of inhibition was calculated according to this equation:

$$\% = [(A_0 - A_1)/A_0] \times 100$$

In the equation, A_0 is the absorbance of the control and A_1 is absorbance of the sample.

4.4.6. Nematicidal activity

4.4.6.1. Method

Nematodes of the species *Bursaphelenchus xylophilus* were supplied by Department of Life Sciences of the Faculty of Science and Technology of the University of Coimbra. The nematodes were maintained in Petri dishes on malt extract agar containing culture of *Botrytis cinerea*. Petri dishes sealed with parafilm were stored in the dark at 25 °C. For the experiment, nematodes were rinsed from the lid of Petri dish by distilled water to another sterile Petri dish.

Different concentrations of essential oils ranging from 5,00 to 0,32 $\mu\text{L}/\text{mL}$ were prepared by serial dilution with distilled water containing Triton X-100 (non-ionic surfactant) in final concentration of 5 mg/mL). The experiment was performed in 20 well microplates. Each well contained 1mL of essential oil dilution and was supplied with 15 nematodes. The plates were maintained at 25 °C in a dark place. Results were read after 1, 3, 6, and then regularly after 24 hours, for 3 days using stereomicroscope. The total number of nematodes and the number of dead nematodes was registered. The nematodes were considered dead when their bodies were straight and they did not move, even after being touched by a needle. Data on mortality was converted to percentage cumulative mortality, with reference to distilled water with Triton X-100 control. Data derived from the 72 hour observation were subjected to probit analysis using IBM SPSS Statistics v.19.

4.4.7. Insect repellency

4.4.7.1. Material

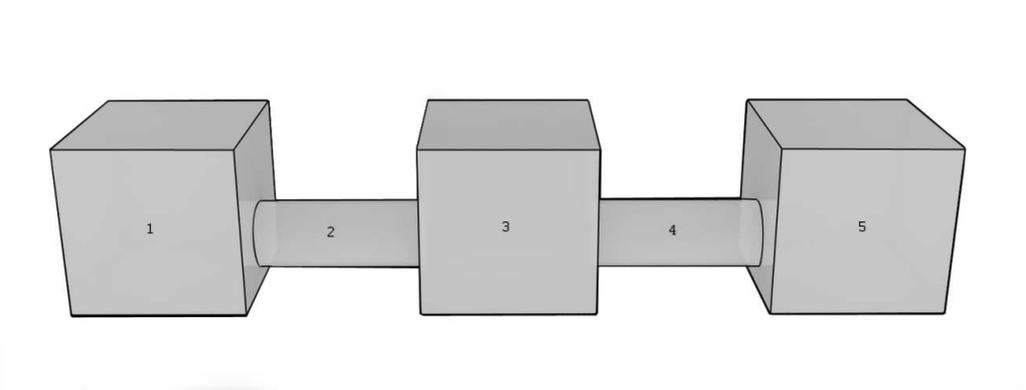
Rhynchophorus ferrugineus beetles were gained during cutting down an infected tree by the municipal authority. A rotten palm tree, blocks of the palm and some palm leaves were also taken. 15 beetles were treated in a dark plastic box (60,0 x 30,0 x 30,0 cm) filled with blocks of palm tree, fresh leaves and rotten palm wood.

4.4.7.2. Method

For the experiment, the special apparatus was created (Pic. 3). Bottom of each box was filled with rotten palm wood, boxes number one and five contained the same amount of fresh palm leaves and small piece of cotton wool. Essential oil (20 μL) was applied on one cotton wool in the box number one, the fifth one was oil free.

Ten beetles were placed in the middle box (n3). Their movements were observed and the results were recorded every thirty minutes during four hours. The experiment took part in the morning (9:00 a.m.) because of the most intensive activity of the beetles.

Picture 3: Repellency experiment



5. RESULTS

5.1. Essential oil isolation and characterization

Identification of components was gained from retention times of SPB-1 column and their mass spectra. Retention times were compared with samples saved in laboratory database. Compounds were listed in order to their elution on the SPB-1 column.

