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Title of the thesis

“Evaluation of the effects of the *Citrus limon*, *Rosmarinus officinalis* and
Lavandula angustifolia essential oils on mitochondrial function”

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LIST OF ABBREVIATIONS

ABTS: 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)

ADP: Adenosine Diphosphate

ANT: Adenine Nucleotide Translocase

ATP: Adenosine Triphosphate

BAT: Brown Adipose Tissue

BHT: Butylated hydroxytoluene

BSA: Bovine Serum Albumin

CRC: Calcium retention capacity

CVD: Cardiovascular Diseases

DMSO: Dimethyl Sulfoxide

DNP: 2,4-dinitrophenol

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EDTA: EthyleneDiAmineTetraAcetic acid

EGTA: Ethylene Glycol bis (2-aminoethyl) Tetra-Acetic acid

EO: Essential Oil

ETC: Electron Transport Chain

FCCP: carbonilcyanide p-triflouromethoxyphenylhydrazone

GC: Gas Chromatography

HFD: High Fat Diet

IMM: Inner Mitochondrial Membrane

IPP: Isopentenyl Diphosphate

ISO: International Standard Organization

LDH: Lactate Dehydrogenase

LXR β : Liver X Receptor β

MgATP-SMP: SubMitochondrial Particles

MIS: Mitochondrial Isolation Solution

MRM: Measuring Respiration Medium

MS: Mass Spectrometry

NAD⁺: Nicotinamide Adenine Dinucleotide

NADP⁺: Nicotinamide Adenine Dinucleotide Phosphate

NMR: Nuclear Magnetic Resonance

NQO1: NAD(P)H:quinone oxidoreductase
pAMPK: AMP-activated protein kinase
PEP: Phospho-Enol Pyruvate
PK: Pyruvate Kinase
PPAR α : Peroxisome Proliferator Activated Receptor- α
PPAR γ peroxisome proliferator activated receptor- γ
PPAR δ peroxisome proliferator activated receptor- δ
PTP: Transition Pore
RCR: Respiratory Control Ratio
ROS: Reactive Oxygen Species
SB: Sonication Buffer
T2DM: Type 2 Diabetes Mellitus
UCP: Uncoupling Proteins
Vmax: Maximum enzyme velocity
WAT: White Adipose Tissue
EGTA: Ethylene Glycol bis (2-aminoethyl) Tetra-Acetic acid
MIS: Mitochondrial Isolation Solution
DMSO: Dimethyl Sulfoxide
BSA: Bovine Serum Albumin
MgATP-SMP: SubMitochondrial Particles
EDTA: EthyleneDiAmineTetraAcetic acid
SB: Sonication Buffer
MRM: Measuring Respiration Medium
FCCP: carbonilcyanide p-triflouromethoxyphenylhydrazone
PEP: Phospho-Enol Pyruvate
LDH: Lactate Dehydrogenase
CRC: Calcium retention capacity
pAMPK: AMP-activated protein kinase
PTP: Transition Pore
BHT: Butylated hydroxytoluene
RCR: Respiratory Control Ratio

ABSTRACT

Several species of medicinal plants have long been used for the complementary treatment of numerous diseases, including diabetes and obesity. Essential oils are composed of volatile and semi-volatile compounds and include antioxidants such as terpenes, terpenoids and phenolic components. Their antioxidant activity can contribute to the food preservation, but also to the prevention of diseases that involve oxidative stress. Indeed, essential oils have multiple biological effects, including antifungal, antibacterial, anti-inflammatory, anticancer activity, in addition to anti-obesity effects. The attention is now focused in defining the cellular targets of these complex mixtures. Mitochondria, given the lipophilic nature of essential oils, are one of the possible targets. These organelles are the powerhouses of the cell and oxidise the nutrients to produce energy in form of ATP. However, mitochondria are also the main producers of radical species in the cell and their dysfunction can lead to oxidative stress. They are thus important targets for drug development, including the treatment of obesity and related complications. The goal is to decrease the metabolic efficiency by increasing nutrients oxidation without producing ATP, i.e. generating a mild mitochondrial uncoupling. Due to their chemical nature, essential oils appear to be interesting candidates.

The purpose of this thesis has been to assess the antioxidant properties of fresh and preserved samples of three essential oils, *Citrus limon*, *Rosmarinus officinalis* and *Lavandula angustifolia*, and their chief compounds and the effects of these compounds on mitochondrial bioenergetics, in order to identify novel bioactive molecules with potential anti-obesity effects. Phytochemical characterization has been performed by GC-MS analyses and the structural identification of the main components by one dimensional NMR analyses. Evaluation of the antioxidant properties of the essential oils and their chief components has been done by in vitro DPPH and ABTS radical scavenging assays. Finally, the effects of fresh and preserved essential oils and their chief components on the mitochondrial function have been evaluated by measuring the oxygen consumption under basal and stimulated conditions and the FOF1ATP synthase activity in isolated mitochondria.

The essential oils of lemon and rosemary are both known to have an anti-obesity action, but the results presented in this thesis show that they act through different mechanisms that could have a different impact on human health. Based on its effects on mitochondrial bioenergetics, only lemon essential oil caused a slight uncoupling of the oxidative phosphorylation system, leading to propose its possible use for the anti-obesity treatment. Conversely, the rosemary essential oil induced a strong mitochondrial uncoupling, while the lavender essential oil did not affect the mitochondrial bioenergetics. These results demonstrate that, despite their antioxidant properties, the biological activity of these essential oils are quite different, at least on isolated mitochondria, leading to the conclusion that further studies are needed to reach a definitive recommendation for the use of essential oils for human health.

CHAPTER 1

INTRODUCTION

1.1- The essential oils

Officinal plants are natural resources used since ancient times for their peculiar properties for personal care, additives and preservatives for food and for their applications in pharmaceutical and medical fields. For some decades the interest in these species has increased considerably, in consideration of the fact that the demand for products derived from them has been increasingly expanding, in harmony with the widespread need to improve the quality of life also through the use of natural substances.

This trend represents a new extensive field of research focused on the identification and development of the substances produced by plants and displaying beneficial health effects. The term "officinal plants" refers to all the aromatic and medicinal species, namely all the plants capable of supplying drugs which can in turn be destined for direct consumption or for the transformation of the active ingredients.

Essential oils (EOs) have been known since antiquity for their flavour properties and their used in a wide variety of consumer goods, such as detergents, soaps, toilet products, cosmetics, pharmaceuticals, perfumes, confectionery food products, soft drinks, distilled alcoholic beverages (hard drinks) and insecticides. Besides EOs or their components have been shown to exhibit antibacterial^{1,2,3} antiviral⁴, antimycotic^{5,6,7,8}, antitoxigenic^{9,10} antiparasitic¹¹ and insecticidal¹² properties. Nowadays, EOs are mainly used in food as flavourings, in perfumes and in pharmaceuticals for their functional properties¹³ and also for their potential therapeutic applications in the prevention of cancer.¹⁴ Essential oils, also known as essences, volatile oils, etheric oils, or aetheroleum, are complex mixtures of over 3000 compounds, about 300 of which are of commercial importance.^{15, 13} . Some essential oils appear to exhibit particular medicinal properties that have been claimed to cure organ dysfunction and systemic disorder^{16,17,18}.

According to the International Standard Organization on Essential Oils (ISO 9235: 2013) and the European Pharmacopoeia (Council of Europe 2004) an essential oil is defined as the product obtained from plant raw material by hydrodistillation, steam distillation or dry distillation or by a suitable mechanical process, for example cold pressing without heat is usually used for Citrus fruit oils

They are obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots)¹⁹. EOs can be biosynthesized in different parts of the plant anatomy (in the leaves, flowers, fruits, pericarp of the fruit, seeds, in the bark, and rhizomes, whether stored in glands of oils, glandular hairs, or dissolved in resins, suggesting that their characteristics are highly variable. The chemical composition of these EOs varies widely depending upon the geographical location, botanical origin,

genetics, time of collection, bacterial endophytes and extraction techniques. Indeed, for some plants, essential oils extracted from different parts of the same plant can differ qualitatively and quantitatively. Some plant families are particularly rich in secondary metabolites useful for the production of essential oils; Apiaceae, Asteraceae, Lamiaceae, Rutaceae, Liliaceae, Magnoliaceae, Cupressaceae, Pinaceae, Hypericaceae, Fabaceae, Malvaceae, Myrtaceae, Oleaceae, Rosaceae and Rutaceae.²⁰ Essential oils are usually soluble in alcohol, ether and fixed oils, but are insoluble in water. The main constituents of EOs are volatile to semi-volatile compounds with a density between 0.8-1.2, usually with a strong odour and rarely coloured^{21,22}. These compounds are also frequently optically active. The extraction methods used are dependent on the nature of the material, in accordance with the characteristics of the essential oil¹⁹. Since essential oils are complex mixtures of compounds, they are required to comply with some sets of standards. Physicochemical properties/indices have been used in determining the quality of essential oils¹⁹. EOs are volatile and therefore need to be stored in airtight containers in the dark in order to prevent compositional changes. Monoterpenes and sesquiterpenes are usually the main group of compounds found in EOs and it has been estimated that there are more than 1000 monoterpene structures¹⁵. Many thousands of compounds belonging to the family of terpenes have so far been identified in essential oils²³. Terpene and terpenoid compounds include alcohols, aldehydes, esters, phenols, oxides of aromatic and aliphatic constituents, characterized by low molecular weight. In addition, phenylpropanoids are also very frequent. Moreover, some essential oils may also contain fatty acids and their esters and, more rarely, nitrogen and sulphur derivatives^{15,22}. Terpenes are compounds obtained by combinations of the 5-carbon-base (C5) isoprene unit. The biosynthesis of the terpenes entails the synthesis of the isopentenyl diphosphate (IPP) precursor, repetitive addition of IPPs to form the prenyldiphosphate precursor of the various classes of terpenes, modification of the allylic prenyldiphosphate by terpene specific synthetases to form the terpene skeleton and finally, secondary enzymatic modification (redox reaction) of the skeleton to attribute functional properties to the different terpenes¹⁵. The monoterpenes are formed from the coupling of two isoprene units (C10). They are the most representative molecules constituting 90% of the essential oils and some compounds are also optically active. The acyclic terpene alcohols such as geraniol, linalool e citronellol are used as fragrance and aromas. The sesquiterpenes are formed from the assembly of three isoprene units (C15). The extension of the chain increases the number of cyclisations, affording a wide variety of structures, including chiral centers (table 1.1). Almost all compounds presented in the essential oils, such as monoterpenes, possess antioxidant properties and the activity of cyclic monoterpene hydrocarbons with two double bonds can be also comparable to the activity of phenols. However, some medicinal plants that are known for their strong medicinal benefits should not be used as food additives without previous detailed proof of their safety, especially if used in large additions.

Table 1.1 Example of terpenes, sesquiterpenes and aromatic compounds found in essential oils		
Monoterpenes		
Chemical classification	Molecular form	Example
Hydrocarbons	acyclic	ocimene, myrcene
	monocyclic	limonenes, p-cymene, terpinenes
	bicyclic	camphene, sabinene, pinenes
Alcohols	acyclic	linalool, geraniol, nerol
	monocyclic	carveolo, terpineolo
	bicyclic	borneol, fenchol
Aldehydes	acyclic	geranial, citronellal
Ketones	acyclic	tegetone
	monocyclic	menthones, carvone, pulegone
	bicyclic	camphor, fenchone, thujone
Esters	acyclic	linalyl acetate or propionate, citronellyl acetate
	monocyclic	α -terpinyl acetate
	bicyclic	isobornyl acetate
Ethers		1,8-cineole
Peroxydes		ascaridole
Phenols		thymol, carvacrol
Sesquiterpenes		
Hydrocarbons		β -bisabolene, cadinenes, β -caryophyllene, logifolene, curcumenes, elemenes, farnesenes, zingiberene
Sesquiterpenoids		
Alcohols		bisabol, farnesol
Ketones		germacrone
Epoxides		caryophyllene oxide, humulene epoxides
Aromatic compounds		
Aldehydes		cinnamaldehyde
Alcohols		cinnamic alcohol
Phenols		chavicol, eugenol
Methoxy derivatives		anethole, elemicine, estragole, methyleugenols

Methylenedioxy derivatives		apiole, myristicine, safrole
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1.2 Antioxidant properties

Essential oils include antioxidants such as terpenoid and phenolic components. The antioxidant property of several essential oils and components has been verified *in vitro* by physical–chemical methods²⁴ and it is strongly affected by the presence of different types of antioxidants. In general, care should be taken before assuming that the antioxidant property of EOs is simply that sum of the characteristic components. In some case the most effective antioxidant components are the dominant ones and the overall oxidative protection offered by the oil is due to such components.²⁵ The overall performance as antioxidant is the result of the complex interplay among components, depending on the exact EO composition and experimental conditions and synergistic or antagonistic effects are also expected. In general, by considering the composition one can roughly predict the antioxidant potential. For example, EO with high amounts of phenolics and cyclohexadiene-like components (such as γ -terpinene) have great antioxidant capacity while, oil without or with low content in phenolic and cyclohexadiene-like components offer modest or no protection.²⁵ The lavender essential oils shows relatively low antioxidant activity against DPPH radical and this could be attributed to the high percentage of non-phenolic monoterpenes present in the investigated essential oils. In particular, the major components of oils, such as linalool and its derivatives among the others, belong to the group of oxygenated monoterpenes with well-documented low activity against DPPH radical^{26,24}. A number of essential oils have been shown to possess several biological properties, including anti-inflammatory, detoxifying, and cancer-protecting, mainly attributed to their antioxidant activity²⁷. It was instead clarified that this arises in living systems from transcriptional induction of antioxidant enzymes such as the NAD(P)H:quinone oxidoreductase (NQO1), glutathione peroxidase (GPx), glutathione reductase (GR), thioredoxine reductase (THR), and others, through the Keap1-Nrf2-antioxidant responsive element (ARE) signaling pathway, with modulation of cellular glutathione levels²⁵.

1.3 Essential oils as potential anti-obesity agents

Obesity is defined as a condition of abnormal or excessive fat accumulation in adipose tissue, to the extent that health is impaired²⁸. In general, obesity is associated with a greater risk of disability or premature death due to type 2 diabetes mellitus (T2DM) and cardiovascular diseases (CVD) such as hypertension, stroke and coronary heart disease as well as gall bladder disease, certain cancers

(endometrial, breast, prostate, colon) and non-fatal conditions including gout, respiratory conditions, gastro-oesophageal reflux disease, osteoarthritis and infertility. Obesity also carries serious implications for psychosocial health, mainly due to societal prejudice against fatness. Several natural bioactives show different activities in the prevention of obesity or obesity-related diseases, such as inhibitors of enzymes implicated in digestion and absorption of dietary fat, inhibitors of adipogenesis and adipogenic factors and appetite suppressants, have been described^{15,29}.

The further development of such natural bioactives may potentially lead to novel strategies to maintain a healthy weight and to combat the obesity epidemic. In addition, the combination of several of these natural bioactives may result in synergistic effects leading to an improved bioavailability profile. Moreover, the simultaneous modulation of multiple different molecular targets in a dual bioactive approach has an advantage with respect to drugs specific for a single target and may result in higher efficacies with a lower dose of each individual bioactive administered.

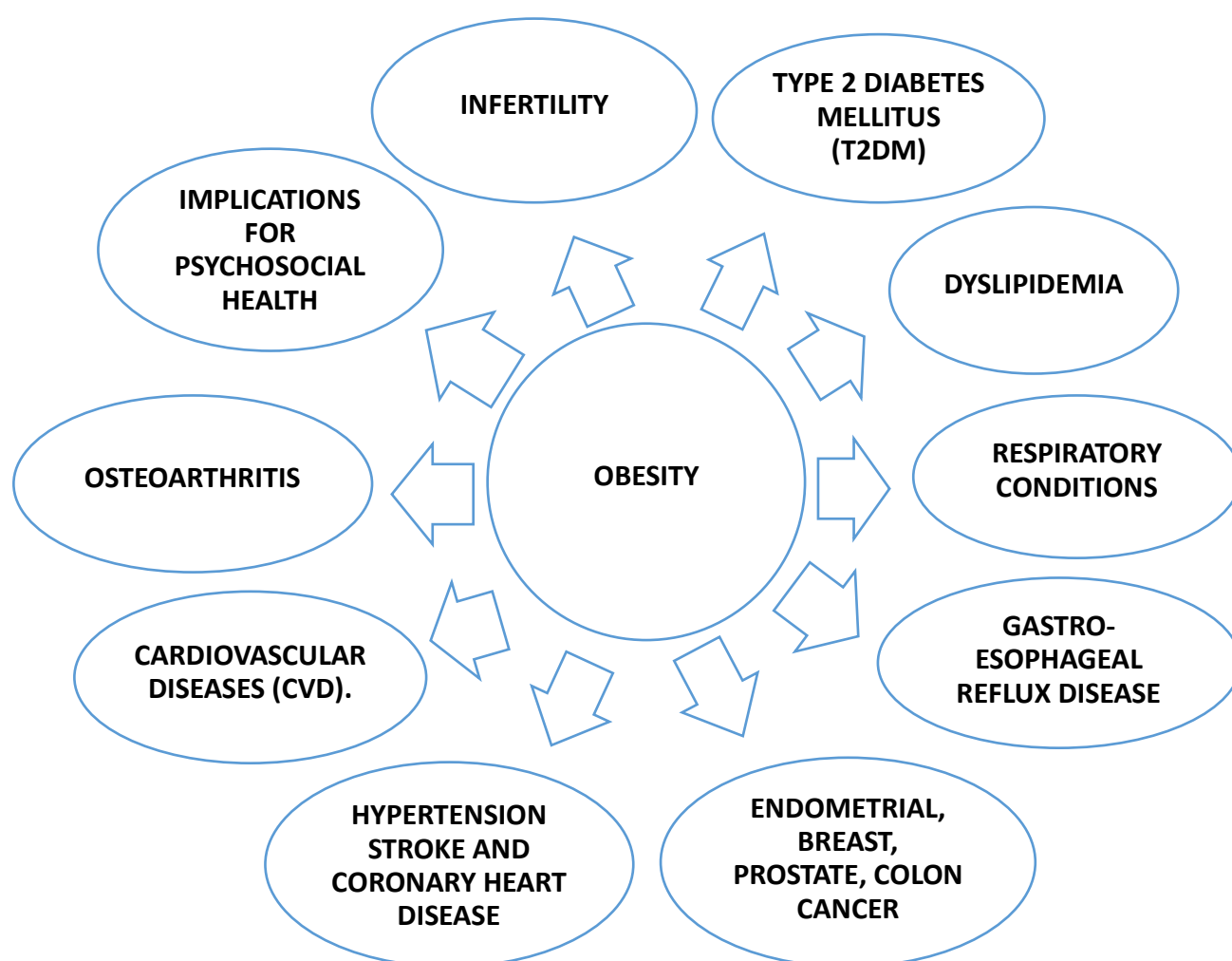


Figure 1.1 Overweight and obesity may increase the risk of many health problems, including diabetes, heart disease and certain cancers.

Several species of medicinal plants have long been used for the complementary treatment of obesity-diabetes in various systems of medicine³⁰. The essential oils have been known to have anti-obesity properties³¹. Most of the recent studies on the treatment of obesity have focused on the potential role of plants used for obesity and its metabolic disorders treatments, exerting a positive effect on lipid and glucose metabolism and anti-inflammatory activity. Terpenes and terpenoids are generally well absorbed by the body (through oral and transdermal pathways, or by inhalation) and are easily metabolized by oxidation, hydroxylation and conversion in glucuronides³². Elimination in the urine (75–95%) and faeces (<10%) is quickly observed and is usually completed after 1–3 days³³. During this time, EO components transported by the blood may exert their action in the original form or through their metabolites. It has been widely reported by the media that citrus essential oil is one the most common oil used in aromatherapy for weight loss program³¹. Among the most studied and promising essential oils for the treatment of obesity and its comorbidities are *Citrus limon*, *Rosmarinus officinalis* and *Lavandula angustifolia*. These three essential oils showing different modes of action likely correlated at the differences in the phytochemical profile. Appetite suppression, lipid metabolism regulation, and increase of energy expenditure are the main mechanisms by which anti-obesity effects are exerted. Plants represent the most studied natural source of anti-obesity bioactives. Since there are many reported studies on the medicinal use of essential oils as anti-obesity agents, it is important to further investigate the worthiness of these claims.

1.3.1 *Citrus limon*

Citrus oils are used for food and beverages and they are Generally Recognized as Safe (GRAS), being a good starting point for the use of EOs as antimicrobials within the food industry, by citrus EOs.

Citrus limon belongs to the Rutaceae family and it is the third most important Citrus species after orange and mandarin. The most commercially important citrus species are sweet oranges (*Citrus sinensis* L. Osbeck) and tangerines (*Citrus unshiu* Marc., *Citrus nobilis* Lour., *Citrus deliciosa* Ten., *Citrus reticulata* Blanco and their hybrids) (more than 80%), followed by lemons (*Citrus limon* L. Burm. f.), limes (*Citrus aurantifolia* Christm. Swing.), grapefruits (*Citrus paradisi* Macf.) and Lemon plant (*Citrus limon* L.).



Figure 1.2. *Citrus limon*

The citrus EOs are present and can be extracted from peels, flowers and from young citrus shoot, buds and leaves. EOs extracted from citrus peel contain numerous compounds of different chemical classes. These compounds can be divided into volatile fraction, which is the most representative and ranges between 85 and 99% and the non-volatile residue, containing fatty acids, sterols, carotenoids, waxes, coumarins, and polymethoxylated flavonoids (2-6% of the oil), which range between 1 and 15%³⁴.