Table 1: Composition of the essential oil of *Lavandula angustifolia*

Compound	[%]	Compound	[%]
α -pinene	0.5	cuminaldehyde	2.3
camphene	0.2	carvone	0.7
sabinene	0.1	geraniol	1.9
β -pinene	0.4	Linalyl-acetate	25.3
myrcene	0.1	phellandral	0.7
limonene	0.2	cuminic alcohol	1.1
1,8-cineol	0.7	lavandulyl acetate	6.4
<i>cis</i> - β -ocimene	0.8	neryl acetate	1.0
linalol	24.7	geranyl acetate	1.1
camphor	6.2	<i>trans</i> -caryophyllene	1.1
pinocarvone	0.1	<i>trans</i> - α -bergamotene	0.6
borneol	4.3	<i>trans</i> - β -farnesene	2.4
lavandulol	1.2	germacrene D	1.1
cryptone	1.4	γ -cadinene	0.5
<i>p</i> -cymene-8-ol	0.5	caryophyllene oxyde	2.1
terpinen-4-ol	1.2	T-cadinol	1.2
α -terpineol	2.0	bisabolol oxyde	0.3
<i>trans</i> -carveol	0.6	β -bisabolol	1.3
others	3,7		

5.2. Antifungal activity

The results for antifungal activity which are determined by their minimal lethal and minimal inhibitory concentrations.

Table 2: Antifungal activity (MIC and MLC) of the essential oil of *Lavandula angustifolia* for *Candida*, dermatophyte and *Aspergillus* strains

Strains	<i>L. angustifolia</i>		Fluconazole		Amfotericin B	
	MIC♦	MLC♦	MIC★	MLC★	MIC★	MLC★
<i>Candida albicans</i>	1.25	1.25	1	>128	N.T	N.T
<i>Candida tropicalis</i>	1.25-2.5	1.25-2.5	4	>128	N.T	N.T
<i>Candida krusei</i>	1.25-2.5	2.5	64	64-128	N.T	N.T
<i>Candida guilliermondii</i>	1.25	1.25	8	8	N.T	N.T
<i>Candida parapsilopsis</i>	1.25-2.5	1.25	<1	<1	N.T	N.T
<i>Cryptococcus neoformans</i>	0.32-0.64	0.64	16	128	N.T	N.T
<i>Trichophyton mentagrophytes</i>	0.64	0.64	16-32	32-64	N.T	N.T
<i>Trichophyton mentagrophytes</i> var. <i>interdigitale</i>	0.64	1.25	128	≥128	N.T	N.T
<i>Trichophyton verrucosum</i>	1.25	1.25	>128	>128	N.T	N.T
<i>Microsporum canis</i>	0.64	0.64	128	128	N.T	N.T
<i>Trichophyton rubrum</i>	0.32	0.32-0.64	16	64	N.T	N.T
<i>Microsporum gypseum</i>	0.64-1.25	1.25	128	>128	N.T	N.T

<i>Epidermophyton floccosum</i>	0.64	0.64	16	16	N.T	N.T
<i>Aspergillus niger</i>	1.25	10-20	N.T	N.T	1-2	4
<i>Aspergillus fumigatus</i>	1.25	2.5-5	N.T	N.T	2	4
<i>Aspergillus flavus</i>	2.5	5	N.T	N.T	2	8

♦ MIC and MLC were determined by a macrodilution method and expressed in $\mu\text{L}/\text{mL}$ (v/v).

★MIC and MLC were determined by a macrodilution method and expressed in $\mu\text{g}/\text{mL}$ (w/v).