The volatile constituents are a mixture of monoterpene (limonene) and sesquiterpene hydrocarbons and their oxygenated derivatives including aldehydes (citral), ketones, acids, alcohols (linalool) and esters³⁵. The terpenoids were suggested to be implicated in the process of bacterial inhibition. It has been reported that EOs of lemon, orange, and bergamot possessing bactericidal effect against *Campylobacter jejuni*, *E. coli* O157:H7, *L. monocytogenes*, *Bacillus cereus*, *S. aureus* and *Acrobacter butzlei*³⁶ on foods. In addition, antifungal activities against *Penicillium digitatum*, *Penicillium italicum*³⁷ have been reported. Lemon has also been shown to suppress diet-induced obesity by suppressing body weight gain and body fat accumulation.

The lemon polyphenols have been shown to be antiobesity in action by increasing beta oxidation through upregulation of mRNA level in liver and WAT³⁸.

Obesity research studies indicate that dietary lemon polyphenols extracted from lemon peel (0.5 % w/w) on high-fat diet-induced obesity in C57BL/6J mice for 12 weeks suppressed body weight gain (44 %) and body fat accumulation (36 %)³⁸. One anti-obesity mechanism reported for lemon is by up-regulation of peroxisomal β -oxidation through the increase mRNA level of acyl-CoA oxidase in the liver and white adipose tissues, which was likely mediated via up-regulation of the mRNA levels of peroxisome proliferator activated receptor- α (PPAR α)³⁸. Additionally, it has been reported that C.

limon has effects on metabolic alterations caused by obesity. Instead, high-fat diet-induced obesity in mice significantly improved hyperlipidaemia (serum triglycerides -18 % , total cholesterol -26 % , and serum free fatty acids -5 %), hyperglycaemia (insulin -65 % and faster glucose -26 %), and insulin resistance (-75 %) with respect to the obese controls³⁸.

Similarly, Naim *et al.*³⁹ reported that hexane extracts from lemon peels showed antidiabetic activity in alloxan-induced diabetic rats, detecting reduced blood glucose level of 44.6, 76.0, 95.4, and 98.1 % in 24, 48, 72, and 96 h, respectively, compared with counterpart controls. Finally, Jing *et al.*⁴⁰ showed that (R)-limonene protects against the development of dyslipidaemia and hyperglycaemia in HFD-fed mice and, in obese mice, (R)-limonene ameliorates insulin resistance and regulates lipid profiles. These effects appear to be mediated through the activation of PPAR α and the inhibition of LXR β signalling.

These data suggest that lemon essential oil could play an important role in preventing weight gain side effects and could be useful in the treatment of obesity and related diseases.

1.3.2 *Rosmarinus officinalis*

R. officinalis L., popularly known as rosemary, is a plant belonging to the family Lamiaceae and originated from the Mediterranean region. It is an evergreen perennial shrub widely used as a spice, food supplement and for cosmetic applications^{41,42}.



Figure 1.3. *Rosmarinus officinalis*

Traditionally, *R. officinalis* has been used in renal colic as an antispasmodic, with anti-nociceptive⁴³. Several studies report the effect of *R. officinalis* to promote weight loss experimented with *R. officinalis* leaf extracts administered for 50 days at doses to 200 mg/kg of body weight in mice fed a high-fat diet, which induced a significant reduction of weight and fat mass gain (64 and 57 %, respectively)⁴¹. Several phytochemicals presenting pharmacological activities may be isolated from essential oils of *R. officinalis*. The main anti-obesity activity reported for *R. officinalis* is correlated with the increase of fecal fat excretion without decreasing food intake⁴¹, suggesting that *R. officinalis* in vitro, had high affinity for pancreatic lipase and hormone sensitive lipase with inhibitory effect. These results suggest that rosemary is a good natural alternative for obesity and its metabolic alteration (table 1.2).

Table 1.2 Anti-obesity and related comorbidity effects of <i>R. officinalis</i>		
Effect	Main findings	References
Control of body weight and dyslipidaemia	<ul style="list-style-type: none"> • Inhibition of the body weight gain • Scavenging of free radical • Antioxidant action • DNA-protective effect 	44
Cardiac remodelling after myocardial infarction	<ul style="list-style-type: none"> • Attenuation of cardiac remodeling • Improvement of metabolism and reduction of oxidative stress 	45
Neuroprotective effect on cerebral ischemia	<ul style="list-style-type: none"> • Absence of dyslipidaemia effect • Reduction of acute ischemic stroke lesion 	46

Evidence suggesting an anti-inflammatory activity of the monoterpene 1.8-cineol which acts suppressing arachidonic acid metabolism and cytokine production in human monocytes⁴⁷.

R. officinalis has a promising future in the medical field, especially in the treatment and prevention of various cancers, infectious diseases and increasingly emerging diseases such as depression, Alzheimer's and Parkinson's diseases. In fact, there are 80 clinical studies on *R. officinalis*, from which 32 are still open studies⁴⁸.

1.3.3 *Lavandula angustifolia*

True lavender, also known as ordinary (*Lavandula angustifolia*, *Lavandula officinalis*, *Lavandula vera*) is an evergreen plant, belonging to the family Lamiaceae as rosemary.

In aromatherapy lavender essential oil is used to prolong the duration of sleep. In patient treat with sleeping pills associated with lavender essential oil, it has been reduced the doses ⁴⁹. Lavender also exhibits anxiolytic action ⁵⁰ and antispasmodic effect⁵¹. Health effects associated with sleep problems include decreased well-being, fatigue, anxiety, depression, cardiovascular disease, hypertension, inflammation, obesity, diabetes, and impaired glucose tolerance⁵². The treatment with lavender essential oils could be preventing the onset of obesity through indirect pathways interfering with the autonomic nervous system functions. The emotional hunger due to stress is one of the major causes for the development of wrong eating behaviours and overfeeding.



Figure 1.4. *Lavandula angustifolia*

The main component of most lavender essential oils is linalool, an acyclic monoterpene compound. Linalool is recognized by olfactory receptors and it can modulate ion channel receptor potentials, e.g. the transient receptor potential channels (TRP) and potentiate γ -aminobutanoic acid receptor A

(GABAA-receptor) response in the central nervous system⁵³. Therefore, linalool has sedative, anxiolytic and calming properties.

1.4 Mitochondria: novel therapeutic target for natural anti-obesity compounds

Alterations of mitochondrial function, dynamics, and biogenesis have been observed in various metabolic disorders, including aging, cancer, diabetes, and obesity. However, the mechanisms responsible for the mitochondrial damage and the pathways leading to metabolic disorders remain to be fully elucidated. Positive energy balance resulting from an excess of food intake, reduced energy expenditure, and dysfunction of adipose tissue biology can lead to obesity and overweight development, which is associated to an increased risk of complications such as atherosclerosis, insulin resistance, diabetes, and cancer. Current approved clinical approaches for the treatment of obesity include diet and exercise, medical therapies aimed at reducing caloric intake or absorption and bariatric surgery for extremely obese individuals. However, medical therapies are still of limited effectiveness and only a small portion of individuals following dietary and/or exercise programs maintain a long-term weight loss⁵⁴.

In the presence of excess calories, cells are programmed to actively catabolize the available calories and use the energy for cellular growth, maintenance, repair, and—in the case of proliferative cells—to replicate their DNA and divide. Thus, a prime anti-obesity rationale is to reduce food intake and increase energy expenditure. Based on the current knowledge of brown fat and muscle bioenergetics and given that mitochondria as the primary combustion machinery in cells are responsible for burning fuels (e.g., fat, glucose) for cellular energy (i.e., ATP) or thermogenesis (energy dissipation in the form of heat) four potential therapeutic approaches have been proposed:

1. increasing brown fat differentiation from progenitor cells;
2. activating brown fat thermogenesis;
3. promoting skeletal muscle thermogenesis;
4. increasing general mitochondrial uncoupling⁵⁵.

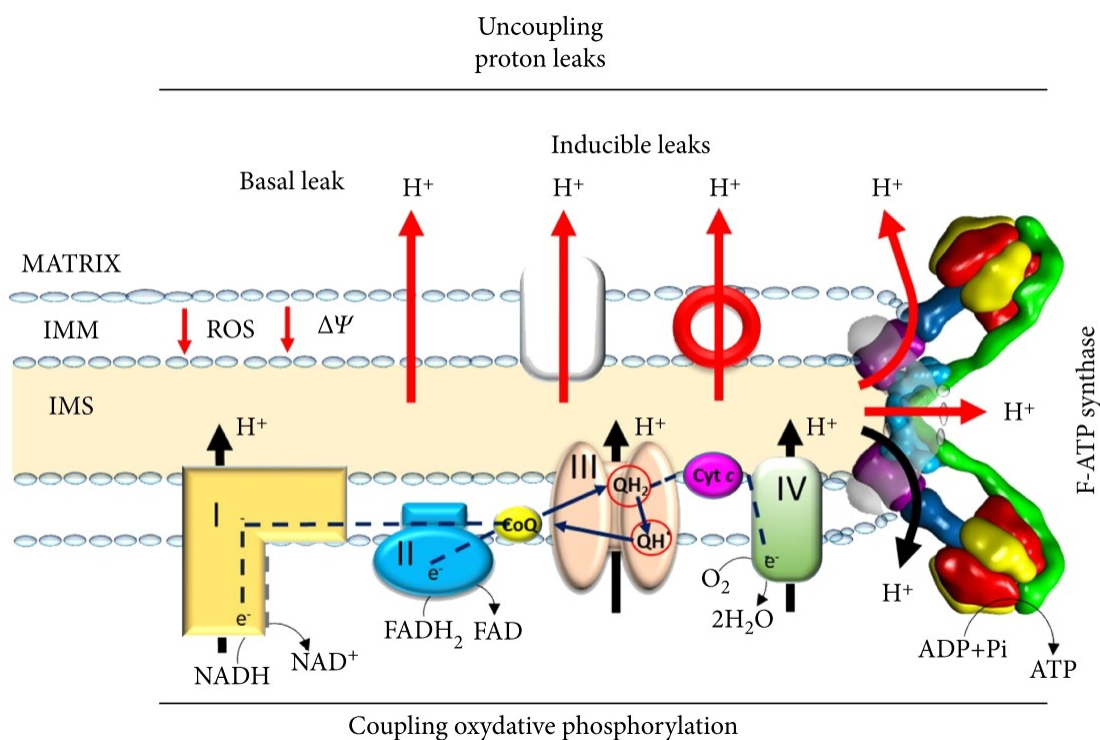


Figure 1.5: The proton circuit across the inner mitochondrial membrane. During the oxidative phosphorylation, the redox reactions of the four respiratory chain complexes are indirectly coupled to ATP synthesis by the F-ATP synthase dimers through the electrochemical proton gradient across the IMM. Return of protons into the matrix independent of ATP synthesis through the basal leak pathway, or through inducible leaks, decreases the electrochemical proton gradient and leads to mitochondrial uncoupling protecting mitochondria against ROS production. Proton back-leaks through the F_0 sector of F-ATP synthase, independent of the synthesis of ATP, lead to dissipation of the proton gradient, thus transforming F-ATP synthase into an energy-dissipating structure. Red arrows correspond to proton leaks. Figure adapted by Lippe *et al* (2019)⁵⁶.

Mitochondria are the powerhouses of cells and generate most cellular energy through the electron transport system coupled to ADP phosphorylation to ATP (figure 1.5). These organelles not only produce chemical energy in the form of ATP, but also liberate energy through a basal thermogenic uncoupling and are a major source of reactive oxygen species (ROS). In addition, mitochondria participate in intermediary metabolism and are involved in self-degradative processes such as autophagy, and in the early stages of cell death⁵⁷. Indeed, the majority of cellular oxygen (O_2) that enters into mitochondria is reduced to water by the four complexes of the respiratory chain, but a fraction can be converted to potentially cytotoxic ROS, such as superoxide anion radical ($O_2^{\cdot-}$)⁵⁸. Moreover, mitochondria are involved in maintaining the fine regulatory balance between Ca^{2+} concentration and production of ROS.

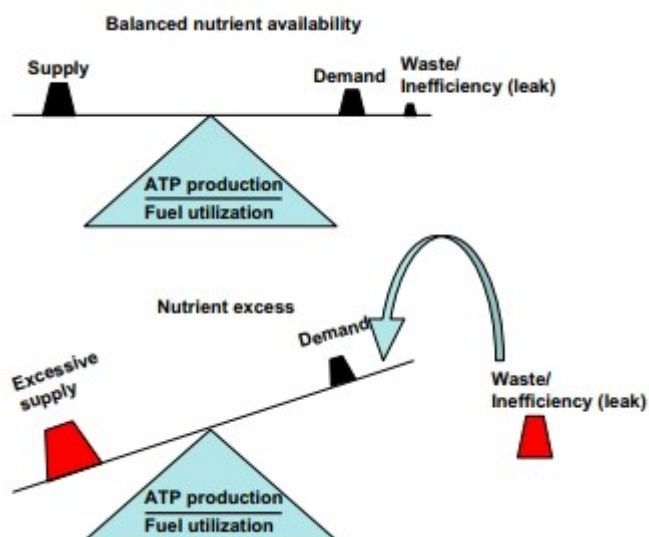


Figure 1.6: Regulation of Cellular Bioenergetic Efficiency under Conditions of Nutrient Excess.

Nutrient excess is characterized by surplus of substrate supply low energy demand. ATP demand is lower than the available energy and this is compensated by the activation of mechanism involve in dissipation of energy in form of heat. The major mechanism for inefficiency/waste in the form of heat is mitochondrial proton “leak.” This mechanism can slow down nutrient accumulation and prevent the development of reductive stress (accumulation of NADH) and ROS production. Figure adapted by Torres-Fuentes *et al* (2014)⁵⁹.

As adaptation to excess of nutrients, mitochondria utilize nutrients first for storage and then for waste like heat generation. This observation has suggested that an appropriate way to waste energy and keep healthy is reducing mitochondrial bioenergetic efficiency, i.e. reducing the ATP produced per molecule of oxidized nutrient and increasing the heat production. An approach is to uncouple the oxygen consumption by the respiratory chain from the synthesis of ATP by the FOF1ATP synthase complex by activating inducible leaks (figure 1.6), thereby dissipating the energy as heat (figure 1.6). Decreased bioenergetic efficiency may also serve as a protective mechanism inducing reduction of ROS production and an enhanced removal of excess of nutrients and of their potentially cytotoxic metabolites. On the other hand, mitochondria elongate in response to sustained deprivation of nutrients and escape from massive autophagy⁶⁰ that would ultimately be detrimental for the cell. Currently there is no approved pharmacological protocol targeting the stimulation of mitochondrial energy expenditure, making the use of herbal medicines an attractive alternative⁶¹.

During physical activity at higher intensity, the body, particularly the skeletal muscles and the cardiovascular system, requires an extra supply of energy, thus stimulating mitochondrial energy expenditure. Mitochondria, which are the main energy suppliers and coordinate ATP production, reactive oxygen species (ROS) production, and calcium signalling, are indeed fundamental for sustaining body activity during exercise⁶².

An important emerging area in the translational aspects of redox biology and bioenergetics are the new findings that implicate mitochondrial dysfunction as a key player in the etiology of metabolic syndrome and diabetes. Metabolic syndrome is a group of metabolic abnormalities which are associated with high blood sugar, elevated blood pressure, excess body fat, and abnormal cholesterol levels, which can ultimately lead to plethora of diseases, such as diabetes, obesity, cardiovascular disease and cancer⁶³. The progression to heart failure is associated with a gradual but progressive decline in the activity of mitochondrial respiratory pathways leading to diminished capacity for ATP production⁶⁴. Energy deficiency can be a cause and an effect of heart failure. Many of the mitochondrial metabolic regulatory events that dictate fuel selection and capacity for ATP production in the normal and failing heart occur at the level of gene expression. On the other hand, decrease in the capacity to transduce energy leads to dysregulation of cellular processes which damage the cardiac pump function, including Ca²⁺ handling and contractile function. This results in an increased energy demand and diminished function⁶⁴.

1.5 Mitochondrial uncouplers such a promising starting point for designing safer molecules for obesity therapy.

Mitochondria have long been known to be sensitive to many compounds and are also known to play important role in drug metabolism and are thus important targets for drug development⁶⁵.

Mitochondrial uncouplers are compounds that impair mitochondrial substrate oxidation from ATP synthesis inducing mitochondrial dysfunction and leading to decreases in ATP levels.

Even normal mitochondrial function, however, is accompanied by unavoidable proton and electron leaks within mitochondria.

Two type of uncoupling mechanism are possible:

1. Chemical uncoupling, characteristic of lipophilic weak acids that picks up protons from the cytosol, diffuse across the mitochondrial inner membrane into the matrix, thus abolishing the membrane potential.
2. Uncoupling mediated by activation of specific proteins such as the uncoupling proteins (UCPs) and the adenine nucleotide translocase (ANT).

Several lipophilic weak acid uncouplers are known, among them 2,4-dinitrophenol (DNP) has been used for weight control in human during last century⁵⁶. DNP, at 3–5 mg/kg led to a 20–30% increase in energy expenditure, apparently without causing adverse effects but, its use has been abandoned for its narrow therapeutic window and serious adverse effects related to overdose. On the other hand, if

safety can be proven, this type of mitochondrial uncoupling may represent a potential therapeutic approach.

The second type of uncoupling is actually more attractive. Physiological uncoupling is typically mediated in mammals by the finely regulated uncoupling protein 1 (UCP1), an integral membrane protein of the brown adipose tissue (BAT) that mediates the leak of protons across the IMM, dissipating the proton gradient and inducing heat production⁶⁶. Physiological uncoupling also enables fine-tuning of insulin secretion by uncoupling protein 2 (UCP2) in pancreatic β cells⁶⁷ and regulation of fatty acid metabolism by uncoupling protein 3 (UCP3), which is specific to skeletal muscle, BAT, and heart, although UCP2 and UCP3 functions are not clearly established⁶⁸. Among the molecules till now identified there are lipophilic acids, which activate proton cycling through a high-affinity interaction with the mitochondrial ANT that causes significant but limited uncoupling at extremely low concentrations, providing a promising starting point for designing safer uncouplers for obesity therapy⁶⁹. For example, benzoic acid uncouples only through the ANT, explaining its ability to cause only mild uncoupling over a wide range of concentrations. The properties of benzoic acid that allow it to uncouple only through the translocase could be exploited, and it could be used as a starting compound for the design of molecules that have the desired affinity and V_{\max} for the translocase with no further uncoupling at higher concentrations⁶⁹.

Uncoupling at the translocase is also caused by substituted triphenylphosphonium molecules, which are not anion and cannot protonate⁶⁹. Recently, there is an increased interest in the use of essential oils as preventive and therapeutic agents for treatment of various diseases, including obesity²⁹ but the molecular targets are still to be defined in the majority of cases¹⁵. For example, thymol, chemically known as 2-isopropyl-5-methylphenol is a colorless crystalline monoterpene phenol. It is one of the most important dietary constituents in thyme species. For centuries, it has been used in traditional medicine and has been shown to possess various pharmacological properties including antioxidant, free radical scavenging, anti-inflammatory, analgesic, antispasmodic, antibacterial, antifungal, antiseptic and antitumor activities. The protective effects of thymol in metabolic disorders such as diabetes mellitus and obesity are proposed (figure 1.7).

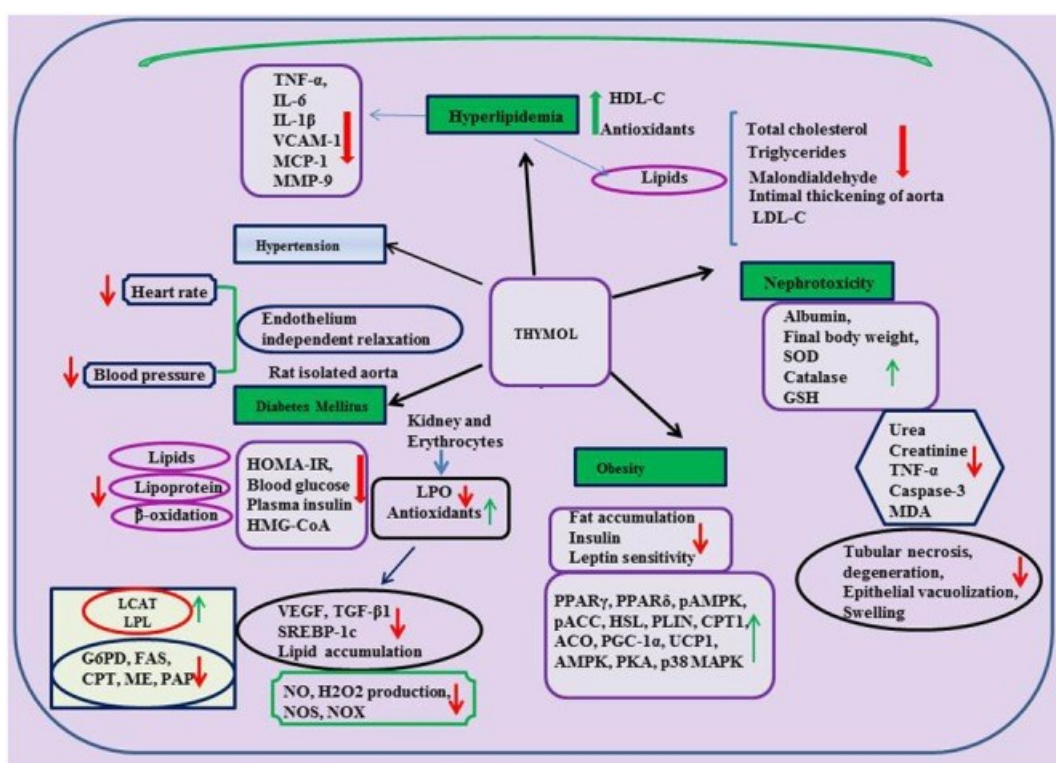


Figure 1.7: The protective effects of thymol in metabolic disorders such as diabetes mellitus and obesity. Figure adapted by Nagoor Meeran *et al* (2017)⁷⁰

Thymol (30 mg/kg) was shown to inhibit the accumulation of visceral fats, enhance insulin and leptin sensitivity and improve lipid lowering action as well as augment antioxidant status in HFD-induced obesity in murine models⁷¹. Thymol (20 μ M) has been shown to promote the biogenesis of mitochondria in the browning of white adipocytes (3T3-L1 white adipocytes) by increasing the expression of peroxisome proliferator activated receptor- γ (PPAR γ), peroxisome proliferator activated receptor- δ (PPAR δ), phospho AMP-activated protein kinase (pAMPK), uncoupling protein 1 (UCP1).