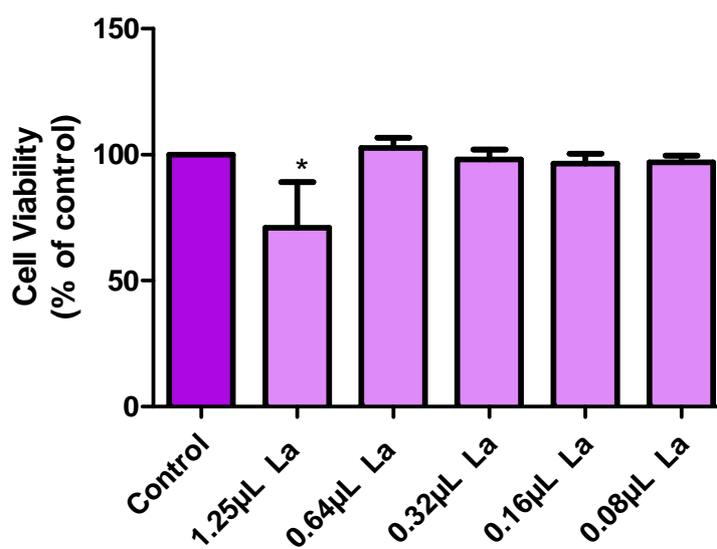
N.T- not tested.

Results were obtained from three independent experiments performed in duplicate.

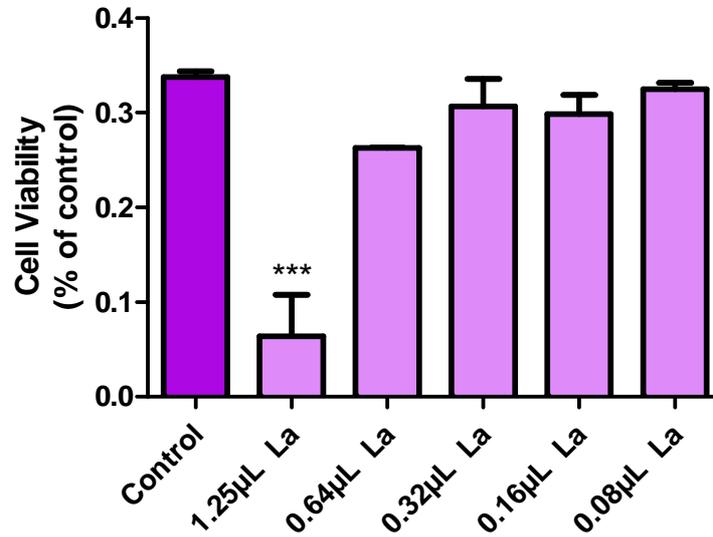
5.3. Cytotoxicity

Results for cell viability of two types of cell - macrophages RAW 264.7 and lung carcinoma cells A549 are represented by two graphics, separated for each cell line. Cytotoxicity for the tested cell lines was very low, except for the highest used concentration of *Lavandula angustifolia* essential oil.

Graph 1: Macrophages RAW 264.7



Graph 2: Lung carcinoma cells A549

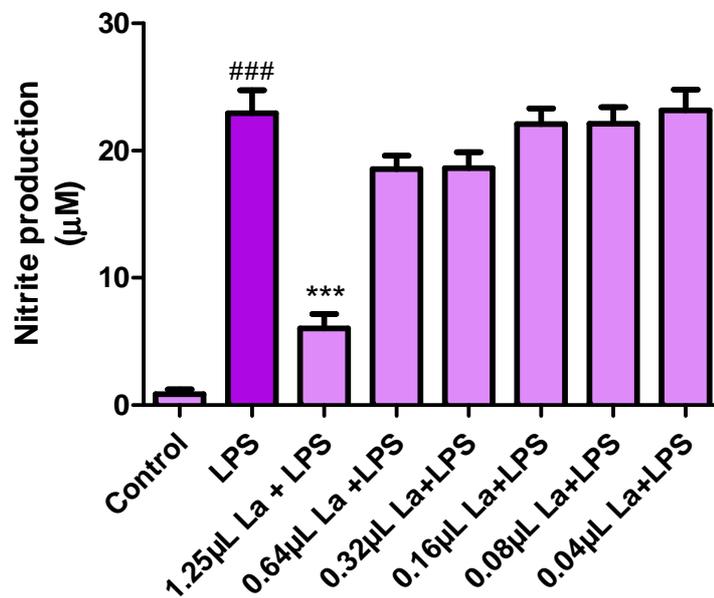


Results are expressed as a percentage of MTT reduction by control cells. Each value represents the mean \pm SEM from 3 independent experiments, performed in duplicate. Means were statistically compared using two-sided unpaired t-test (###p <0.001, compared to control) and one-way ANOVA test, with a Dunnett's post-test (*p<0.05, **p<0.01, ***p< 0.001, compared to LPS)