Uncoupling of F-ATP synthase is another important example of uncoupling mediated by activation of specific proteins and has been associated with a variety of pathological conditions, such as cardiovascular⁷² and neurodegenerative diseases⁷³, obesity and type 2 diabetes⁷⁴, and cancer⁷⁵. In the mitochondrial energy-converting membranes, F-ATP synthase catalyses the aerobic synthesis of ATP by using the proton gradient across the inner mitochondrial membrane. The complex is of about 600 kDa and consists of a roughly globular, water-soluble F₁ head, composed of three $\alpha\beta$ dimers, and a membrane-embedded F₀ subcomplex comprising the a subunit, a ring of multiple c subunits and a subcomplex of 5 conserved proteins b , $A6L$, f , g , e . These moieties are connected by two stalks: the lateral or peripheral stalk, which is structurally part of the F₀ moiety and is formed by the OSCP, b C-terminal, F6, and d subunits., and the central stalk, which is associated to the F₁ sector and comprises the γ , δ and ϵ subunits⁷⁶. All types of F-ATP synthases function as nanometer-scale rotary

machines consisting of two motors linked by a rotor, which comprises the *c*-ring and the central stalk. The motor located in the F_0 sector generates movement of the rotor at a rate of ~ 100 revolutions/s by consuming the proton motive force; the other, located in the F_1 moiety, uses energy transmitted by the rotor to synthesize ATP. The synthetic motor can work in reverse, driving the rotor backward with energy from ATP, releasing ADP and phosphate, and generating a membrane potential⁷⁷.

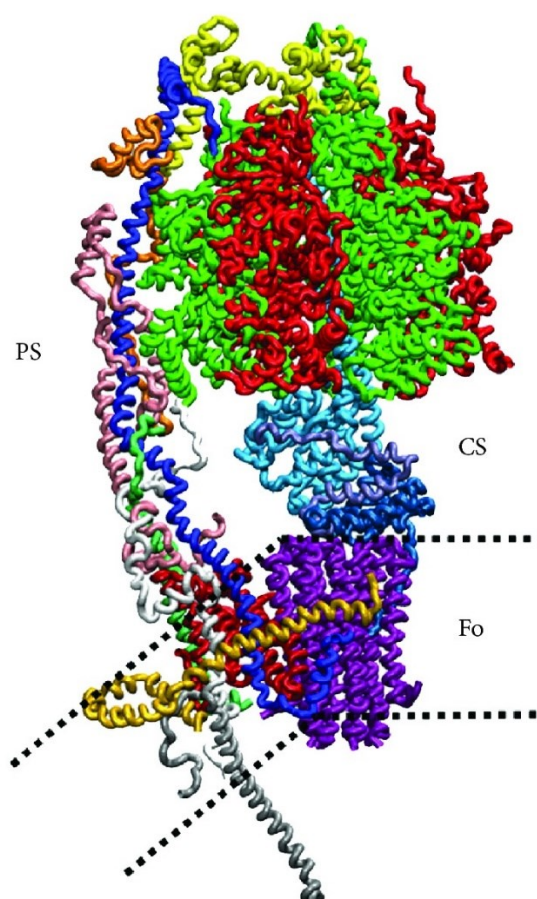


Figure 1.8 Structure of the mitochondrial F-ATP synthase. Subunits are shown in colors as follows. F1 is shown with the alternating α (green) and β subunits (red). On the left, the peripheral stalk (PS) includes the OSCP (yellow), b (dark blue), F6 (orange), and d (pink) subunits. The central stalk (CS) connecting the $\alpha 3\beta 3$ subcomplex to the *c*-ring composed of 8 identical subunit *c* (purple) includes the γ (cyan), δ (blue), and ϵ (ice blue) subunits. The F_0 membrane sector includes the subunits a (dark red, mostly covered in the picture by other subunits), f (white), A6L (emerald, mostly covered by other subunits), g (light orange), and e (silver). In the membrane region, which is delineated by dotted lines, the subunits e and g, with the N-terminal part of subunit b, create a subdomain that bends the inner mitochondrial membrane. Figure adapted by Lippe *et al* (2019)⁵⁶.

This F_0 - F_1 coupling is disrupted by the antibiotic oligomycin, which binds the *c*-ring, preventing it to rotate in either directions⁷⁸. Malfunction of F-ATP synthase including its, has been associated with a variety of pathological conditions^{73,76}. Despite such important evidence, F-ATP synthase has only recently been used as an effective drug target for disease conditions and for the regulation of energy

metabolism, despite the fact that more than 300 natural and synthetic molecules are known to bind and inhibit this complex⁵⁶.

Moreover, the recent finding that F-ATP synthase is involved in formation of the permeability transition pore (PTP) may make this complex a viable target for future therapy in a variety of diseases⁵⁷. PTP is indeed a high conductance pore whose opening leads to the collapse of the mitochondrial proton gradient, with the consequent drop in ATP synthesis, and to induction of apoptosis⁵⁷.

Attention has recently focused on dietary phytochemicals with antimicrobial properties, a variety of which inhibits the bacterial F-ATP synthase to a variable degree depending on the type and positioning of the functional groups⁵⁶. Both F1 and FO subunits have been identified as contributing to the binding sites for such inhibitors⁷⁹. Because dietary phytochemicals also inhibit the mitochondrial F-ATP synthase, attempts are being made to modify the functional groups of these compounds, making them more potent and selective inhibitors of the bacterial ATP synthases⁸⁰. On the other hand, many of these phytochemicals exhibit diverse activities, such as antioxidant, anticancerogenic, and antiobesity actions⁸⁰. The essential oils component D-limonene, which is proposed as a potential chemotherapeutic agent against pancreatic cancer and breast cancer⁸¹ and induces apoptosis via the mitochondrial death pathway in several human cell lines⁸², has also anti-obesity activity, inducing browning in white adipocytes⁸³. Interestingly, the essential oils component *p*-cymene directly induces a mild uncoupling of F-ATP synthase, leading to a proton leak through the FO moiety that decreases mitochondrial bioenergetics⁸⁴.

CHAPTER 2

OBJECTIVES OF THESIS WORK

General objectives

The aim of this doctoral thesis was to assess the effects of fresh and preserved samples of three essential oils, *Citrus limon*, *Rosmarinus officinalis* and *Lavandula angustifolia* and their main compounds on mitochondrial functions, with particular regard to the antioxidant effects in vitro and the ability to generate a mild uncoupling without affording loss in mitochondrial function. The goal was also the identification of novel bioactives from natural resources with potential anti-obesity effects in order to contribute to the development of therapeutics and functional foods that can be used in the prevention and/or treatment of obesity.

Specific objectives

- Phytochemical characterization of fresh and preserved samples of *Citrus limon*, *Rosmarinus officinalis* and *Lavandula angustifolia* via GC-MS analysis.
- Structural identification of main component via one dimensional NMR analysis
- Evaluation of antioxidant property of selected essential oils and chief components via DPPH and ABTS radical scavenging assay
- Evaluation of the effects of fresh and preserved essential oils and their chief components on the oxygen consumption and the FOF1ATP synthase activity of isolated mitochondria.
- Evaluation of the Ca²⁺ retention capacity (CRC) of HeLa cells in the presence of (S)-limonene or (R)-limonene.

CHAPTER 3

MATERIALS AND METHODS

3.1) Chemical characterization of essential oils

3.1.1) Raw materials

Certified organic essential oil of *Citrus limon*, *Rosmarinus officinalis* and *Lavandula angustifolia* of two different lots were acquired from Flora s.r.l. Italian certified companies. The essential oils can be ingested and are therefore suitable for food flavouring.

1) Lemon from *Citrus limon* c.t. limonene (lot 150758, 2015; lot 182284, 2018); Extract by cold pressing from the peel of the fruit; origin: Italy / Argentina;

(2) Lavender from *Lavandula angustifolia* c.t. linalool (lot 150251, 2015; lot 180192, 2018); Extract by steam distillation from flowers; origin: Italy / Bulgaria.

(3) Rosemary from *Rosmarinus officinalis* c.t. cineol (lot 151629, 2015; lot 182327, 2018) Extract by steam distillation from the whole flowering plant.; origin: Tunisia.

(4) Limonene derivatives: perillyl alcohol and perillic acid

The essential oils purchased with the 2015 lots were preserved for two years at 4 °C and in dark.

3.1.2 Enzymes, cells and chemical compounds

Enzymes, terpenes, terpenoides used in the assay procedures were purchased from Sigma Aldrich, Italia. HeLa cells were obtained from the American Tissue Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Basel, Switzerland) supplemented with fetal calf serum (10%), glutamine (4 mM), and penicillin and streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). Cells were free of contamination with mycoplasma.

3.1.3 GC-MS analyses

GC-MS analyses of *L. angustifolia* (lavender), *R. officinalis* (Rosemary) and *C. limon* (Lemon) essential oils were carried out using a Trace GC Ultra gas chromatograph equipped with a flame ionization detector on a SLB-5 MS (Supelco, Sigma-Aldrich) 30 m × 0.25 mm i.d., 0.1 µm film thickness and coupled to an ion-trap mass spectrometer (ITMS) detector Polaris Q (Thermo Scientific) with a split-splitless injector. According to the method reported by Sparkman *et al.*⁸⁵ with same modifications, the oil was introduced in the chromatograph through direct injection of a dilution 1: 10000 in organic solvent acetone⁸⁶. The oven temperature was programmed to rise from 60 °C to 250 °C at 3 °C/min, then hold at 240 °C for 20 min. Injector and detector temperatures were

maintained at 220 °C and 280 °C, respectively. The oil samples were dissolved in acetone (1:10000) and 1 µL aliquots were injected in split mode with split ratio of 1:10 using helium as the carrier gas (1 mL/min). Each peak was carefully investigated across its profile to establish its homogeneity. The individual constituents were identified by the retention indices of the compounds known from literature data⁸⁵ and also by comparing their mass spectra with those of the known compounds or those available from the mass spectral library (NIST). Furthermore, each recorded spectrum was carefully examined to establish the nature of the eluted component. The relative amounts of the components were calculated based on GC peak areas without correction factors. The mass spectra were recorded under electron impact at 70 eV electron energy with a mass range from m/z 50 to 1000 and a scan rate of 0.8 scan sec^{-1} . Whenever possible, to ensure definitive positive identification, the GC retention time of the components were checked against the retention time of an authentic specimen obtained from commercial sources or prepared synthetically. The percent of essential oils constituents was determined by integration of the peak area and quantitative determination was based on the total ion count detected by GC-MS.

3.1.4 Nuclear magnetic Resonance (NMR) analyses

NMR spectra have been recorded with a Nuclear Magnetic Resonance Spectrometer Bruker 400 Avance III HD (^1H at 400MHz and ^{13}C at 101 MHz), software Bruker TopSpin, δ (ppm), J in Hz and the residual CDCl_3 solvent signal has been used as reference ($\delta = 7.28$ in ^1H and $\delta = 77.0$ in ^{13}C). Spectra analysis was processed with MestReNova® spectral data analysing software and was compared with the data reported in Spectral Database for Organic Compounds (SDBS), an integrated spectral database system, to confirm the results for organic compounds.

3.1.5 Radical scavenging activity

3.1.5-a 2,2-Diphenyl-1-picrylhydrazyl free radical-scavenging capacity (DPPH assay)

The 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity (DPPH) was performed following the method previously reported⁸⁷ with same modifications. Briefly, methanol solutions of the essential oil of lemon, rosemary, lavender (10, 30, 60, 100 and 150 ppm) and their major terpenes and tepenoides (limonene, linalool, 1,8 cineole, perillic acid, perillyl alcohol) at concentration of 10, 30, 60, 75 ppm have been prepared. Then, 100 µL of solution was mixed with 900 µL of freshly made DPPH solution (100 µM methanol). The absorbance of the mixture was recorded at 517 nm after incubation for 30 minutes, in the dark and at room temperature, with a ultraviolet-visible Shimadzu UV-VIS-2501PC spectrophotometer. DPPH is a stable free radical and has a dark violet colour. It has

a maximum absorption at 517 nm and the peak of the DPPH radical is decreased in the presence of a hydrogen donor that acts as a free radical-scavenging antioxidant. Thus, we investigated the free radical-scavenging effects of essential oils by evaluating the decrease in the peak height of the DPPH radical at 517 nm. Initial absorbance readings for DPPH, used as control, were 0.96 ± 0.05 . Antioxidant activity was expressed as percentage of inhibition, calculated by the following equation:

$$\text{DPPH} \cdot \text{ Radical scavenging capacity (\%)} = (1 - \text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}}) \times 100$$

Where A_{sample} is the absorbance of DPPH mixed with the sample and A_{control} is the absorbance of DPPH in which the sample was replaced with methanol. Butylated hydroxytoluene (BHT, 10, 30, 60, 100 and 150 ppm) was used as positive control.

3.1.5-b 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity (ABTS assay)

The ABTS assay was measured according to the protocol⁸⁸ reported, with slight modifications. The ABTS radical solution was allowed to stand for 12-16 hours in the dark, at room temperature, to produce the ABTS radical proton. ABTS working solution was prepared by mixing equal volume of 7 mM ABTS-water solution and 140 mM water-potassium persulfate. Immediately before the analysis, the ABTS working solution was diluted in ethanol obtaining an absorbance of 0.70 ± 0.025 at 734 nm (control). After that, 1000 μL of diluted ABTS radical solution was mixed with 10 μL of different concentrations of essential oil solutions. After 5 minutes incubation, the absorbance was read at 737 nm with UV/VIS spectrophotometer. The free radical-scavenging activity was expressed as percentage of inhibition and calculated as reported for the DPPH method.

$$\text{ABTS} \cdot \text{ Radical scavenging capacity (\%)} = (1 - (\text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}})) \times 100$$

Where A_{sample} is the absorbance of remaining ABTS in the presence of the sample and A_{control} is the initial absorbance of diluted solution of ABTS. Butylated hydroxytoluene (BHT, 10, 30, 60, 100 and 150 ppm) was used as positive control.

3.2 Functional assessment of isolated beef heart mitochondria in vitro

3.2.1 Isolation of mitochondria from beef heart

Mitochondria were isolated according to the method reported by P. Constantini *et al* (1995)⁸⁹. Briefly, the heart was taken from young beef (9 months older) immediately after slaughter. It was kept on ice during the transport and processed within 1 hour. 2 L of fresh ice-cold mitochondrial isolation solution (MIS) buffer was prepared using 250 mM sucrose, 10 mM Tris-HCl pH 7.4 and 0.1 mM ethylene glycol bis (2-aminoethyl) tetra-acetic acid (EGTA) and used during all steps. The ventricular tissue was cut to small pieces which were carefully trimmed with removal of fat, ligaments and connective tissue. After that it was passed in mincer, blended in mixer at medium speed for three time and blotted with gauze to eliminate residual fat particles. After adjusting pH to 7.6 with 2 M Tris-base (hydroxymethyl)aminomethane, the homogenate was centrifuged at 700 x g for 10 minutes (4 °C) to remove unbroken cells and nuclei. The supernatant containing mitochondria and other organelles was centrifuged at 7000 x g for 10 min in a RC2B refrigerated centrifuge kept at 4 °C. The mitochondrial pellet was carefully suspended in ice-cold MIS buffer and spun at 9.000 x g for 5 minutes at 4 °C. The mitochondrial pellet was suspended in buffer MIS to give a protein concentration of about 50 mg/mL and immediately used for functional analyses or stored at -80 °C when used for preparing the sub-mitochondrial particles.

3.2.2 Preparation of submitochondrial particles (MgATP-SMP)

Ultrasonic disintegration of mitochondria produces inverted submitochondrial particles, in which the substrate binding sites for both the ATP synthase and the respiratory chain are on the outside. Different types of submitochondrial particles can be prepared depending on the medium used during the ultrasonic disintegration. In the MgATP-SMP the interaction between the F1 and the FO domains of the FoF1ATP synthase complex is well coupled⁹⁰. Mitochondria stored at -80 °C were thawed under cold tap water and suspended at 15 mg/mL with the sonication buffer (SB) composed of 250 mM sucrose, 10 mM Tris/HC, 1 mM succinate and 0.2 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.4). MgCl₂ and ATP were added to reach a final concentration of 15 mM and 1 mM respectively. Aliquots of 3 mL were exposed to sonic oscillation for four 30 sec cycles separated by 30 sec intervals in a Labsonic oscillator. The entire treated batch was centrifuged at 19.000 x g for 20 min at 4 °C, followed by a second centrifugation of the supernatant for 30 m at 100.000 x g. The pellet was suspended in a small amount of 0,25 M Sucrose, 10 mM Tris/HCl, pH 7.3 and the protein content was determined according to the Lowry method. Typically, the yield of particles was 17-22% of the mitochondrial protein⁹⁰.

3.2.3 Protein quantification

The Lowry method⁹¹ is particularly suitable for membrane protein estimation thanks to the presence of a detergent. The method consists of two steps:

1. The starting interaction between the proteins and cuprous ions in a basic solution;
2. The reduction of phosphotungstic and phosphomolybdic acid to blue tungstate and blue molybdate by the complex Cu-proteins and by tryptophan and tyrosine residues present in the proteins.

The reaction is followed spectrophotometrically at 750 nm. Aliquots of mitochondria or submitochondrial particles were suspended in 0.2 mL of 10% (w/v) sodium deoxycolate (Na-DOC) solution in 0.01N NaOH and shaken. Then, 1 mL of reagent A (50 parts of 2% (w/v) Na₂CO₃ in 0.1 N NaOH and 1 part of 0.5% (w/v) CuSO₄ 4H₂O in 1% (w/v) of Na-K tartrate) were added. After an incubation of 10 min at 25 °C, 50 µL of reactive B (1:1 Folin-Ciocalteu and H₂O) were added and the samples were shaken. The following incubation was carried on for 30 min at room temperature and then the absorbance at 750 nm was measured. 2-64 µg of bovine serum albumin (BSA) was used to establish standard calibration curves for each assay.

3.2.4 Samples preparation for use in biological assays

For all biological assay, the samples have been prepared in five different concentrations with dimethyl sulfoxide (DMSO) as vehicle. The concentrations used for essential oils were 10, 30, 60, 100 and 150 ppm instead for terpenes and terpenoides were 10, 30, 60, 100 ppm.

3.2.5 Measuring mitochondrial respiration

The measure of mitochondrial respiration was evaluated according the Petronilli *et al*⁹² protocol with minimal modifications. A 8 mL oxygraph (Yellow Springs Instrument (YSI), Oxygraph Model 5300) chamber was thermostatically controlled and maintained at 30 °C with a bath assembly. Before each experiment, the chamber was washed with 70% ethanol, rinsed 3 times with distilled water, then filled with measuring respiration medium (MRM) containing 250 mM sucrose, 50 mM Hepes, 10 mM KCl, 3 mM KH₂PO₄, 1.5 mM MgCl₂, 1 mM EGTA, 0.5 mg/mL BSA (pH 7.4). The chamber was allowed to equilibrate with an ambient gas phase at 37 °C with a stirrer speed of 750 rpm for 3 minutes to allow air saturation of the MRM to 398 nmol O₂/mL of dissolved oxygen. Intact beef heart mitochondria (mitochondrial that retains structure and respiratory functions^{93,94}) suspension containing approximately 0.5 mg mitochondrial protein is added to 2 mL of MRM and the oxygen

consumption rate was measured using a polarographic oxygen electrode (Yellow Springs Instrument (YSI), Oxygraph Model 5300). During assay reagents were added to the chamber, through access slot on the plunger using Hamilton Microliter removable needle syringes. Oxygraph was connected with serial cable to a voltage-input data logger (Picolog ADC-20/24, Pico Technology) and a computer serial port. Control experiments were performed in the presence of 0.3% DMSO. Oligomycin was added when state 4 of mitochondrial respiration (the rate after ADP is phosphorylated) begun and state O (state 4 inhibited with oligomycin) was measured. FCCP stimulated respiration was induced with the addition of 2 μ M FCCP added 2 minutes after mitochondria energization⁸⁴.

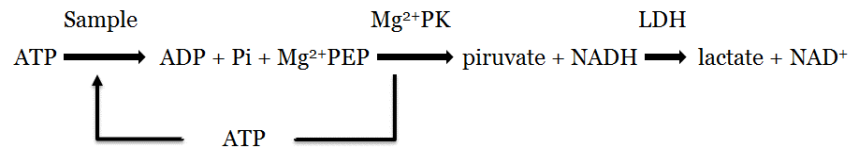
A protocol involving serial additions of various substrates, inhibitors, and uncouplers allowed the assessment of mitochondrial function, stepwise as described below:

1. *Equilibration with ambient oxygen.* After addition of MRM the oxygen concentration was measured while in equilibration with a gas phase for air saturation.
2. *Baseline respiration.* 0.5 mg/mL of fresh isolated beef heart mitochondria was added to the chamber and respiration was measured in the absence of exogenous substrates.
3. *State 2 respiration (state 2; V_2).* The complex I substrate glutamate-malate, respectively 5 and 2.5 mM, was injected in the chamber and the oxygen consumption was recorded.
4. *State 3 respiration (State 3; V_3)* After addition of 250 μ M ADP state 3 respiration was induced, i.e. the oxygen used for the synthesis of ATP from ADP.
5. *State 4 respiration.* When all ADP was phosphorylated, mitochondria returned to state 4 respiration.
6. *State O respiration (State O; V_o).* The mitochondria sample was added to 2.5 μ M oligomycin which inhibited respiration by blocking the F_o ATPsynthase proton channel and effectively eliminating ATP synthesis. The residual oxygen consumption in the absence of ADP phosphorylation is attributable to proton leak across the inner mitochondrial
7. *Uncoupled respiration (State FCCP; V_{FCCP}).* Finally mitochondria were added with 2.5 μ M carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) to induce an uncoupled state by dissipating the proton gradient across the inner mitochondrial membrane.