5.4. Anti-inflammatory activity

Anti-inflammatory activity was tested according to the NO production assay. It can be seen that our essential oil showed no significant effect against LPS produced inflammation in non-toxic concentrations.

Graph 3: Anti-inflammatory activity on macrophages RAW 264.7



Results are expressed as a percentage of nitrite production by control cells. Each value represents the mean \pm SEM from 3 independent experiments, performed in duplicate. Means were statistically compared using two-sided unpaired t-test (###p < 0.001, compared to control) and one-way ANOVA test, with a Dunnett's post-test (*p < 0.05, **p < 0.01, ***p < 0.001, compared to LPS).

5.5. Antioxidant activity

Table 3: Antioxidant activity of the essential oil, BHA and BHT in different concentrations using TBARS assay without ABAP.

EO/substance	Concentration, mean±SE			
	90 µg/5 ml	67,5 µg/5 ml	45 µg/5 ml	22,5 µg/5 ml
BHA	64,10±2,20	60,57±1,92	58,03±2,09	52,81±0,66
BHT	95,35±0,75	93,20±0,89	89,62±0,88	84,65±1,59
Essential oil of LA	-1,19±12,15	0,91±5,36	3,67±2,10	3,91±5,03

SE, standart error

Table 4: Antioxidant index of the essential oil, BHA and BHT in different concentrations using TBARS assay with ABAP.

EO/substance	Concentration, mean±SE			
	90 µg/5 ml	67,5 µg/5 ml	45 µg/5 ml	22,5 µg/5 ml
BHA	89,94±1,66	88,56±2,22	86,25±2,27	79,94±1,39
BHT	71,77±1,37	68,34±0,87	66,14±1,10	63,99±1,51
LA	-0,68±4,94	3,47±5,23	1,95±2,06	0,88±4,96

SE, standart error

5.6. Nematicidal activity

The graph shows that oil concentration 21,611 $\mu\text{L}/\text{mL}$ causes mortality by 50 % (LC50), after 72 hours of exposure.

Graph 4: Probit plot of the effect of exposure for 72 hours to different concentrations of *Lavandula angustifolia* essential oil on *Bursapehlenchus xylophilus* mortality.