Respiration rates was normalized to the amount of mitochondrial protein to allow comparison across groups and mitochondrial respiration rate was expressed in % change % change of the activity respect the control (100%).

3.2.6 Measurement of ATP hydrolysis

The ATP hydrolysis rate was determined using an ATP regenerating system as shown in the scheme 95.



the mitochondrial samples are added to the assay buffer, the decrease of absorbance due to NADH oxidation is directly proportional to the amount of hydrolysed ATP. As ATP is continuously regenerated, the rate of ATP hydrolysis remains constant over time.

The assay buffer consists of 30 mM sucrose, 50 mM Tris/HCl pH 7.4, 50 mM KCl, 2 mM EGTA, 4 mM MgCl₂ and 2mM phospho-enol pyruvate (PEP), 2 mM ATP/Tris pH 7.4, 0.3 mM NADH; 4 U/mL pyruvate kinase (PK) plus 3 U/mL lactate dehydrogenase (LDH) ⁹⁶.

The assay was carried out at 30 °C adding appropriate aliquots of beef heart mitochondria or MgATP-SMP to a final volume of 1 mL using a plastic cuvette. In the case of samples containing mitochondria 10 μM alamethicin was also added.

The decrease in absorbance of NADH at 340 nm was measured over a period of 3-5 min. The specific activity in μmol/min/mg of protein was calculated using the following equation:

$$\text{ATPase activity} = \frac{\Delta A_{340}/\text{min}}{\epsilon \times l \times c}$$

ΔA₃₄₀/min: decrement in the absorbance measured after interval of 1 minute.

ε: molar extinction coefficient of NADH at 340 nm 6.22 x 10³ M⁻¹ cm⁻¹

c: protein concentration express as mg/mL

3.2.7 Calcium retention capacity (CRC) assay

Calcium retention capacity (CRC) assay of permeabilized HeLa cells was performed according to V. Giorgio et al⁷⁷ with Calcium-Green® 5 N.

HeLa cells (2.5 × 10⁶ cells/ml, harvested by trypsinization) were suspended in high potassium buffer composed of 130 mM KCl, 1 mM K₂HPO₄, 10 mM Tris-MOPS, 10 μM EGTA, 5 mM succinate, pH adjusted to 7.4 with KOH. 30 μM digitonin and 0.30 μM Calcium-Green® 5 N were added to the

samples as permeabilizing agent and Calcium indicator, respectively. After that digitonin-treated HeLa cells were exposed to exogenously added 2.5 μM CaCl_2 pulses repeated after 30 s for 6 minutes. Indirect measurement of mitochondrial Ca^{2+} uptake was recorded using a Fluoroskan Ascent FL (Thermo Electron, Waltham, MA, USA) plate reader. When indicated, 50, 75, 150 μM (R)-limonene or (S)-limonene were added to permeabilized HeLa cells.

CHAPTER 3

RESULTS

4.1 Chemical composition

The technique currently widely used for the analysis of volatile and semi-volatile compounds in essential oils is the gas chromatography coupled to mass spectrometry (GC-MS). Phytochemical profile, determined and confirmed through NMR, showed an interesting compositional variability of essential oils. Dilutions of three fresh and three preserved essential oils of *Citrus limon*, *Rosmarinus officinalis* and *Lavandula angustifolia* were analysed to identify their major components. The results of GC-MS analysis study were reported in tables below and, the identified compounds, were listed according to their retention index on SLB 5 MS column.

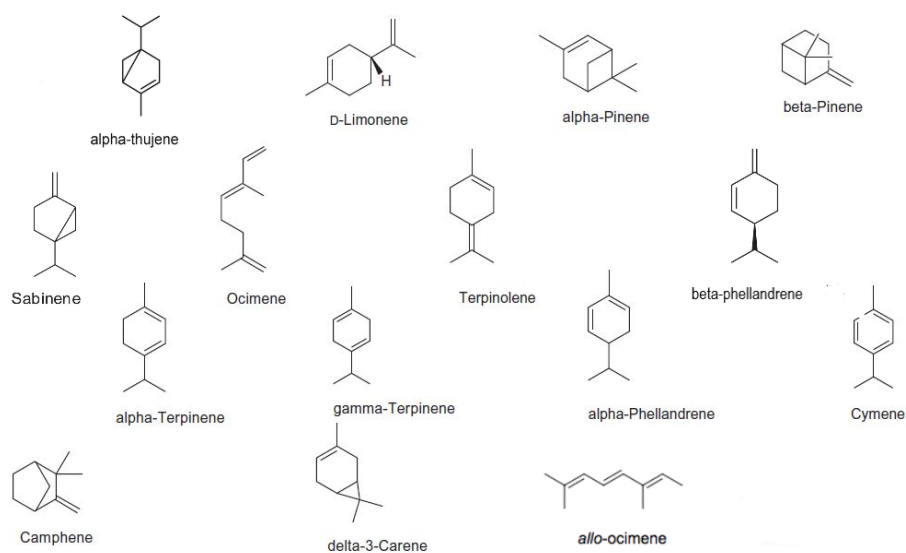
The GC-MS analysis has allowed identified 107 different total compounds: 18% were monoterpenes, 36% were sesquiterpenes and the other were t, named terpenoids, such as alcohols, aldehydes, ketones and acids. Monoterpene hydrocarbons and oxygenated monoterpene alcohols also tend to be among the most copious groups.

The essentials oils of *R. officinalis*, *C. limon* and *L. angustifolia* had showed a higher concentration of a few components that characterize the plant chemotype. The derivatives 1.8-cineol, limonene, linalool and linalyl acetate were found to be the main components in *R. officinalis* ct 1.8-cineol, *C. limon* ct limonene and *L. Angustifolia* ct linalool, respectively. The latter in particular showed a rather similar concentration of two terpenoides, linalool and linalyl acetate, in both fresh and preserved sample.

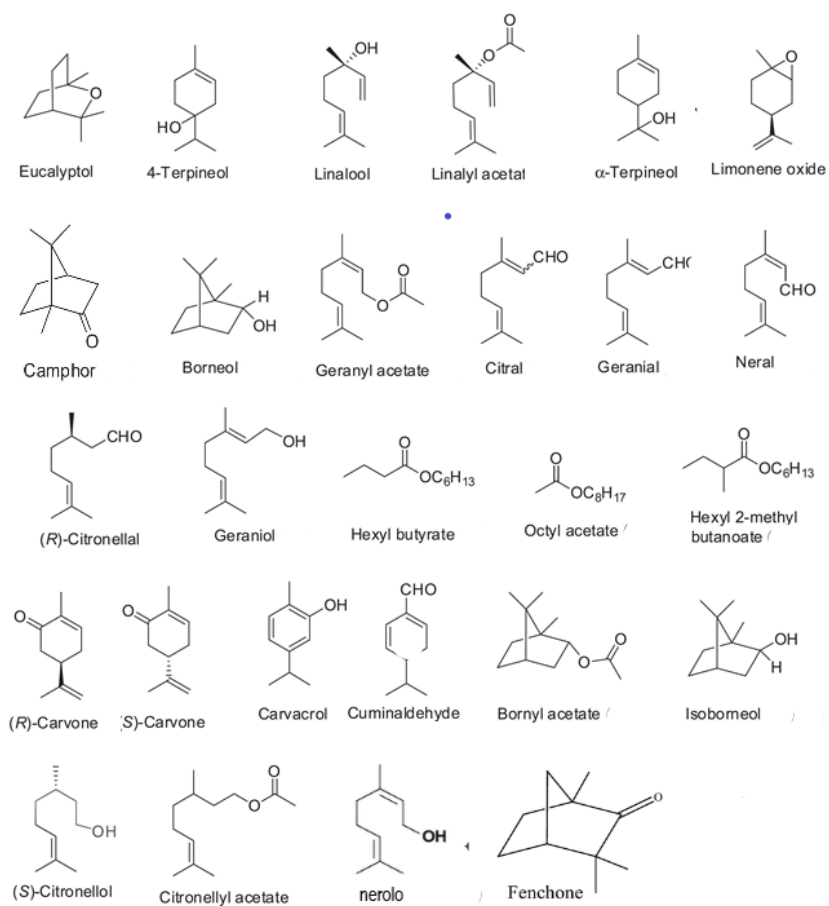
Rosemary EO showed the major variability with 22 different molecules, with respect to the 13 compounds found in lemon and 10 in lavender EO.

Monoterpenes and oxygenate derivatives, such as α -thujene, α -pinene, camphene, sabinene, p-cimene and camphor, were founded into all essential oils. Lemon essential oil presented around 85% of terpenes hydrocarbons. α -Pinene, camphene and p-cymene were preponderant in rosemary EO and particularly in preserved products with a concentration of 14.0, 4.02 and 3.0%, respectively. Conversely, both the samples of fresh and preserved lemon EO showed a high concentration of sabinene, 8.3 and 8.4%, respectively. Lavender EO showed a low presence of sesquiterpenes, with a concentration of β -caryophyllene of 3.2% in the fresh oil, while in preserved lavender Eo. and also in lemon samples β -caryophyllene was present only in small concentration (0.9% and 0.2% respectively). Finally, the fresh and preserved Rosemary eo. gave 2.8 and 1.9% of β -caryophyllene, respectively.

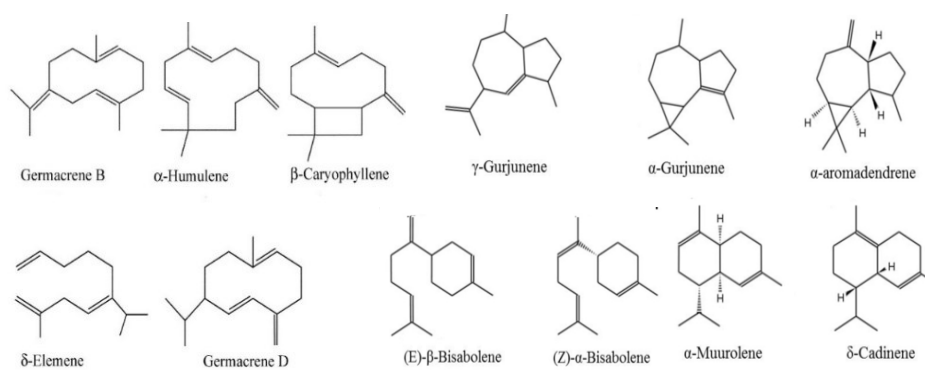
Monoterpenes



Terpenoides



Sesquiterpenes



Sesquiterpenoids

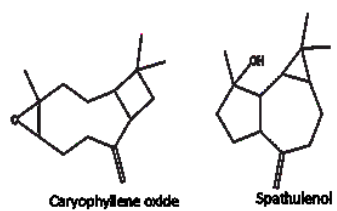


Figure 4.1. Structure of main components found in *C. limon*, *R. officinalis* and *L. angustifolia* commercial essential oils.

4.1.1 *Citrus limon* essential oil

The GC/MS analysis of commercial essential oil of *C. limon* obtained by cold pressing from the peel of the fruit had allowed to identify forty-three volatile organic compounds (table 4.2). The essential oil mainly contains limonene, a monoterpene with a content of 76.19% and 78.74% in fresh and preserved samples, respectively. This result is in line with the data found in the literature.

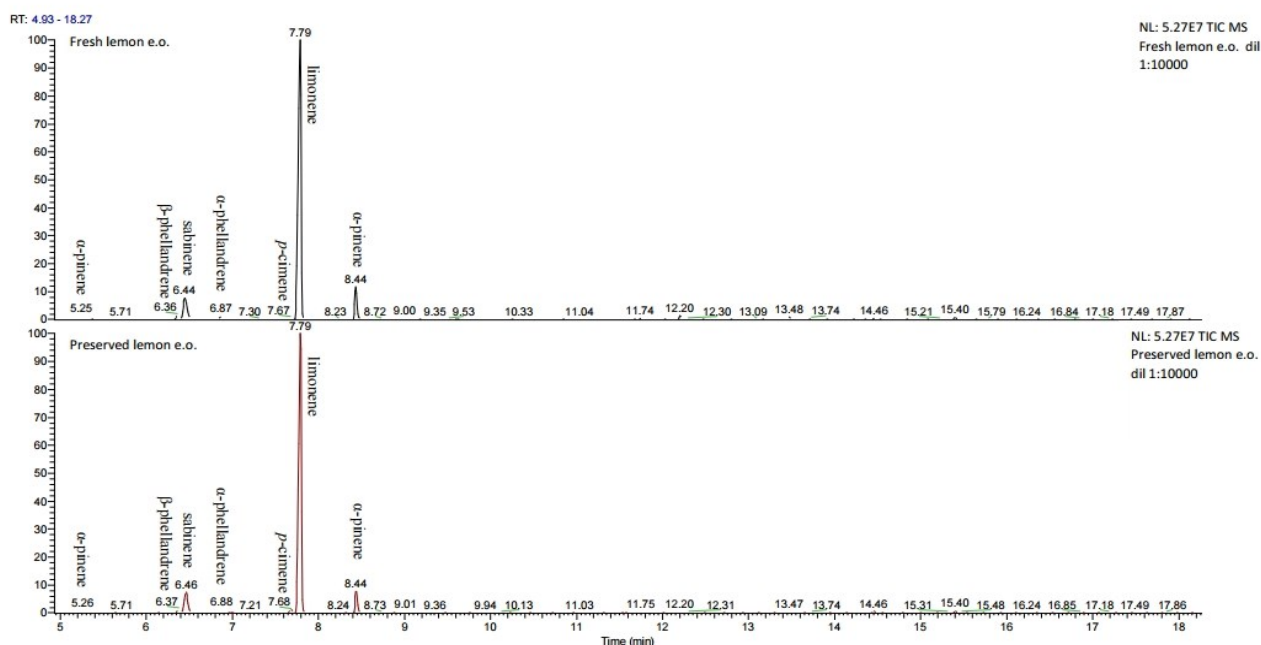


Figure 4.2 Total ion-current chromatograms of fresh and preserved lemon essential oil. Main components in descending order of abundance: 1, limonene; 2, sabinene; 3, γ -terpinene; 4, α -pinene; 5, p-cimene; 6, β -phellandrene; 7, α -phellandrene.

According to the literature, the EOs obtained from *Citrus limon*, always show the limonene, as the most abundant compound, whose concentration generally represents about 60-95% of the oil.⁴⁰ Interestingly, most of the other compounds are monoterpene hydrocarbons or their oxygenated derivatives (12 monoterpene hydrocarbons; 17 terpenoids). The data showed that about 97% of lemon essential oil was composed by monoterpene hydrocarbons. Among these compounds, sabinene (8.29% and 8.44%), γ -terpinene (7.31% and 5.82%), α -pinene (1.51% and 1.41%), β -phellandrene (1.45% and 1.34%) and p-cymene (0.34% and 1.03) represent about 18.9% and 18% respectively in fresh and preserved samples (table 3.1.1). Sesquiterpenes (13 sesquiterpene hydrocarbons; 1 sesquiterpenoid) in lemon essential oils were minor components.

	Rt(min)	d% fresh Cl e.o.	d% preserved Cl e.o.
1 α-Pinene	5,26	1,41	1,51
2 β-Phellandrene	6,37	1,34	1,45
3 Sabinene	6,46	8,44	8,29
4 α-Phellandrene	6,87	1,02	1,00
5 <i>p</i>-Cymene	7,68	0,34	1,03
6 Limonene	7,79	78,74	76,19
7 γ-Terpinene	8,44	5,82	7,31

Components are shown in order of retention time.

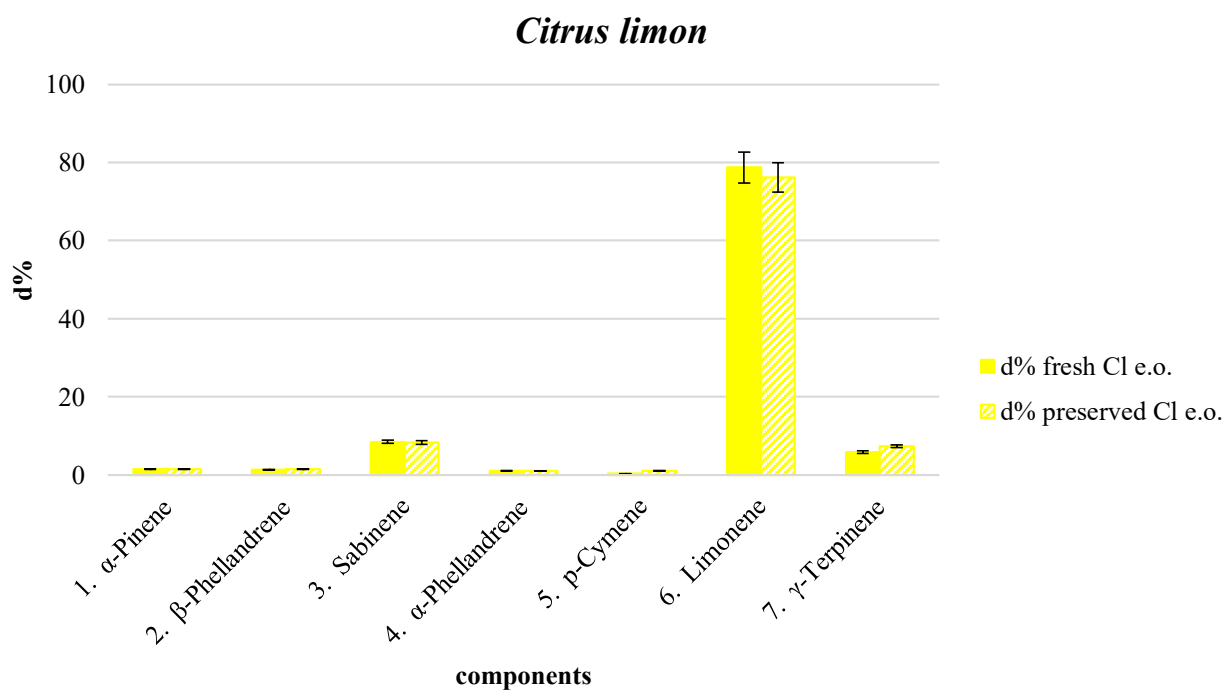


Figure 4.3 Differences among principal components of fresh and preserved samples of *C. limon* essential oils reported in table 3.1. Data were the mean of 3 measurements and the error was referred as standard error.

Table 4.2: Chemical composition of fresh and preserved *Citrus limon* essential oils

number	Components ^a	Rt (min) ^b	d%	
			CI fresh	CI preserved
1	α -Thujene	5,09	0,33	0,27
2	α -Pinene	5,26	1,51	1,41
3	Camphene	5,71	0,04	0,04
4	β -Phellandrene	6,37	1,45	1,34
5	Sabinene	6,46	8,29	8,44
6	α -Phellandrene	7,22	1,00	0,02
7	α -Terpinene	7,49	0,15	0,07
8	<i>p</i> -Cymene	7,68	0,34	1,03
9	Limonene	7,79	76,19	78,74
10	<i>cis</i> -b-Ocimene	8,01	0,02	0,01
11	<i>trans</i> -b-Ocimene	8,24	0,06	0,05
12	γ -Terpinene	8,44	7,31	5,82
13	<i>cis</i> -Sabinene hydrate	8,73	0,02	0,03
14	Terpinolene	9,01	0,31	0,21
15	Linalol	9,36	0,07	0,08
16	<i>cis</i> -Limonene oxide	9,94	0,00	0,06
17	<i>trans</i> -Limonene oxide	10,02	0,00	0,05
18	4,8-Epoxy- <i>p</i> -menth-1-ene	10,13	0,00	0,02
19	Camphor	10,19	0,00	0,01
20	Citronellal	10,33	0,04	0,00
21	Terpinen-4-ol	10,78	0,02	0,01
22	α -Terpineol	11,03	0,10	0,13
23	Nerol	11,54	0,00	0,04
24	Neral	11,75	0,40	0,20
25	Geraniol	11,97	0,00	0,01
26	Geranial	12,2	0,74	0,46
27	Δ -Elemene	13,09	0,01	0,00
28	α -Terpinyl acetate	13,36	0,01	0,01
29	Neryl acetate	13,47	0,38	0,29
30	Geranyl acetate	13,74	0,20	0,10
31	<i>cis</i> - α -Bergamotene	14,2	0,02	0,02
32	β -Caryophyllene	14,28	0,15	0,15
33	<i>trans</i> - α -Bergamotene	14,46	0,28	0,30
34	<i>cis</i> - β -Farnesene	14,74	0,01	0,01
35	β -Santalene	14,79	0,01	0,01
36	Germacrene D	15,1	0,01	0,01
37	Valencene	15,22	0,03	0,00
38	<i>cis</i> - α -Bisabolene	15,31	0,01	0,00
39	<i>tras</i> - α -Bisabolene	15,31	0,02	0,02
40	β -Bisabolene	15,4	0,43	0,43
41	Spathulenol	16,24	0,01	0,05
42	Caryophyllene oxide	16,3	0,00	0,02
43	α -Bisabolanol	17,49	0,01	0,01

^a Identification of components GC-MS based on National Institute of Standards and Technology 05 MS (NIST) library data.

^b *Rt* retention time

From NMR analysis, the characteristic ^{13}C and ^1H NMR signals of the components of *C. limon* are shown in figure 3.3 and 3.4. The presence of limonene confirmed the plant chemotype.

^{13}C NMR of *citrus limon* essential oil

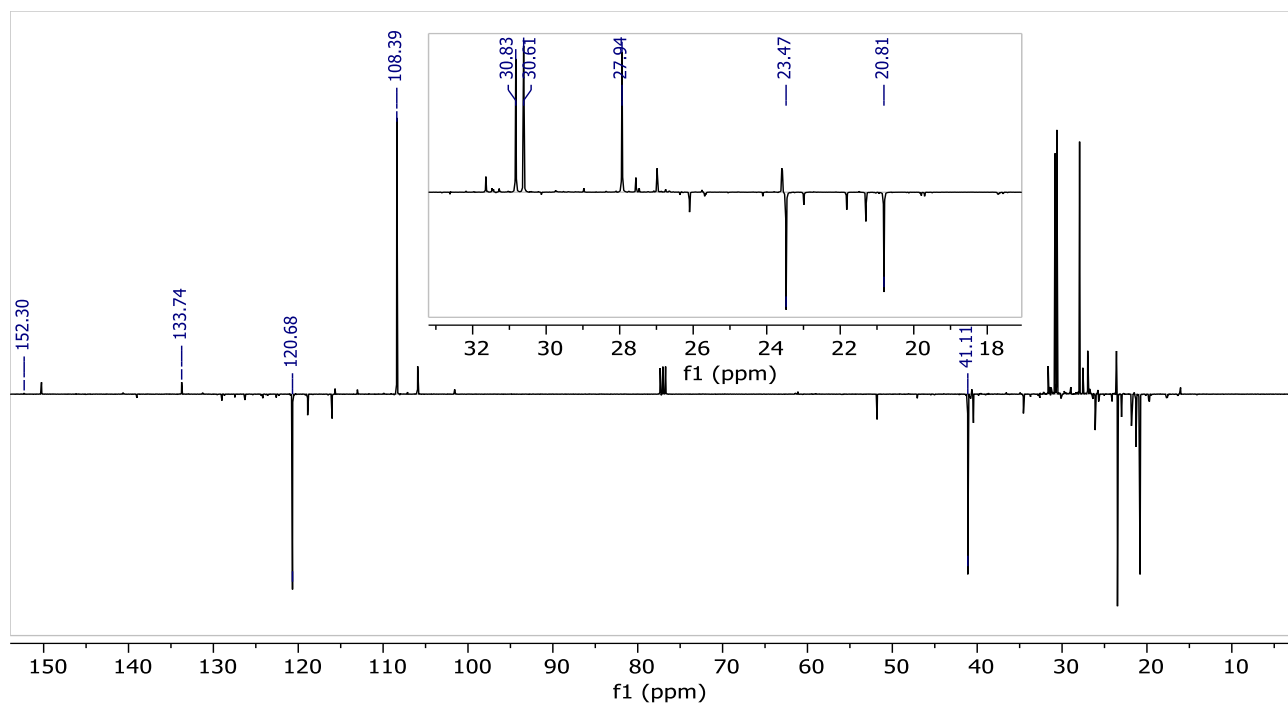


Figure 4.4 ^{13}C NMR spectra of *Citrus limon* essential oil. Signals were assigned to the corresponding carbon atoms of limonene (table3.3). The solvent was CDCl_3

^1H NMR of *C. limon* essential oils

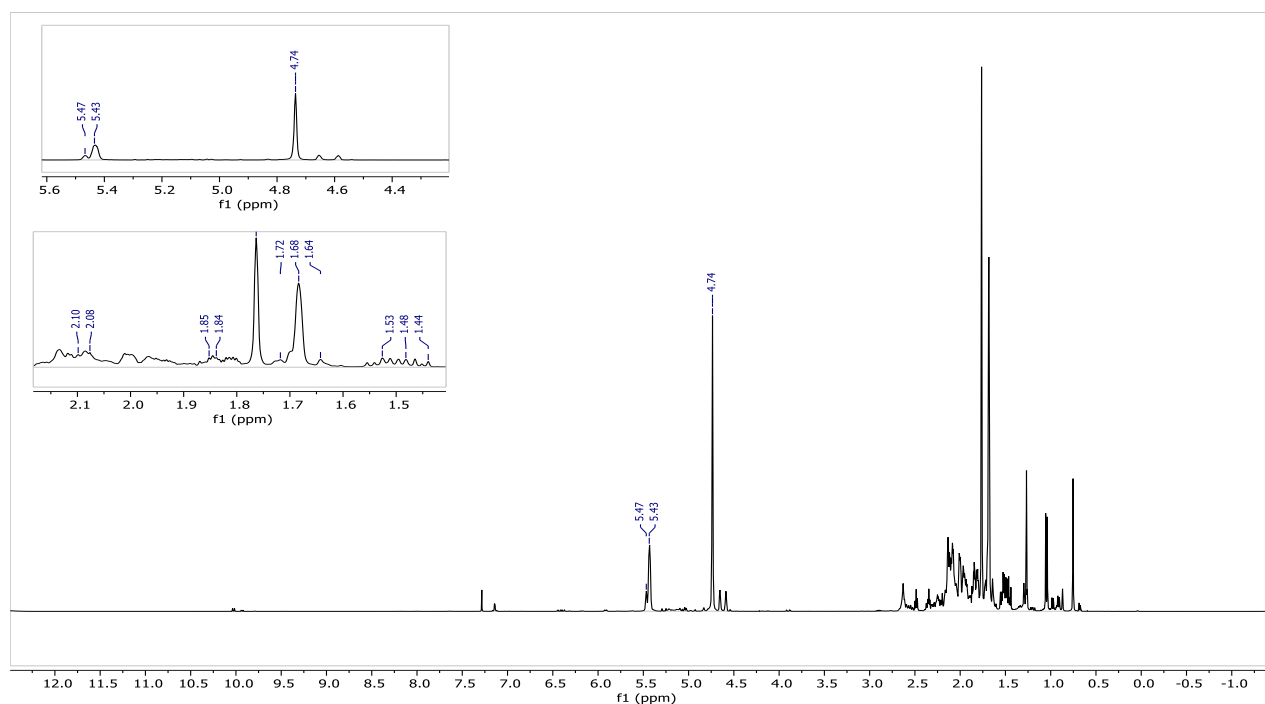
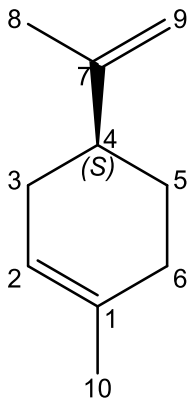


Figure 4.5 One dimensional ^1H NMR spectra of *Citrus limon* essential oil. Signal were assigned to the corresponding protons of limonene (table3.3). The solvent was CDCl_3

Table 4.3: NMR identification of main component of <i>Citrus limon</i> essential oil				
Components	¹³ C NMR (101 MHz, CDCl ₃) δ		¹ H NMR (400 MHz, CDCl ₃) δ	
	number atom	ppm	number atom	ppm
Limonene (<i>C. limon</i>) 	7 1 2 9 4 6 3 5 10 8	152.29 133.73 120.68 108.39 41.11 30.83 30.61 27.94 23.47 20.81	8 5 8 8,9 4 6, 3 9,9' 7 2	1.44 (qd) 1.64 (s) 1.73 (s) 1.76 (m) 1.85 (m) 2.08 (m) 4.73 (s) 5.43 (br.s)

4.1.2 *Rosmarinus officinalis* essential oil

GC-MS measurements of rosemary essential oil allowed to identify 54 different components (table 3.5). The chromatography analysis of the fresh and preserved Ro essential oils revealed nine significant peaks, reported in table 3.4 which shows a comparison between the percentages of more abundant components present in analysed Ro essential oils.

The predominant compound was 1,8-cineol (around 58%) which shows minimal variation in percentages of abundant between the fresh and the preserved sample. The most considerable difference is recorded for the terpene sabinene, whose concentration is much lower in the preserved essential oil, with respect to the fresh sample (0.6% and 6.5%, respectively) (see figure 4.6).

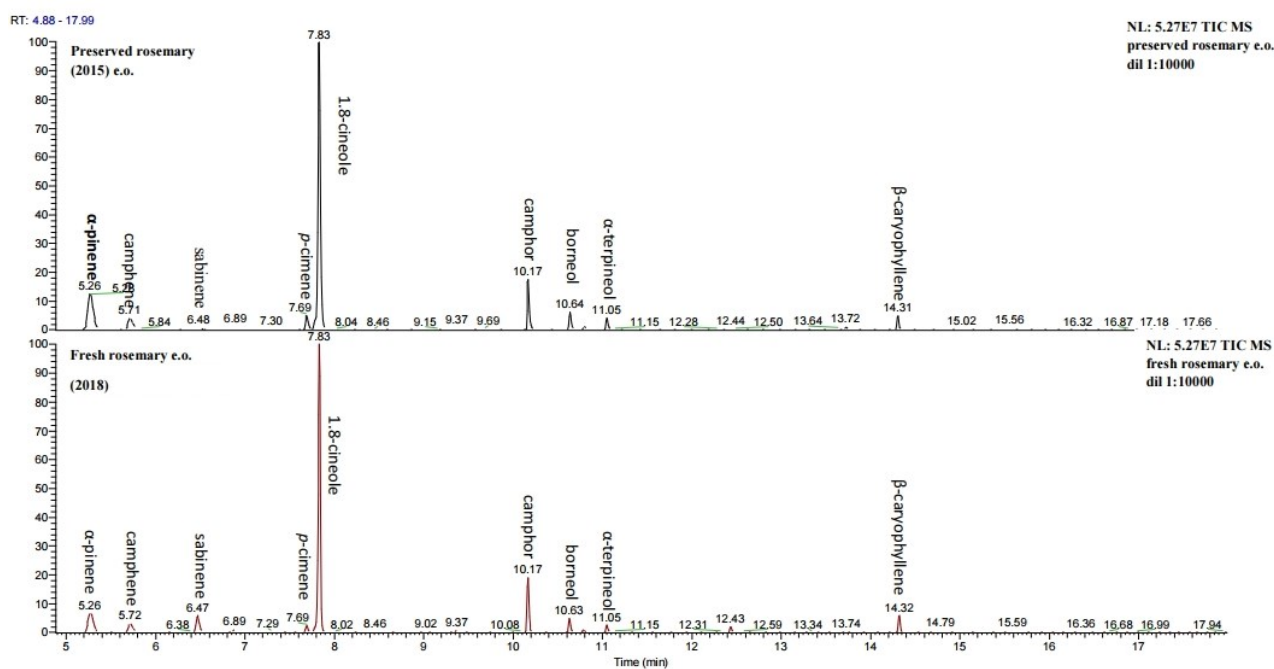


Figure 4.6 Total ion-current chromatograms of fresh and preserved rosemary essential oils. In both samples the major compounds identified were: 1,8- cineol; α -pinene, camphor, camphene; p-cimene, sabinene, borneol, β -caryophyllene α -terpineol.

components	Rt (min)	d% fresh Ro e.o.	d% preserved Ro e.o.
1. α -pinene	5.26	8.79	14.04
2. camphene	5.72	3.60	4.02
3. sabinene	6.47	5.26	0.55
4. p-cimene	7.69	1.70	3.03
5. 1,8-cineole	7.83	57.92	58.08
6. camphor	10.17	9.31	8.11
7. borneol	10.63	2.39	2.70
8. α -terpineol	11.05	1.29	1.76
9. β -caryophyllene	14.32	2.85	1.88

Major compounds of commercial essential oil of *R. officinalis* were identified and quantified, using gas chromatography mass spectrometry (GC-MS). Components are shown in order of retention time.

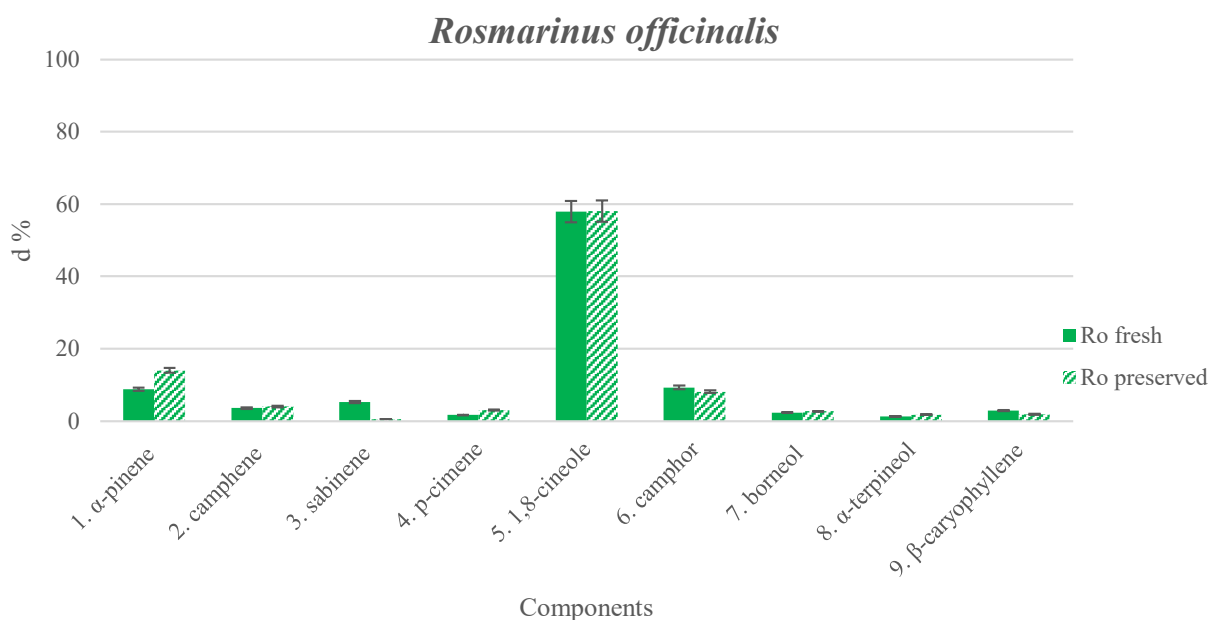


Figure 4.7. Differences among principal components of fresh and preserved samples of *R. officinalis* essential oils reported in table 3.2. Data were mean of 3 measurements and the errors was referred as standard error.

Around 73 % of rosemary components were oxygenated derivatives from terpene hydrocarbons. Among minimum components found sesquiterpenes hydrocarbon and their sesquiterpenoids which represent 4.13 and 3.19% in fresh and preserved sample respectively. The main difference between fresh and preserved samples was the abundance percentage of α -pinene, which is higher in preserved sample than in the fresh one. By contrast, sabinene presented a higher percentage of α -pinene in the fresh sample.

Table 4.5: Chemical composition of fresh and preserved *Rosmarinus officinalis* essential oils

number	Components ^a	Rt (min) ^b	d ^o %	
			Ro fresh	Ro preserved
1	α -Thujene	5,1	0,24	0,00
2	α -Pinene	5,26	8,79	14,04
3	Camphene	5,72	3,60	4,02
4	Sabinene	6,47	5,26	0,55
5	β -Pinene	6,89	0,82	0,77
6	δ -3-Carene	7,29	0,20	0,15
7	α -Terpinene	7,5	0,12	0,12
8	<i>p</i> -Cimene	7,69	1,70	3,03
9	Limonene	7,78	1,24	1,56
10	1,8-Cineole	7,83	57,92	58,08
11	β -Ocimene	8,02	0,02	0,00
12	γ -Terpinene	8,46	0,27	0,03
13	<i>cis</i> -4-Thujanol	8,73	0,08	0,00
14	Terpinolene	9,02	0,17	0,00
15	<i>p</i> -Cimenene	9,14	0,01	0,05
16	Linalol	9,37	0,49	0,40
17	<i>endo</i> -Fenchol	9,68	0,02	0,06
18	<i>cis-p</i> -Menth-2-en-1-ol	9,81	0,01	0,00
19	α -Campholenal	9,86	0,01	0,02
20	<i>trans</i> -Pinocarveol	10,08	0,03	0,02
21	Camphor	10,17	9,31	8,11
22	<i>trans</i> - β -Terpineol	10,33	0,02	0,03
23	<i>p</i> -Mentone	10,38	0,00	0,04
24	Pinocarvone	10,48	0,02	0,00
25	Borneol	10,63	2,39	2,70
26	Terpinen-4-ol	10,78	0,52	0,57
27	<i>p</i> -Cymen-8-ol	10,93	0,03	0,03
28	α -Terpineol	11,05	1,29	1,76
29	Verbenone	11,31	0,01	0,00
30	Bornyl formate	11,57	0,01	0,00
31	<i>trans</i> -Verbenyl acetate	12,31	0,02	0,00
32	Bornyl acetate	12,43	0,93	0,25
33	α -Cubenene	13,34	0,03	0,00
34	α -Ylangene	13,64	0,05	0,08
35	α -Copaene	13,74	0,19	0,34
36	Methyleugenol	14,12	0,02	0,00
37	β -Caryophyllene	14,32	2,85	1,88
38	Germacrene	14,46	0,03	0,00
39	β -Gurjunene	14,57	0,04	0,05

40	Aromadendrene	14,72	0,01	0,03
41	α -Humulene	14,79	0,31	0,22
42	α -Amorphene	15,04	0,14	0,23
43	γ -Amorphene	15,1	0,02	0,03
44	β -Selinene	15,19	0,02	0,02
45	γ -Amorphene	15,24	0,03	0,03
46	α -Selinene	15,29	0,02	0,03
47	α -Muurolene	15,32	0,05	0,07
48	β -Bisabolene	15,45	0,03	0,03
49	γ -Muurolene	15,52	0,10	0,13
50	δ -Cadinene	15,56	0,22	0,26
51	<i>trans</i> -Calamenene	15,59	0,06	0,11
52	α -Calacorene	15,86	0,01	0,03
53	Caryophyllene oxide	16,36	0,18	0,04
54	Humulene epoxide	16,68	0,01	0,00
^a Identification of components GC-MS based on National Institute of Standards and Technology 05 MS (NIST) library data. ^b <i>Rt</i> retention time				

The ^{13}C and ^1H NMR spectra of *R.officinalis* are reported in figure 4.8 and 4.9 and the presence of 1,8-cineol confirmed the plant chemotype.

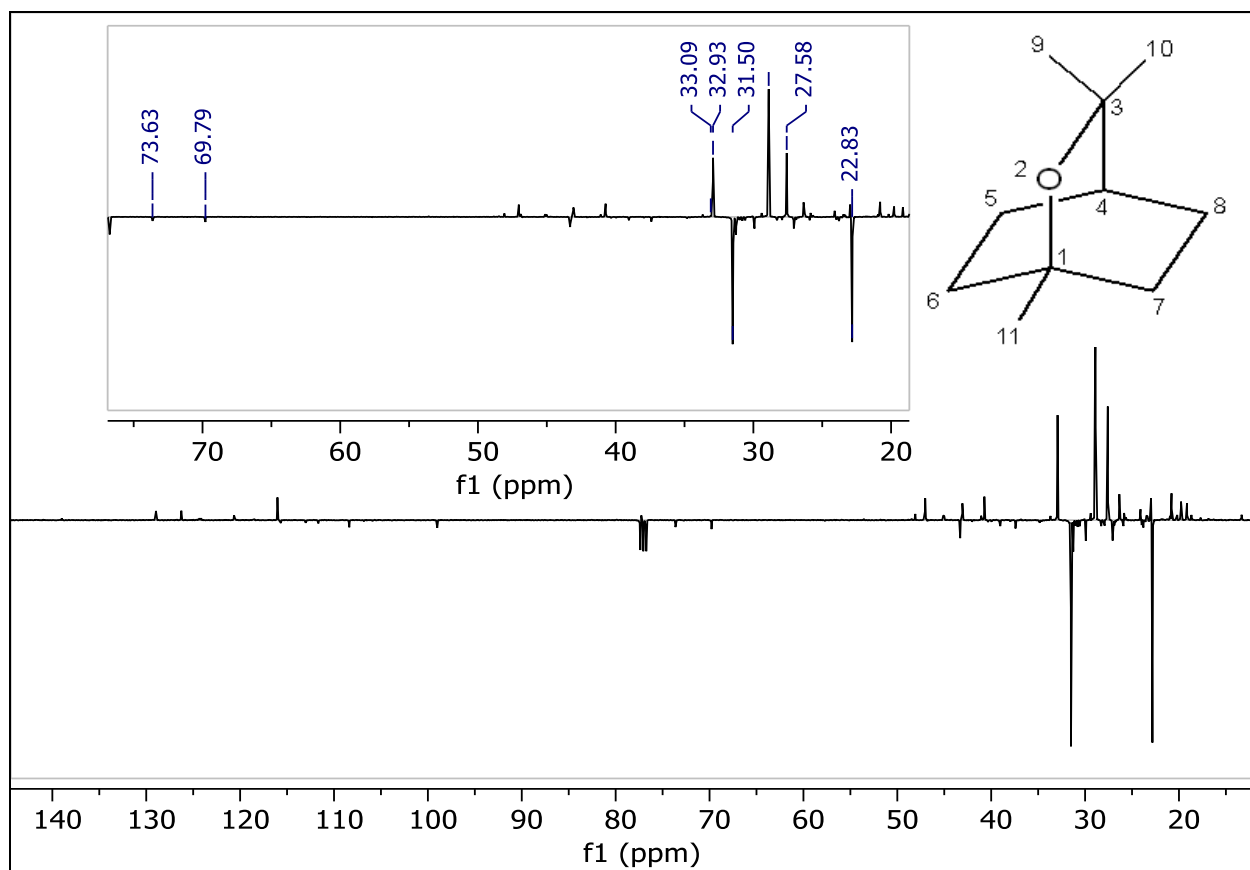
^{13}C NMR of *R. officinalis* essential oil

Figure 4.8 ^{13}C NMR spectra of *R. officinalis* essential oil. Signals were assigned to the corresponding carbon atoms of 1,8-cineol (table 4.6). The solvent was CDCl_3