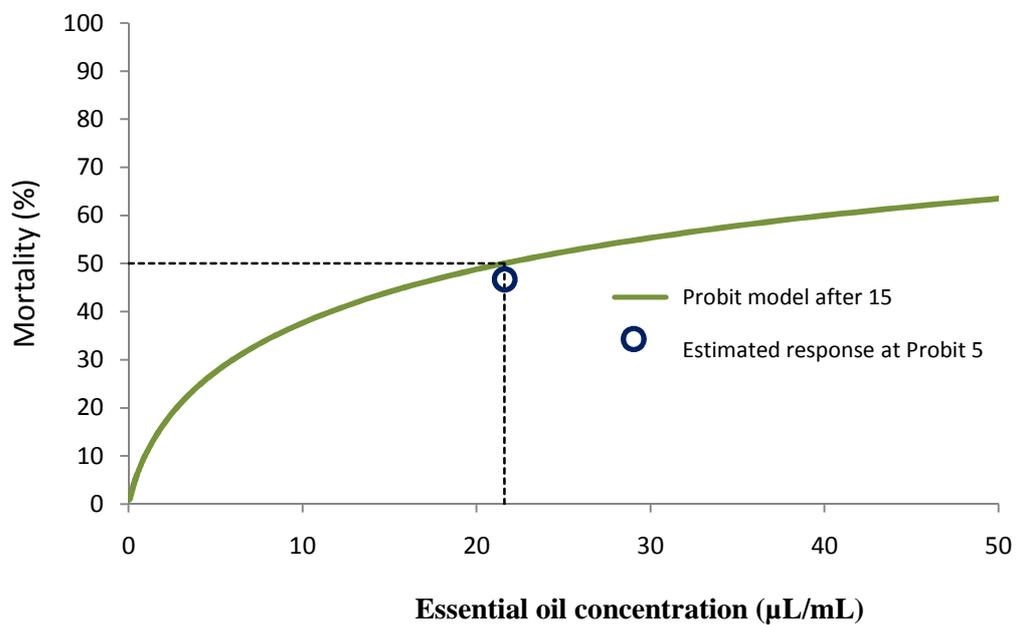


Table 5: Number of alive/dead nematodes in time.

Time / concentration		1 h.	3 h.	6 h.	24 h.	48 h.	72 h.
Aqua	1.	9/0	10/0	13/0	13/0	13/0	13/0
	2.	14/0	16/0	16/0	16/0	16/0	16/0
	3.	8/0	19/0	19/0	19/0	19/0	19/0
	4.	13/0	19/0	19/0	19/0	19/0	19/0
	5.	10/0	11/0	15/0	15/0	15/0	15/0

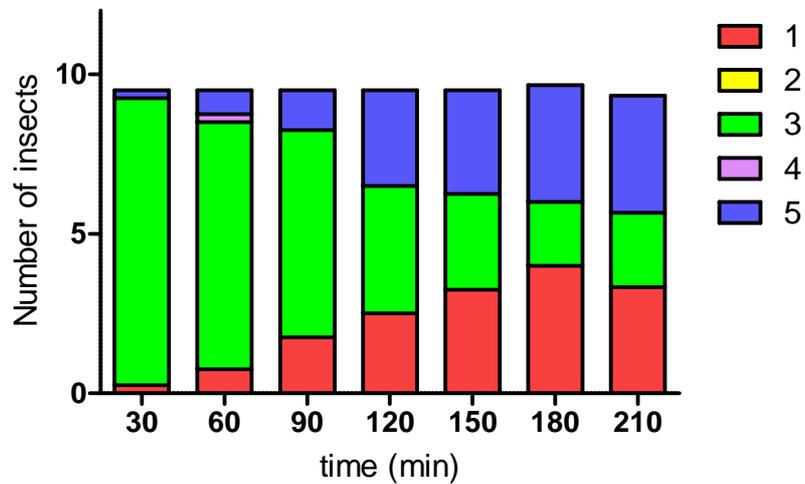
Solvent	1.	8/0	10/0	12/0	12/0	12/0	12/0
	2.	6/0	17/0	20/0	20/0	20/0	20/1
	3.	11/0	17/0	19/0	19/0	19/0	19/0
	4.	11/0	19/0	19/0	19/0	19/0	19/1
	5.	8/0	8/0	12/0	12/0	12/0	12/0
5 $\mu\text{L}/\text{mL}$	1.	6/0	15/5	15/5	15/6	15/6	15/7
	2.	12/0	14/3	14/4	14/4	14/4	14/5
	3.	10/0	15/8	14/6	14/6	14/6	14/6
	4.	9/0	14/3	14/3	14/3	14/4	14/4
	5.	16/0	14/2	15/1	15/1	15/2	15/4
2,5 $\mu\text{L}/\text{mL}$	1.	8/0	14/0	14/0	14/0	14/0	14/2
	2.	9/0	18/2	18/2	18/2	18/2	18/2
	3.	7/0	15/0	15/0	15/0	15/1	15/2
	4.	12/0	17/2	17/1	17/1	17/1	17/2
	5.	11/0	16/1	20/0	20/1	20/1	20/2
1, 25 $\mu\text{L}/\text{mL}$	1.	9/0	16/0	19/0	19/0	19/1	19/2
	2.	10/0	14/0	20/0	15/1	18/1	18/1
	3.	16/0	22/0	27/0	27/0	27/0	27/1
	4.	5/0	26/0	26/0	26/0	26/1	26/2
	5.	9/0	13/0	19/0	19/0	19/3	19/3
0,64 $\mu\text{L}/\text{mL}$	1.	5/0	15/0	19/0	19/0	19/0	19/1
	2.	5/0	14/0	16/0	16/0	16/2	16/2
	3.	11/0	16/0	22/0	22/0	19/0	19/0
	4.	7/0	15/0	18/0	18/0	18/0	18/2
	5.	10/0	10/0	15/0	15/1	15/2	15/3