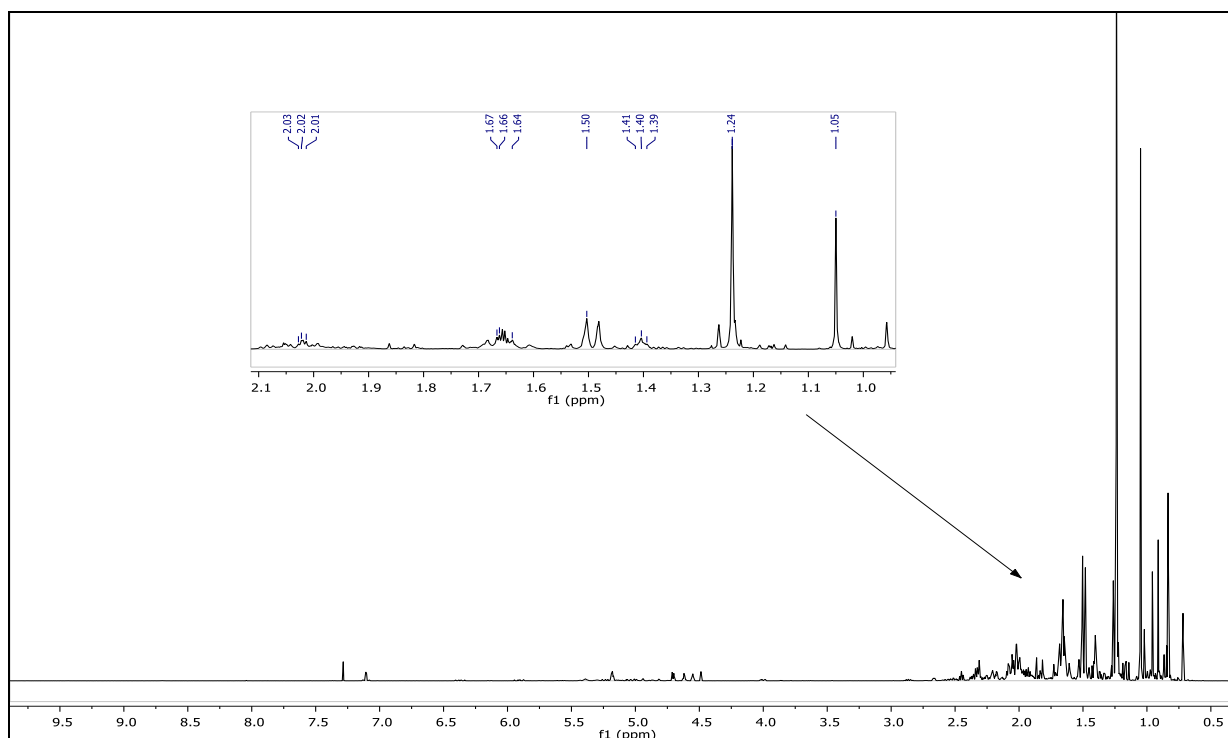
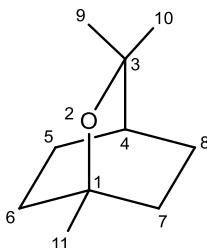
^1H NMR of *R. officinalis* essential oil

Figure 4.9 One dimensional ^1H NMR spectra of *R. officinalis* essential oil. Signals were assigned to the corresponding protons of 1,8-cineol (table 3.6). The solvent was CDCl_3

Table 4.6: NMR identification of main component of *Rosmarinus officinalis* essential oil

Components	^{13}C NMR (101 MHz, CDCl_3) δ		^1H NMR (400 MHz, CDCl_3) δ	
	number atom	ppm	number atom	ppm
1,8-cineol (<i>R. officinalis</i>) 	1	73.6	5;8	2.02 (m)
	3	69.8	6,7	1.66 (m)
	4	33.1	5',6',7',8'	1.50 (m)
	5,8	33.0	4	1.40 (m)
	9,10	31.5	9,10	1.24 (s)
	11	27.6	11	1.05 (s)
	6,7	22.8		

4.1.3 *Lavandula angustifolia* essential oil

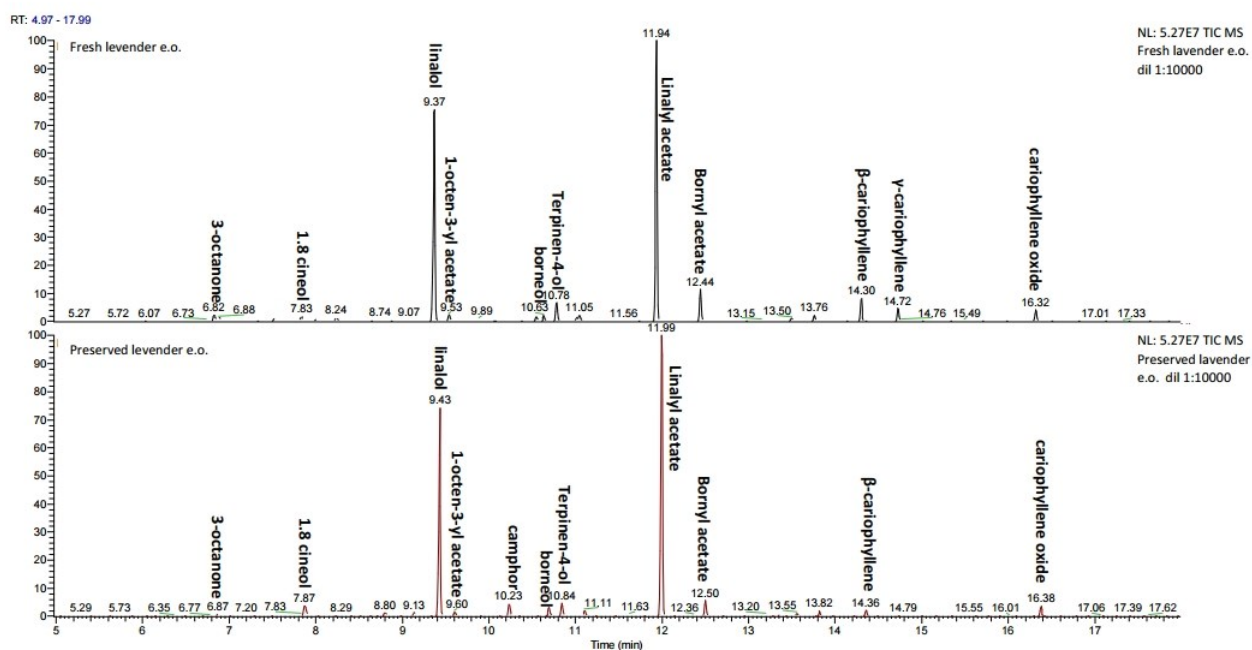


Figure 4.10 Total ion-current chromatograms of fresh and preserved lavender essential oil. Major compounds identified were: linalyl acetate, linalool, 1,8-cineole, camphor, bornyl acetate, borneol, terpinen-4-ol, β -caryophyllene, γ -caryophyllene, caryophyllene oxide, 3-octanone, 1-octen-3-yl acetate and α -terpineol (table 4.7).

Table 4.7 Comparison percentages of abundance between fresh and preserved *L. angustifolia* essential oil.

components	Rt (min)	d% fresh La e.o.	d% preserved La e.o.
1. 3-Octanone	6.87	1.20	0.48
2. 1,8-cineol	7.87	0.87	2.52
3. linalool	9.43	33.90	32.57
4. 1-Octen-3-yl acetate	9.6	1.03	0.67
5. camphor	10.23	0.24	2.29
6. borneol	10.70	0.78	1.48
7. Terpinen-4-ol	10.84	2.71	2.05
8. α -terpineol	11.11	0.87	1.14
9. Linalyl acetate	11.99	38.37	42.95
10. Bornyl acetate	12.5	4.01	2.29
11. β -caryophyllene	14.32	3.22	0.87
12. γ -Caryophyllene	14.79	1.79	0.18
13. Caryophyllene oxide	16.38	1.54	1.80

Major compounds of commercial essential oil of *Lavandula angustifolia* were identified and quantified using gas chromatography mass spectrometry (GC-MS). Components are shown in order of retention time.

L. angustifolia shows high values of linalyl acetate (38.5% and 42.9% respectively in fresh and preserved samples) and linalool (33.9% in fresh sample and 32.6% in preserved one), according to the ISO standard composition range of 25–45% for linalyl acetate and of 25–38% for linalool. These values allowed to define the type of lavender used to produce the essential oil, which is characterized by around 95% of terpenoids. The sesquiterpenes hydrocarbon and the sesquiterpenoids represent around 7.2 and 3.2% in fresh and preserved sample respectively. The main differences between the fresh and preserved samples were in the content of camphor, 1,8-cineol and borneol, whose values were higher in the preserved one. Thus, the camphor was almost 10 times higher, while 1,8-cineol and borneol were 3 and 2 times higher, respectively. Conversely, γ -caryophyllene, β -caryophyllene, 3-octanone, bornyl acetate were around 10, 4, 2 time higher in the fresh sample.

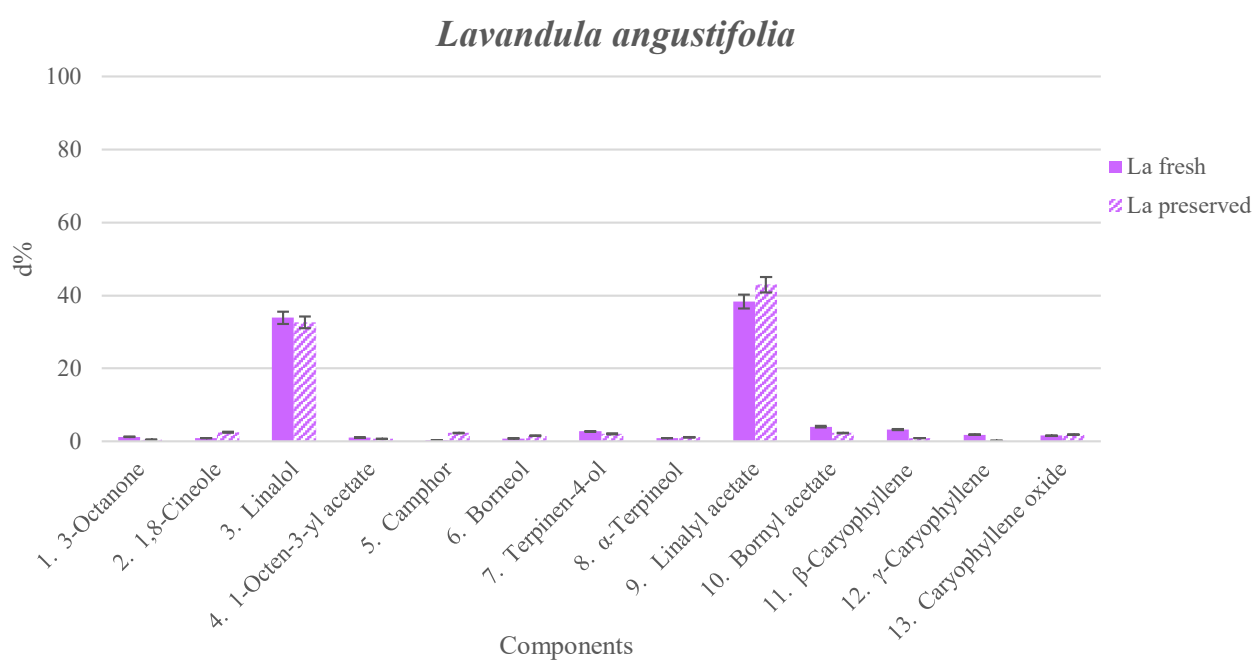


Figure 4.11 Differences among principal components of fresh and preserved samples of *L. angustifolia* essential oils reported in table 4.7. Data were mean of 3 measurements and the errors was referred as standard error.

Table 4.8: Chemical composition of fresh and preserved *Lavandula angustifolia* essential oils

number	Components ^a	Rt (min) ^b	d%	
			La fresh	La preserved
1	α -Thujene	5,11	0,07	0,04
2	α -Pinene	5,29	0,14	0,18
3	Camphene	5,73	0,14	0,16
4	β -Phellandrene	6,41	0,04	0,03
5	Sabinene	6,5	0,04	0,11
6	1-Octen-3-ol	6,77	0,15	0,09
7	3-Octanone	6,87	1,20	0,48
8	β -Pinene	6,92	0,86	0,67
9	Butyl butanoate	7,16	0,10	0,04
10	3-Octanol	7,2	0,28	0,10
11	Δ -3-Carene	7,33	0,07	0,05
12	Hexyl acetate	7,58	0,51	0,14
13	α -Phellandrene	7,62	0,11	0,11
14	<i>p</i> -Cimene	7,73	0,31	0,26
15	Limonene	7,83	0,42	0,30
16	1,8-Cineole	7,87	0,87	2,52
17	<i>cis</i> - β -Ocimene	8,07	0,44	0,06
18	<i>trans</i> - β -Ocimene	8,29	0,65	0,20
19	<i>cis</i> -Linalool oxide	8,8	0,29	0,83
20	<i>trans</i> -Linalool oxide	9,13	0,20	0,67
21	Linalol	9,43	33,90	32,57
22	1-Octen-3-yl acetate	9,6	1,03	0,67
23	3-Octanyl acetate	9,83	0,19	0,06
24	Alloocimene	9,96	0,29	0,03
25	Camphor	10,23	0,24	2,29
26	Hexyl butanoate	10,32	0,07	0,05
27	Lavandulol	10,61	0,64	0,33
28	Borneol	10,7	0,78	1,48
29	Terpinen-4-ol	10,84	2,71	2,05
30	<i>p</i> -Cymen-8-ol	11	0,22	0,14
31	3-Hexenyl butanoate	11,04	0,11	0,27
32	Octanoyl butanoate	11,08	0,46	0,38
33	α -Terpineol	11,11	0,87	1,14
34	β -Pinene	11,5	0,02	0,05
35	Bornyl formate	11,63	0,15	0,23
36	Hexyl-2-methyl butanoate	11,78	0,03	0,04
37	Cumaldehyde	11,88	0,11	0,10
38	Linalyl acetate	11,99	38,37	42,95
39	<i>trans</i> -Verbenyl acetate	12,36	0,12	0,18
40	Bornyl acetate	12,5	4,01	2,29
41	Hexyl tiglate	13,16	0,02	0,04
42	Limonene oxide	13,2	0,09	0,28

43	Limonene dioxide	13,36	0,25	0,66
44	Fenchene	13,55	0,46	0,43
45	Geranyl acetate	13,82	0,75	0,77
46	α -Bergamatene	13,93	0,25	0,11
47	β -Caryophyllene	14,36	3,22	0,87
48	Zinziberene	14,54	0,13	0,11
49	γ -Caryophyllene	14,79	1,79	0,18
50	Isocaryophyllene	15,4	0,00	0,06
51	β -Bisabolene	15,48	0,04	0,06
52	γ -Muurolene	15,55	0,12	0,14
53	Caryophyllene oxide	16,01	0,06	0,08
54	Caryophyllene oxide	16,38	1,54	1,80
55	Δ -Cadinene	17,06	0,07	0,06

^a Identification of components GC-MS based on National Institute of Standards and Technology 05 MS (NIST) library data.

^b *Rt* retention time

The ¹³C and ¹H NMR spectra of *L. Angustifolia* are reported in figure 4.12 and 4.13 and the presence of linalool and linalyl acetate confirmed the plant chemotype.

¹³C NMR of *Lavandula angustifolia* essential oils

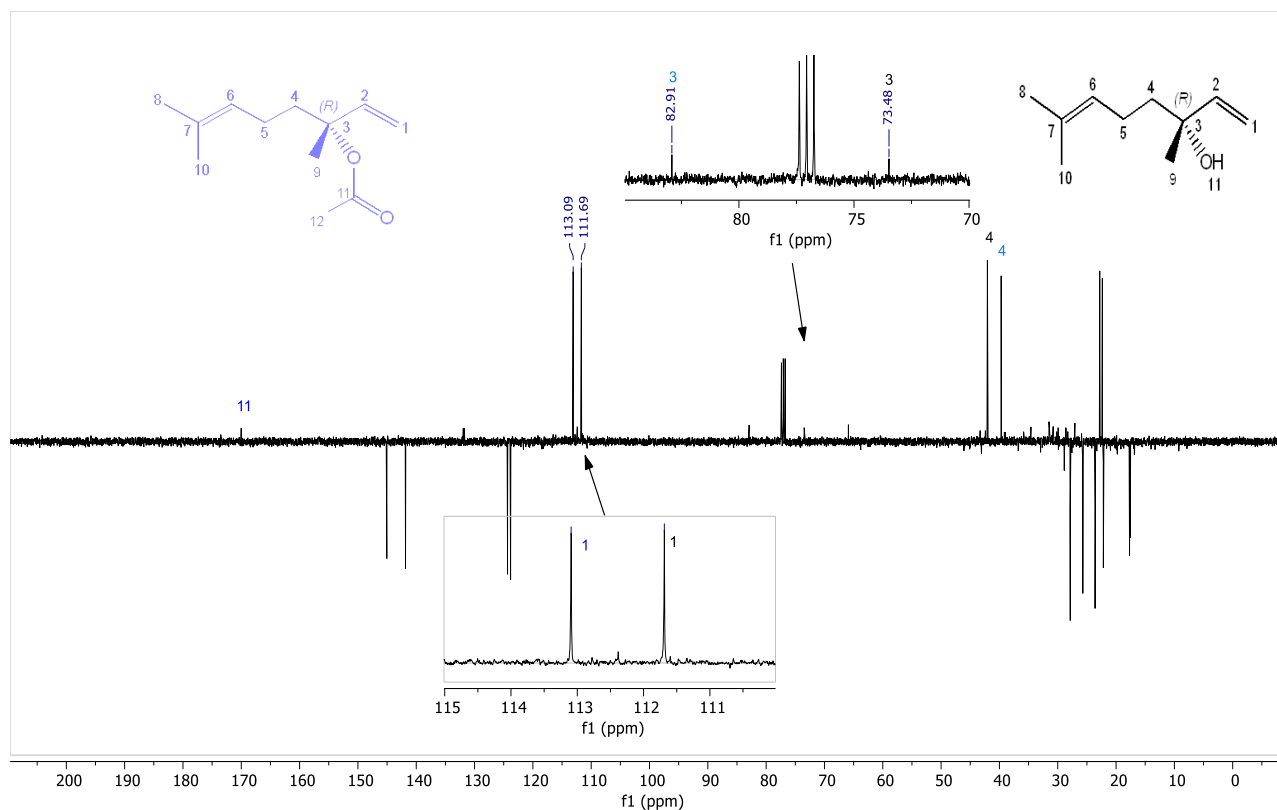


Figure 4.12 ¹³C NMR spectra of *L.angustifolia* essential oil. Signals were assigned to the corresponding carbon atoms of linalool (and linalyl acetate (blue) (table 4.9). The solvent was CDCl₃

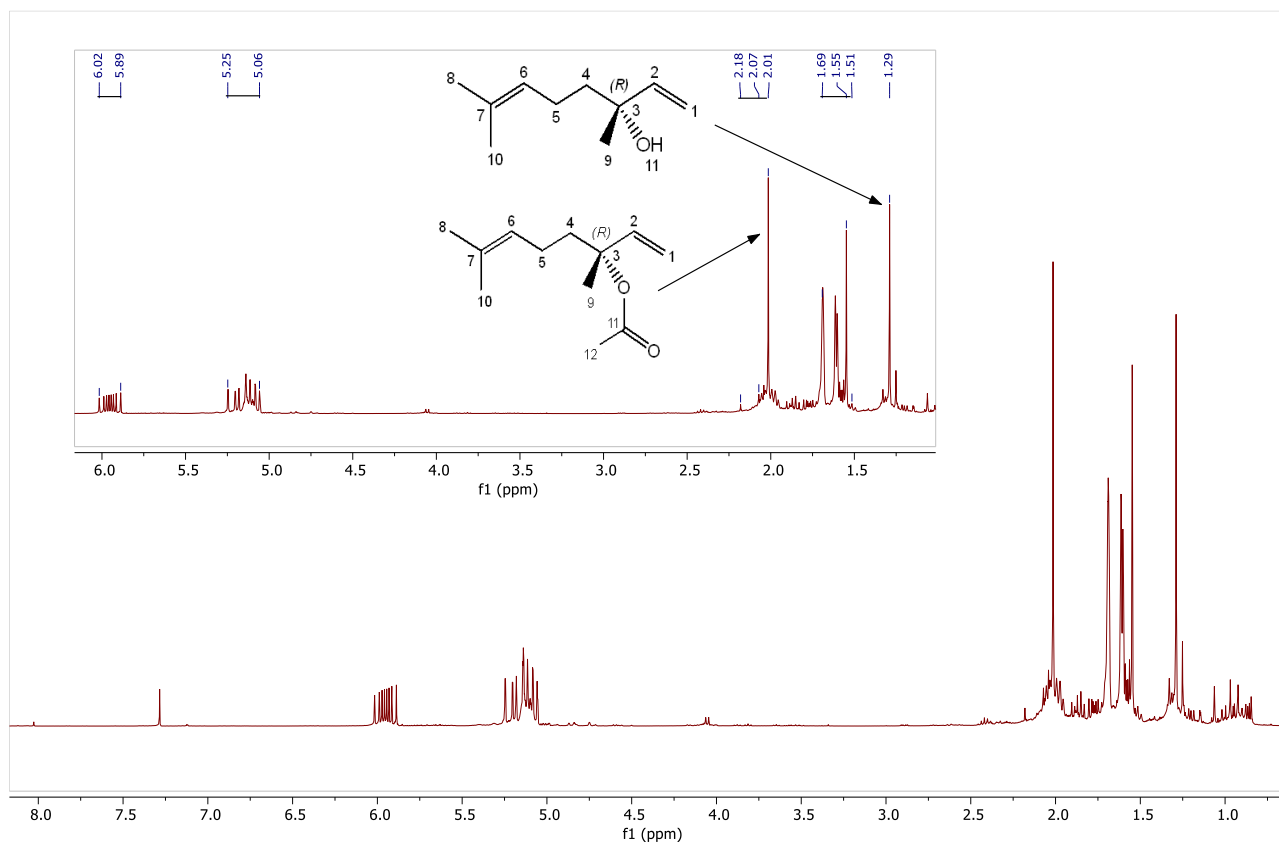
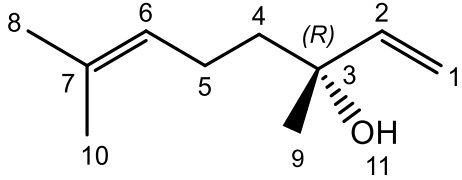
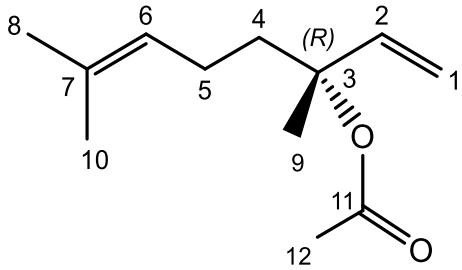
^1H NMR of *Lavandula angustifolia* essential oil

Figure 4.13 One dimensional ^1H NMR spectra of *L. angustifolia* essential oil. The signals were assigned to the corresponding protons of linalool and linalyl acetate. The solvent was CDCl_3 .

Table 4.9: NMR identification of main components of <i>Lavandula angustifolia</i> essential oils				
Components	¹³ C NMR (101 MHz, CDCl ₃) δ		¹ H NMR (400 MHz, CDCl ₃) δ	
	number atom	ppm	number atom	ppm
Linalool (<i>L. angustifolia</i>) 	2 7 6 1 3 4 9 8 5 10	145.0 131.8 124.3 111.7 73.5 42.1 27.8 25.7 22.8 17.6	2 1 6 1' 5,5' 8 10 4,4' 9	5.92 (dd) 5.22 (dd) 5.11 (m) 5.07 (dd) 1.96 (m) 1.68 (br s) 1.60 (br s) 1.59-1.52 (m) 1.29 (s)
Linalyl acetate (<i>L. angustifolia</i>) 	11 2 7 6 1 3 4 8 9 5 10 12	169.7 141.9 131.9 123.8 113.1 82.9 39.7 25.6 23.7 22.4 22.1 17.7	2 1 1' 6 12 4,4',5,5' 8 10 9	5.98 (dd) 5.14 (m) 5.11 (m) 5.10 (m) 2.01 (s) 2.15-1.70 (m) 1.69 (br s) 1.61 (br s) 1.55 (s)

4.2 Antioxidant activity of essentials oils

Antioxidant properties of essential oils and of their main components have been fully investigated and proven with the use of DPPH and ABTS⁺ methods. To examine the relative radical-scavenging activities of the oils the standard antioxidant BHT was employed. The antioxidant activity detected by the ABTS assay is higher than the one of the DPPH assay. Our observations are in line with those indicated by other authors who report that the ABTS inhibition for samples from vegetables, fruits and beverages is higher than DPPH inhibition⁹⁷. The percentage of inhibition values of the oils for DPPH radical were: rosemary < lemon ≤ lavender.

All of the essential oils were found to have scavenging effects on DPPH and ABTS⁺ method. Results showed that fresh and stored sample had no significant differences in antioxidant capacity. The highest DPPH radical-scavenging activity was obtained by the lavender and lemon essential oils. However, they show a relatively low activity against the DPPH respect to ABTS⁺ radical.

Radical-scavenging activity against the ABTS radical was highest in rosemary essential oil while lavender and lemon essential oils showed moderate activity. The percentage of inhibition values of the oils for ABTS radical cation were: lemon \leq lavender < rosemary.

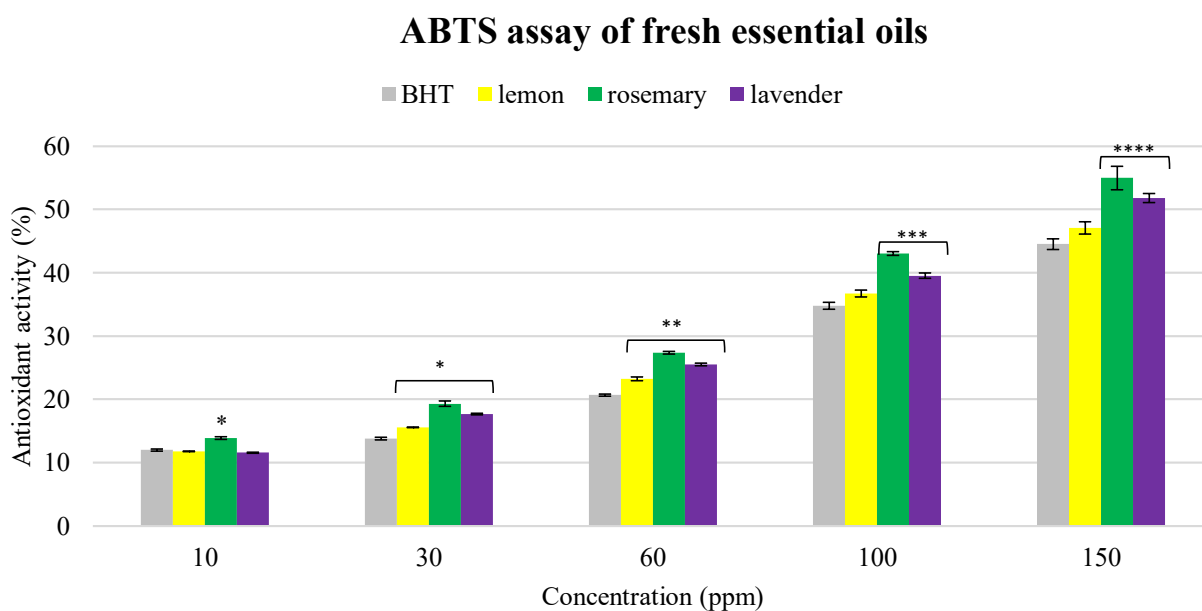
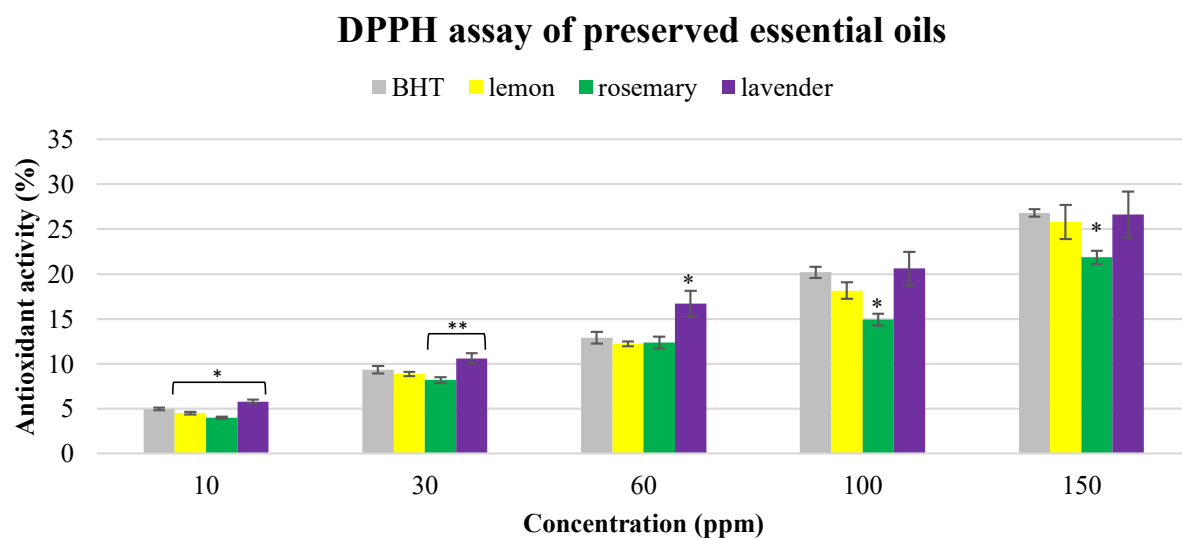
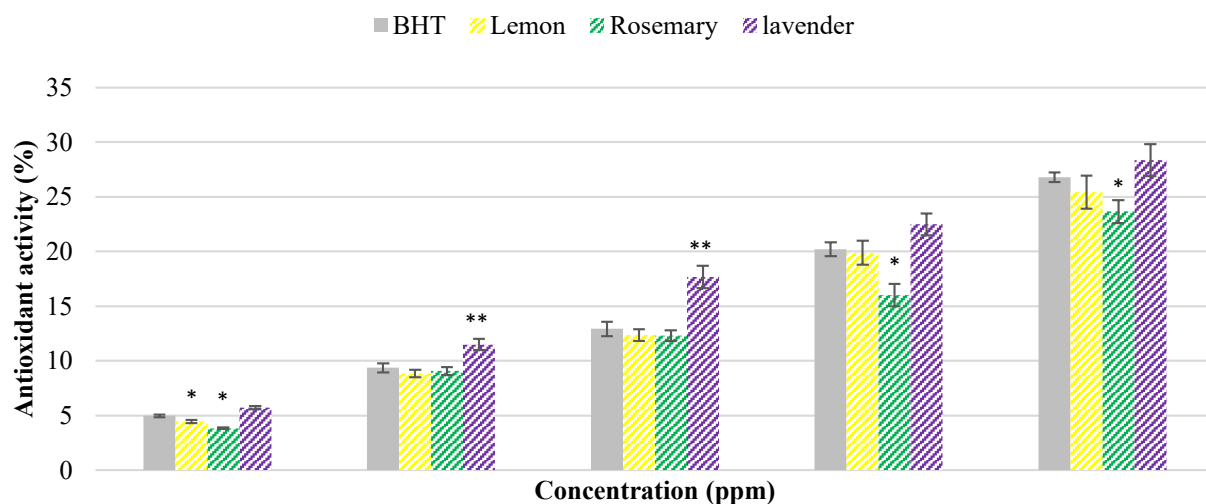


Figure 4.14 Scavenger effect of different concentrations of fresh EOs on the stable 1,1 diphenyl-2-picrylhydrazyl radical (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical, respectively.

Results are expressed as percentage decrement of absorbance at 517 nm with respect to control. Each value represents the mean \pm SD of three experiments. * indicate significant different ($p \leq 0,5$, $n=3$, test T) with respect to positive control (BHT).

DPPH assay of preserved essential oils



ABTS assay of preserved essential oils

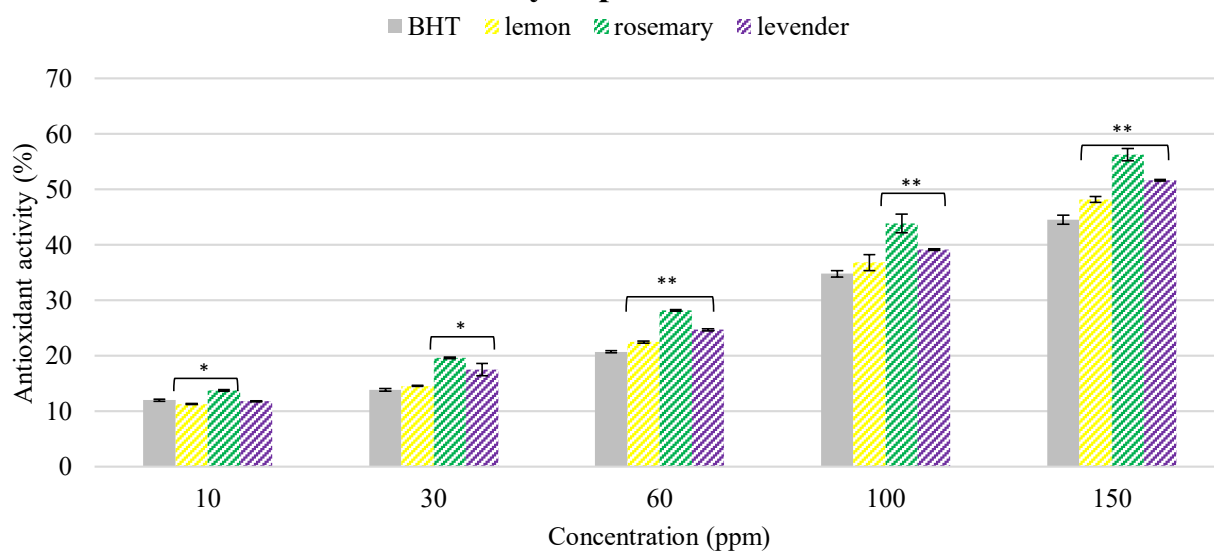
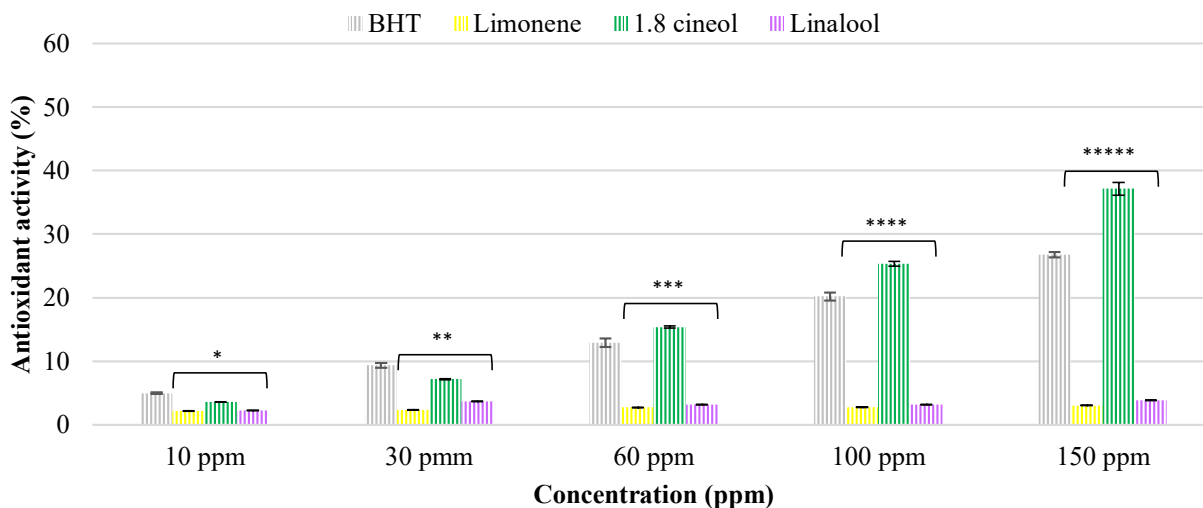


Figure 4.15. Scavenger effect of different concentrations of stored EOs on the stable 1,1 diphenyl-2-picrylhydrazyl radical (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical, respectively.

Results are expressed as percentage decrement of absorbance at 517 nm with respect to control. Each value represents the mean \pm SD of three experiments. * indicate significant different ($p \leq 0,05$, $n=3$, test T) with respect to positive control BHT

DPPH of main components of essential oils



ABTS of main components of essential oils

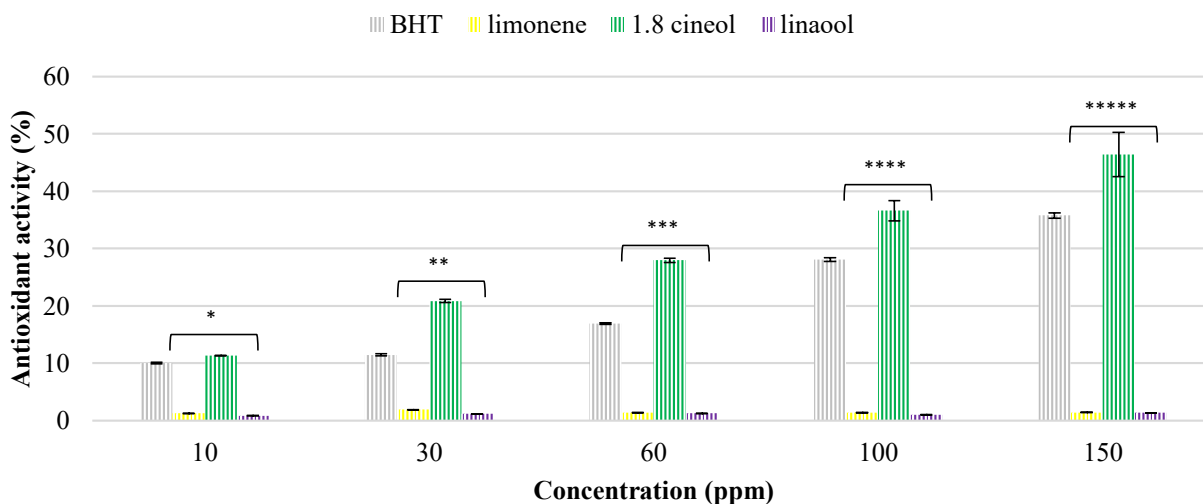


Figure 4.16 -Scavenger effect of limonene, 1,8-cineol and linalool on the stable 1,1 diphenyl-2-picrylhydrazyl radical (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical, respectively.

Results are expressed as percentage decrement of absorbance at 517 nm with respect to control. Each value represents the mean \pm SD of three experiments. *indicate significant different ($p \leq 0,5$, $n=3$, test T) with respect to positive control BHT.

The single main components analysed showed a different reactivity for DPPH and ABTS radical with respect to the essential oils. These results indicated that in essential oils exists a synergistic effect of several components which influences the capacity to scavenge free radicals.

Furthermore, 1,8-cineol present in rosemary essential oil, seems to have a predominant effect as scavenger with respect to other components and was highly reactive versus ABTS radical cation. In both assay the percentage of inhibition values of the characterizing components of essential oils were: 1,8-cineol < lemon ≤ lavender. These results could be explained by the presence of unsaturated compounds, mostly terpenes which display one or more double bonds. The saturation of these bonds would result in a decrease of their planarity and most importantly of their reactivity.⁸⁶ On the other hand, rosemary and lavender essential oils showed a high abundance in derived oxygenated terpenes such as alcohols, aldehydes, ketones and acids. The presence of hydroxyl and carbonyl groups are factors that reduce the reactivity of the compounds towards DPPH radicals⁹⁸.

4.3 Effect of essential oil on mitochondrial functions

Mitochondria from any tissue can provide energy in the form of ATP as a result of nutrient oxidation⁹⁹. Mitochondria activity can be controlled through an acute mechanism by modifying mitochondrial function and with long term transcriptional one, such as increasing in number¹⁰⁰. In response to changes in energy demand and supply, the organism adapts its capacity and/or efficiency of ATP production. Bioenergetic efficiency and ATP synthesis differs among tissue and is correlated to their specific physiology and to availability of nutrient.

Oxidation of nutrient generate electrons to electron transport chain (ETC) in form of NADH and FADH₂. The transfer of electron throughout the four complexes of ETC, extrude protons from matrix to intermembrane space and, energy proton re-entry through complex V or ATP synthase channel, is use for the synthesis of ATP from ADP. Proton re-entry that doesn't involve in ATP synthases via ATP synthase are referred as uncoupled respiration¹⁰¹.

In order to compare the direct effects of fresh and preserved sample of *C. limon*, *R. officinalis* and *L. angustifolia* essential oils on mitochondrial oxidative phosphorylation, the mitochondrial respiration rates in various metabolic states were measured. In a first series of experiments, the effects of different concentrations of DMSO-essential oils solutions on the glutamate and malate (mitochondrial Complex I dependent substrates or NADH-oxidase) oxidation were assessed in freshly isolated beef heart mitochondria (Figure 4.17-4.19).

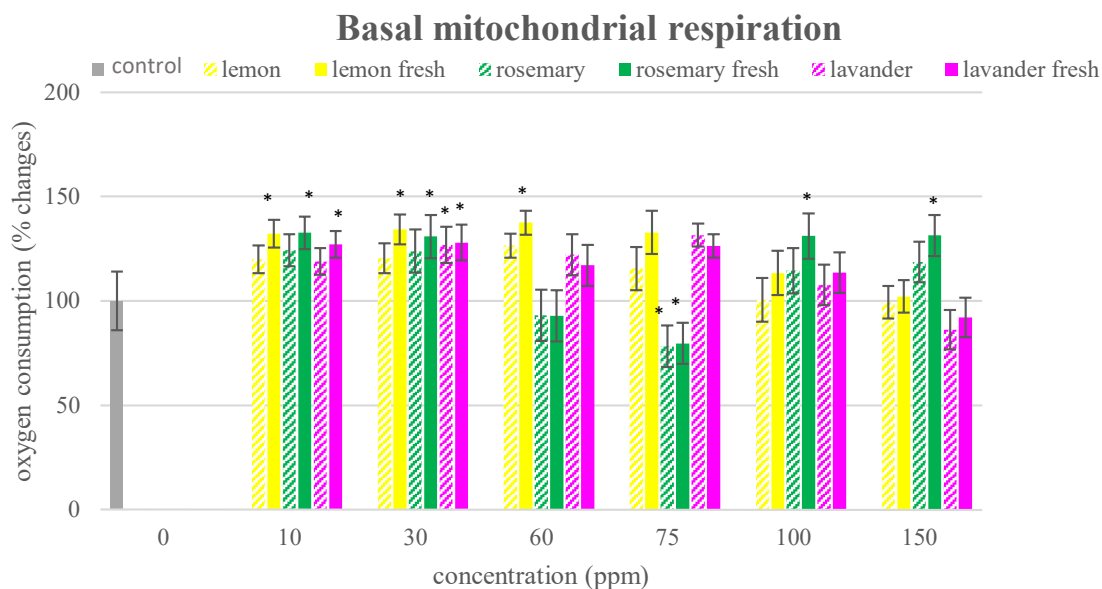


Figure 4.17 Effects of fresh and preserved essential oils on mitochondrial in the second metabolic state. 0.5 mg/mL of fresh beef heart mitochondria were added with 5 mM glutamate + 5 mM malate in the presence or absence of increasing concentrations of the different DMSO-essential oils solutions. O₂ consumption using a Clark-type electrode. Experiments were carried out in triplicate using three mitochondria preparations and expressed as % change of the activity respect the control (100%) of mean \pm standard deviation... * indicates statistical significant difference with respect to control mitochondria, i.e. untreated with essential oils ($p \leq 0,5$, $n=3$, test T).