0,32 $\mu\text{L}/\text{mL}$	1.	12/0	15/0	17/0	17/0	17/0	17/0
	2.	15/0	16/0	21/0	21/0	21/0	21/2
	3.	10/0	17/0	21/0	21/0	21/0	21/1
	4.	16/0	18/0	20/0	20/0	20/0	20/2
	5.	10/0	13/0	18/0	18/0	18/0	18/1

5.7. Insect repellency

In the following graph, distribution of ten beetles during four hours can be seen. Each column contains different colors according to their position in the boxes.

Graph 5: Movement of *Rhynchophorus ferrugineus*



6. DISCUSSION

6.1. Essential oil isolation and characterization

The chemical composition of the essential oil is shown in Table 1, with the compounds listed in order of their elution on a SPB-1 column. The essential oil was obtained in yields of 2 % (v/w). A total of 36 compounds were identified, representing 96,3 % of the total volatile oils in the plant. The oils were characterized by high contents of oxygen-containing monoterpenes. The main constituents of the oils were linalyl-acetate (25.3 %) and linalool (24.7 %).

The tested essential oil belongs to those with lower content of linalyl-acetate and linalool between other lavender oils.

Table 5: Content of linalool and linalyl-acetate

Sample	Linalyl-acetate [%] (3,7-dimethylocta-1,6-dien-3-yl acetate)	Linalool [%] (3,7-dimethylocta-1,6-dien-3-ol)
Castro de Aire, Portugal	25,3	24,7
French Pyrenees, France [77]	43,98	38,47
Uttarakhand, India [78]	47,56	28,06
Xinjiang, China [79]	-	44,54
Invitrogn Ltd. [80]	51,0	35,0
Muggia, Trieste, Italy [26]	43,1	32,7
Ditta Primavera, Pisa, Italy [81]	27,34	32,10

6.2. Antifungal activity

The essential oil was used to evaluate the antifungal activity against several pathogenic strains involved in human diseases. Since the essential oils are mixtures of different compounds, the exact mechanism of action is not known and it is presumed, that the antifungal effect is caused by the combination of the effect of more compounds in the oil. Various degrees of inhibition were registered against all the fungi tested (Table 2). The highest antifungal activity was observed against dermatophyte strains and *Cryptococcus neoformans*, with MIC and MLC values ranging from 0,32 to 1,25 $\mu\text{L}/\text{mL}$. For *Candida* and *Aspergillus* strains, MIC values ranged from 1,25 to 2,5 $\mu\text{L}/\text{mL}$. For most of the dermatophytes, *Cryptococcus neoformans* and *Candida* strains, the MIC was equivalent to the MLC, indicating a clear fungicidal effect of *L. angustifolia* essential oil.

The higher susceptibility of dermatophytes has also been reported for other essential oils.[4, 82]

Because of the fact that essential oils of lavandula species belong to the very often used ones in many departments of medicine, essential oils were also tested for the possibility of antifungal activity.

L. angustifolia showed moderate to low antifungal activities against the human pathogens *Malassezia furfur*, *Trichophyton rubrum*, and *Trichosporon beigelii*. [83]

Lavandula viridis L'Hér and *Lavandula pedunculata* (Miller) Cav.[4] were tested against *Candida*, *Cryptococcus neoformans* (not for *L. pedunculata*), *Aspergillus* strains and *Dermatophytes*. According to the determined MIC and MLC values, the *Dermatophytes* and *Cryptococcus neoformans* were the most sensitive fungi (MIC and MLC values ranging from 0,32 to 0,64 $\mu\text{L}/\text{mL}$), followed by *Candida* species (at 0,64 – 2,5 $\mu\text{L}/\text{mL}$). [82] *Lavandula pedunculata* (Miller) Cav. proved to have a significant antifungal activity against *Dermatophyte* strains.[4] *Lavandula angustifolia* oil

demonstrated a great antifungal effect against *Aspergillus nidulans* and *T. mentagrophytes*. [84]

As far as we know, this is the first report on the antifungal activity of this species against *Dermatophytes* and *Aspergillus* strains. Our results support further investigations of this essential oil for clinical use in the management of superficial and/or mucosal fungal infections (dermatophytosis).

6.3. Cytotoxicity

L. angustifolia essential oil did not affect the viability of the macrophages (Graph 1) and lung cells (Graph 2) at the concentrations lower than 1,25 $\mu\text{L/mL}$. A cell-free control was performed in order to exclude non-specific effects of the oil on MTT. Our results prove that the lavender essential oil cytotoxicity is dose-dependent. Results were similar to the study which also tested the main compounds of lavender essential oil – linalool and linalyl-acetate. Cytotoxicity of essential oil and linalool was found to have equivalent pattern, which leads to the conclusion that the linalool is the active compound of lavender essential oil. On the other hand, the toxicity of linalyl-acetate was more damaging to the fibroblasts. [80]

To the date, there are very few reports on the cytotoxicity of lavender essential oil. Indeed, literature has lavender oil placed in the category of safe oils. As far as we know, there is not much evidence to support this information. Lavender oil has a historical use in wound healing and is commonly applied to skin without dilution, but, similarly, there is no scientific evidence suggesting that lavender accelerates wound healing [85] or reduces scarring. [9] With reports of contact dermatitis associated with lavender oil, there is also a potential for either allergic or skin irritation reactions. Nine years long study in Japan has already found that up to 13.9 % of subjects had contact dermatitis on exposure to lavender oil. [80, 9]

6.4. Anti-inflammatory activity

Lavandula angustifolia essential oil was not able to inhibit NO production on significant level at different concentrations without being cytotoxic to cells. Our results did not prove traditional use of lavender essential oil as an anti-inflammatory agent.

The study which was using another method - carrageena-induced paw edema test - provides different results which say that the essential oil of lavender leaves (major compound 1,8-cineole) had potent anti-inflammatory activity against carrageena.[8] Major components of *lavandula* essential oil were also tested. Both of them, linalool and linalyl-acetate, have demonstrated anti-inflammatory activity in carrageenan-induced paw edema test supporting the theory of *Lavandula angustifolia* essential oil having anti-inflammatory activity.[86]

6.5. Antioxidant activity

Antioxidant activity was tested by TBARS method and according to the results (Table 3), *Lavandula angustifolia* essential oil had no significant antioxidant activity. Other plants from *Lamiaceae* family were also tested. *Thymus camphorathus* essential oil (main components 1,8-cineole, linalool and linalyl-acetate) belonged to the most similar ones to our plant. Thyme was tested by the same method as we used and it showed low, but still important antioxidant activity.[42, 87]

6.6. Nematicidal activity

Pine wilt disease establishes one of the biggest threads to the native species of *Pinus* spp. To control this disease, chemical agents as ethylene dibromide or dibromochloropropane were used, but nowadays, they have been removed from the market due to their non-selectiveness and harmful effects on environment. New possibilities are searched and use of essential oil belongs between them.[89]

General research was made on activity of aliphatic compounds against *Bursaphelenchus xylophilus*. Aliphatic alcohols, aldehydes and acids provided greater efficiency than hydrocarbons and ketones. Very important is the position of double

bond, p.e. 2-alkenals have stronger nematicidal activity compared to alkanals and double bond in α , β - position at aldehydes provides better nematicidal activity as well. Other fact influencing activity of the compound is the chain length – C9-C11 is optimal (at 2-alkenals).[103]

Different essential oils have already been tested on nematicidal activity with good results. Essential oils from *Trachyspermum ammi*, *Pimenta dioica* and *Litsea cubeba* showed nematicidal activity at a concentration of 2 mg/mL (LC50 0,431; 0,609; 0,504 mg/mL). Single components of essential oil were also tested – good activity was pronounced by thymol, carvacrol, eugenol, β -caryophyllen, methyl-eugenol, α -humulene, limonene and 1,8-cineole.[81] Our study showed that *Lavandula angustifolia* doesn't belong to the essential oils with high nematicidal activity with concentration of 21 μ L/mL to kill 50 % of nematodes.

Coriandrum sativum, *Liquidambar orientalis* and *Valeriana walichii* essential oils showed 100% mortal activity at concentration of 2,0 mg/mL (strong nematicidal activity persisted even at concentrations 1,0; 0,8 and 0,6 mg/mL, except for *valeriana* EO, where the activity was below 50 %).[68] *Gaultheria fragrantissima* and *Zanthoxylum alatum* essential oils showed almost 100% nematicidal activity at a concentration 5 mg/mL and high activity at concentration of 2,5 mg/mL. *Citrus reticulata* and *Tasmannia lanceolata* showed lower activity, only 25 %.[36] *Cymbopogon citratus*, *Cinnamomum verum*, *Allium sativu*, *Leptospermum petersonii* and *Eugenia caryophyllata* caused 100% immobility of *Bursaphelenchus xylophilus*, *Asiasarum sieboldi* and *Mentha spicata* inflicted 100% mortality of male and females nematodes, but only 78,3 and 8,55% mortality of juveniles. *Boswellia carterii* and *Pimenta racemosa* killed approximately 70-80 % of nematodes.[32]

6.7. Insect repellency

Rhynchophorus ferrugineus is very serious pest almost all around the world. It is spreading quickly through wood industry and is difficult to eradicate. Different methods were developed in order to protect the plants, but more effective ones are still needed.

Phoenix canariensis, *Washingtonia robusta*, *Washingtonia filifera* and *Trachycarpus fortunei* are susceptible plants to infection of RF. The usage of imidacloprid oil dispersion as a drench provided very good results in both preventive and curative treatment.[70] Other effective method is the use of traps which can be filled with femomone and/or food or can be bait free. Beetles can be better trapped in liquid filled trap and there is also the need to change the traps every seven days to assure attractiveness for the beetles. Other interesting fact needed to be explained is that female beetles are more attracted to the baits than male ones. Both methods showed great results and are suggesting further use in efforts to protect palm trees from this parasite.[88, 89, 90] By using acoustic measurements we can recognize infected palm trees with no visual symptoms and then provide treatment to them.[89]

There was also *Boxus chinensis* oil tested against ten days old larvae of *Rhynchophorus ferrugineus* with good results. The mortality of the larvae depended on the oil concentration with highest mortality connected to the highest concentration (78% mortality at concentration 1,25 %).[69]

Lavender oil was also tested as a potential repellent to protect *Brassica napus* L. seeds from its major pest - *Meligethes aeneus*. Major component of the oil were tested to identify the most repellent ones and to find the best combination of them to provide the best repellency activity.[91]

7. CONCLUSION

Essential oil was isolated from plant *Lavandula angustifolia* by hydrodistillation. Composition of essential oil was identified by analytical gas chromatography and mass spectrometry, the main compounds were recognized as linalool and linalyl-acetate. Tested essential oil belonged to the ones with lower content of these constituents.

The aim of work was also to test several biological activities such as antifungal, antioxidant, anti-inflammatory, cytotoxicity, nematocidal and repellency ones.

Tested oil did not prove to have significant anti-inflammatory, antioxidant, nematocidal or repellency activity. On the other hand, toxicity to macrophages and lung carcinoma cells was low and it indicates that the oil can be used for human patients. The antifungal activity was strongest for *Dermatophyte* strains and *Cryptococcus neoformans*.

This research shows that *Lavandula angustifolia* does not have such a strong potential as we hoped, but still the low toxicity and antifungal properties indicates that the oil should be tested further.

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