As it can be seen in Figure 4.17, all the essential oils studied influenced oxygen consumption under basal conditions or state 2. Fresh and preserved essential oils showed very similar activity suggesting that the characteristic components were involved in the modulation of state 2 of respiration.

Lavender and lemon oils had a similar behaviour, characterized by a slight activation of the oxygen consumption up to 75 ppm, followed by a lack of effects at higher concentrations. The observed effect suggests that the respiratory complexes activity can be differently influenced depending on the rosemary oil/membrane protein ratio, i.e. that multiple targets of the oil components may exist in the respiratory complexes.

After adding 0.25 mM ADP the mitochondrial respiration rate was induced in the state 3 and the effects of the three essential oils are shown in Figure 4.18.

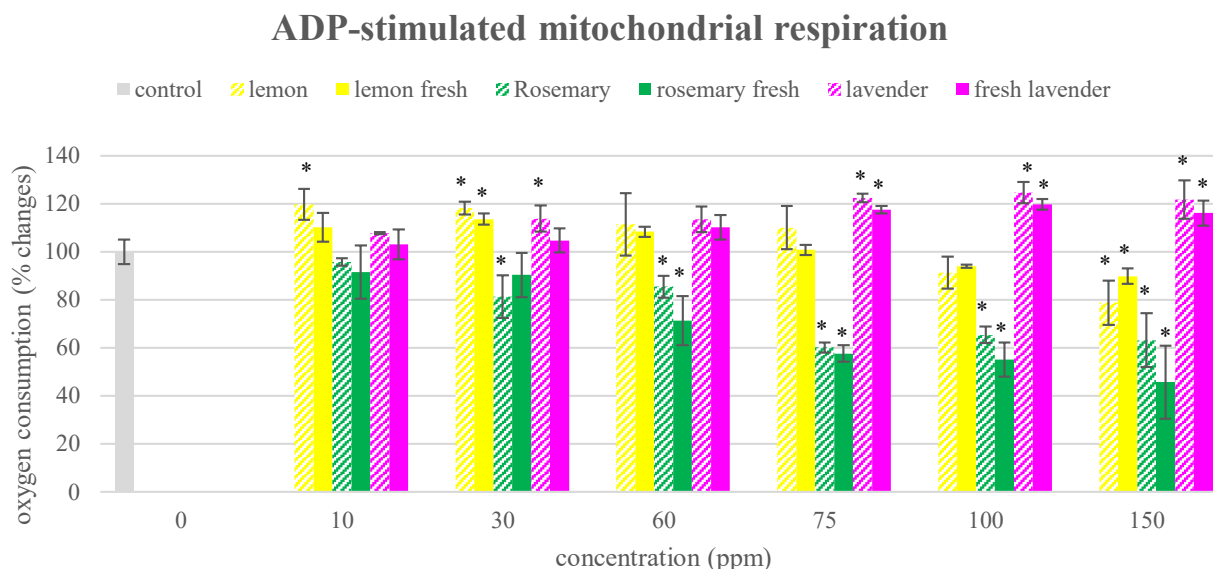


Figure 4.18 Effects of fresh and preserved essential oils on state 3 of mitochondrial respiration. 0.5 mg/mL of fresh beef heart mitochondria were energized with 5 mM glutamate + 5 mM malate and added with 0.25 mM of Mg^{2+} -ADP in the presence or absence of increasing concentrations of the different DMSO-essential oils solutions. O_2 consumption was followed using a Clark-type electrode. Experiments were carried out in triplicate using three mitochondria preparations and expressed as % change of the activity respect the control (100%) of the mean \pm standard deviation. * indicates statistical significant difference with respect to control mitochondria, i.e. untreated with essential oils ($p \leq 0,5$, $n=3$, test T).

Fresh and preserved essential oils induced similar effects on the state 3 mitochondrial respiration, except the rosemary essential oil, of which the fresh one caused at 150 ppm a significantly more marked inhibition than the preserved oil. The lemon essential oil did not affect state 3 respiration up to 100 - 150 ppm, where it caused a reduction of 10% and 21%, respectively. Conversely, the rosemary essential oil significantly reduced the oxygen consumption in a concentration-dependent manner up to 44% at 150 ppm. The lavender essential oil, on the other hand, caused a slight activation of the state 3 respiration at higher concentrations.

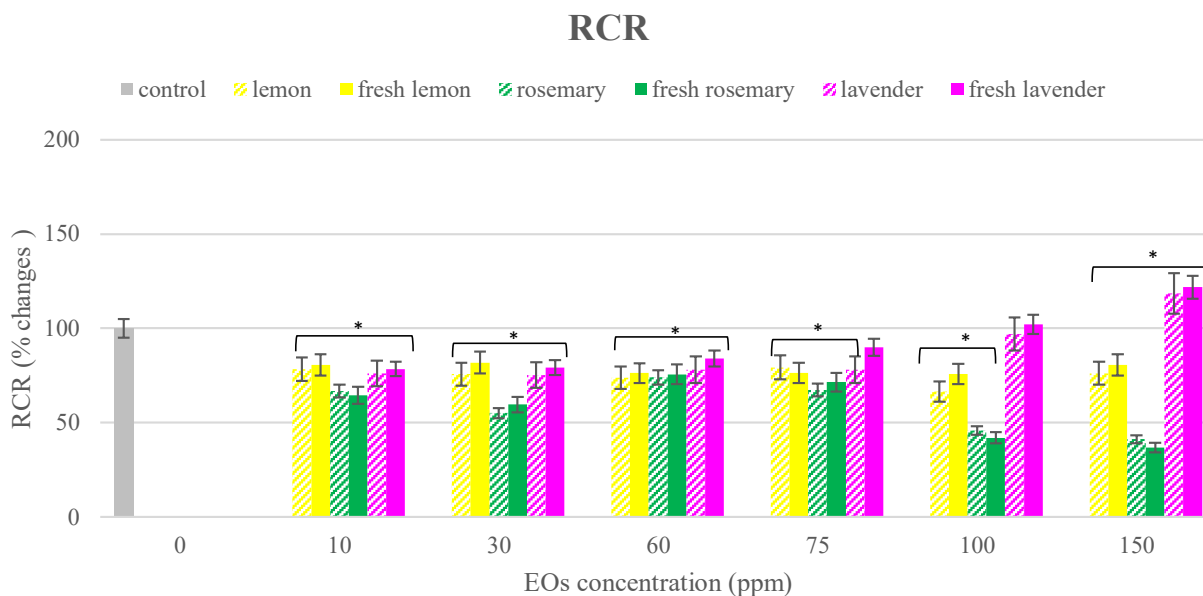


Figure 4.19 Effects of the essential oils on the respiratory control ratio (RCR). Results are expressed as percentage change of the RCR value of the untreated mitochondria (control). The mean values \pm standard deviation are reported. * indicates statistical significant difference with respect to control ($p \leq 0,5$, $n=3$, test T).

When all ADP was phosphorylated, mitochondria returned to state 4 respiration, where oxygen consumption rates similar to those measured in state 2 were observed (data not shown).

By calculating the ratio between state 3 and state 4 respiration, the RCR values were obtained

As seen in figure 20 in mitochondria treated with fresh or preserved samples of the three essential oils affected the values of the respiratory control ratio (RCR) compared to control mitochondria, although at different extent. Rosemary essential oil markedly decreased the RCR in a concentration-dependent manner, as a consequence of inhibition of the state 3 respiration, thus suggesting the induction of a proton leak through the components of the inner mitochondrial membrane. Conversely, the lemon essential oil only slightly affected mitochondrial function even at high concentrations, inducing a mild uncoupling, while the lavender essential oil did not induce any uncoupling of the oxidative phosphorylation system, even slightly increasing the RCR value at 150 ppm, possibly as a result of inhibition of the state 4 inhibition.

Mild mitochondrial uncoupling has been proposed as a therapeutic strategy under conditions that involve tissue damage associated with mitochondrial ROS formation¹⁰². Preventing mitochondrial ROS production through mild uncoupling seems a more effective antioxidant strategy than attempting to remove these species after they are formed using classical antioxidants. In fact, a number of approaches aiming to delay ageing and increase animals life span through increases in ROS scavenging capacity produced disappointing results^{103,104} and reviewed in¹⁰⁵. Conversely, calorie restriction has been widely shown to decrease mitochondrial ROS production^{106,107,108,109}, most probably because it paradoxically leads to mitochondrial uncoupling and biogenesis^{106,110}.

Mitochondrial uncoupling proteins and the adenine nucleotide translocator (ANT) have been shown to be activated by increases in levels of mitochondrial generated ROS and/or thiol oxidation¹¹¹ This suggests that these proteins can act as physiological redox sensors in mitochondria, maintaining the balance between low respiratory rate states, in which ROS production is high, and slightly increased respiratory states, in which ROS levels are low.

The lemon essential oil thus appears a promising treatment in reducing mitochondrial ROS production by both scavenging activity and mild uncoupling.

To clarify the effects of the three essential oils on the respiratory changes, further experiments were performed with the FOF1-ATPsynthase inhibitor oligomycin, a macrolide antibiotic isolated from *Streptomyces*. This antibiotic binds to the surface of c ring in the FO baseplate⁷⁸ thereby preventing protons from passing back into the mitochondria during ATP synthesis. The addition of oligomycin during the basal respiration decreased the oxygen consumption stimulated by the three essential oils, including the lemon and rosemary essential oil. These results suggest that the uncoupling effects induced by these essential oils are probably due to the formation of proton leaks, although of different extent, through the FO sector of the FOF1-ATPsynthase complex that is reverted by oligomycin.

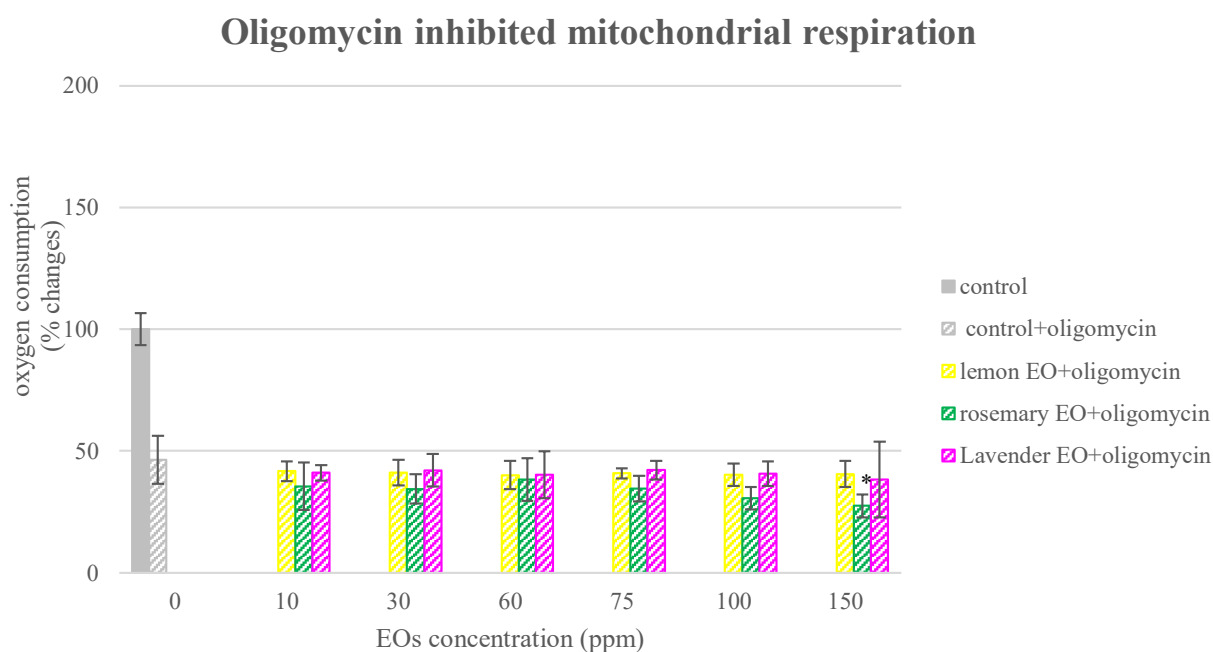


Figure 4.20 Effect of the essential oils on the oligomycin-inhibited mitochondrial respiration. 0.5 mg/mL of fresh beef heart mitochondria were incubated for 5 minutes with 2 μ M of oligomycin after that the analysis was carried out with the polarographic method. Results are expressed as percentage change of the untreated mitochondria (control), taken as 100%. The mean values \pm standard deviation are reported. * indicates statistical significant difference with respect to Control ($p \leq 0,5$, $n=3$, test T).

The maximal capacity of the mitochondrial electron transport chain was estimated by promoting protonophore-induced maximal oxygen consumption ¹¹².

Freshly isolated mitochondria were titrated with 2 μM of carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) in the presence of different concentrations of the three essential oils. As no effect was observed, the results suggest that the tested essential oils affected the basal respiration through a mechanism different from that of a protonophore like FCCP. Considering that the essential oils are mixtures of weakly acidic lipophilic molecules, it could be hypothesized that these compounds enter the mitochondria and mediate their effects through interactions with the oxidative phosphorylation complexes, including the FOF1-ATP synthase complex.

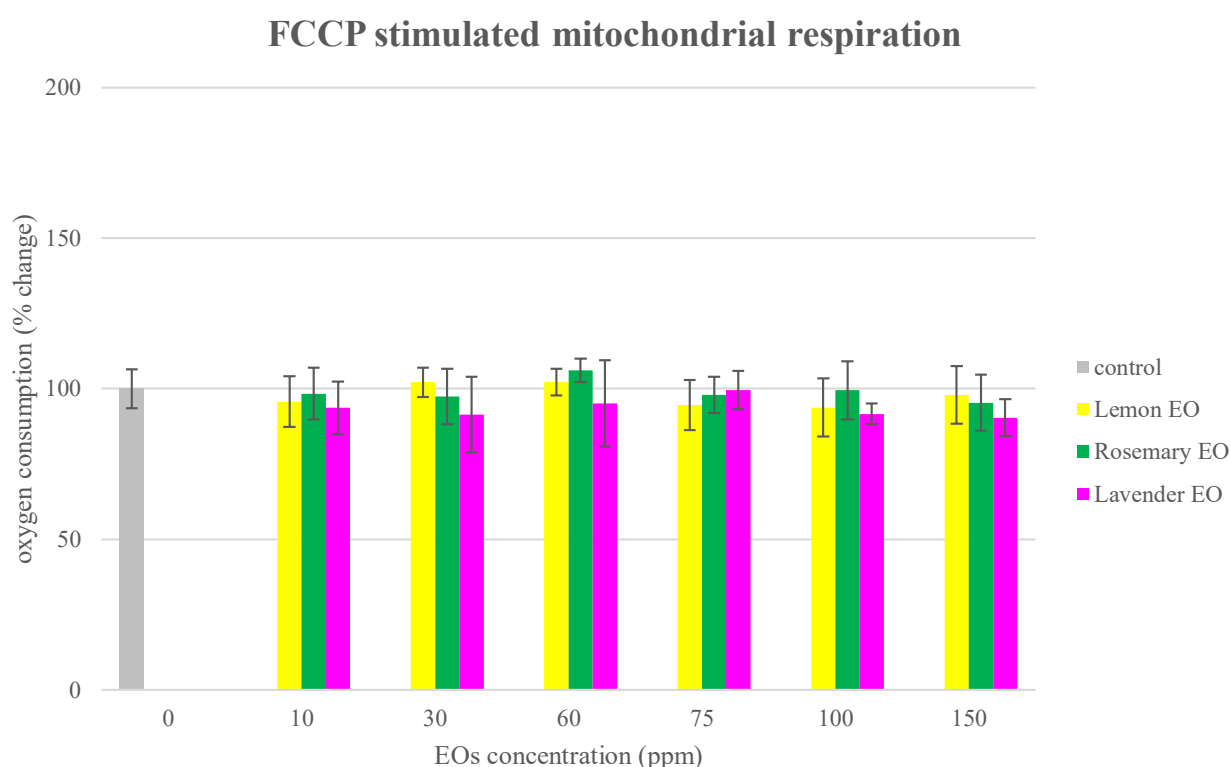


Figure 4.21 Effects of the essential oils on mitochondrial respiration maximally stimulated with FCCP. 0.5 mg/mL of fresh beef heart mitochondria were incubated for 5 minutes with 2 μM of FCCP after that the analysis was carried out with the polarographic method. Results are expressed as percentage change of the oxygen consumption of the untreated mitochondria (Control) taken as 100%. The mean values \pm standard deviation are reported. * indicates statistical significant difference with respect to Control ($p \leq 0,5$, $n=3$, test T).

As FOF1-ATP synthase can also operate in the reverse, hydrolysing ATP and pumping protons in the opposite direction, to examine the effects of the three essential oils on the FOF1-ATPase/ATP synthase activity, the ATP hydrolysis rate, also named ATPase activity, was followed in the bovine

heart sub-mitochondrial particles (SMP), in which the substrate binding sites for ATP are on the outside, as a consequence of sonication of the mitochondrial membranes.

Unexpectedly, all essential oils caused a dose-dependent inhibition of ATP hydrolysis, suggesting that in SMP additional targets on the F1 sector have been exposed during mitochondria sonication. Moreover, ATP hydrolysis was any more sensitive to oligomycin when the SMP were treated with more than 30 ppm of the lemon and lavender essential oils. As these essential oils did not affect the oligomycin sensitivity of oxygen consumption in whole mitochondria (figure 3.20), their behaviour suggested a further damage to the FO part of the complex. Conversely, the rosemary essential oil did not markedly influence the oligomycin sensitivity of ATP hydrolysis, a result that is consistent with the maintained oligomycin sensitivity of the mitochondrial oxygen consumption.

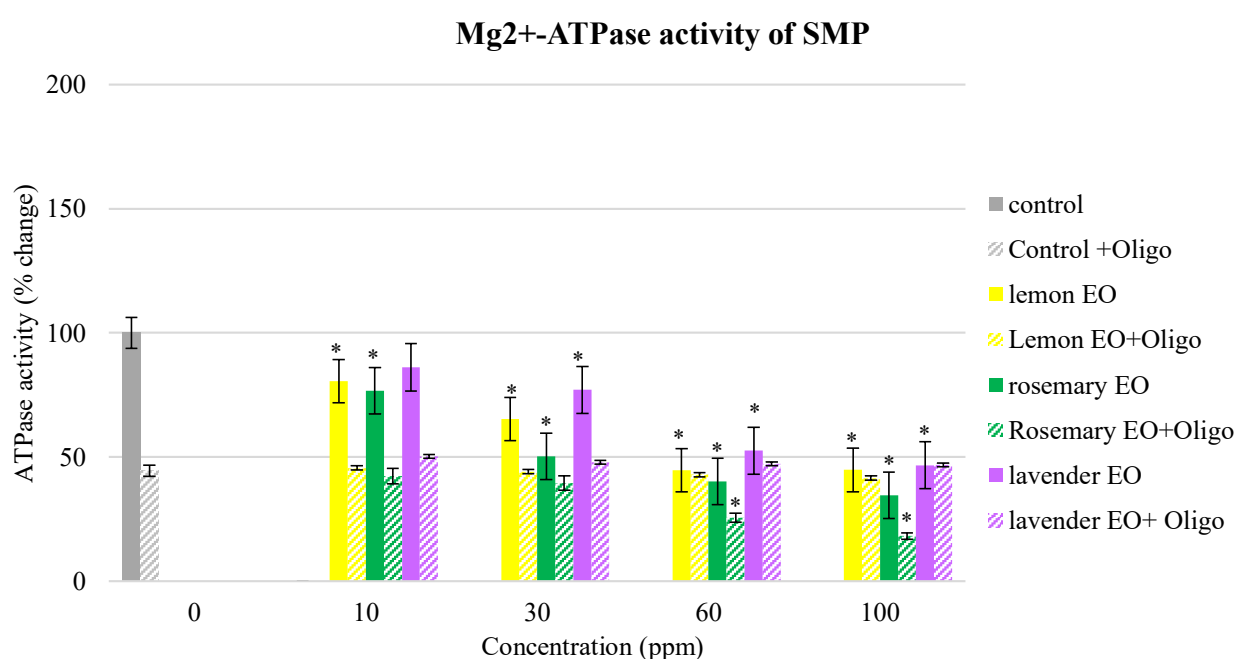


Figure 4.22 Dose-dependent effects of the essential oils on Mg²⁺-ATPase activity and oligomycin-sensitive ATPase activity of SMP. 0.25 mg/mL of SMP, with ant without 2 μM of oligomycin, were treated with increasing concentrations of the different DMSO-essential oils solutions. The activity was determined spectrophotometrically using an ATP regenerating system. All experiments were carried out in triplicate of three SMP preparations and expressed as % of the activity of the control taken as 100%. The mean values ± standard deviation are reported (p ≤ 0,5, n = 3, test T).

4.4 Effects on mitochondrial function of (*R*)- and (*S*)-limonene of the lemon essential oil and of the (*S*)-oxygenated derivatives perillyl alcohol and perillic acid.

The limonene structure has a chiral center, and thus it is found in nature as two enantiomers, i.e. the (*R*)- and (*S*)-limonene. Isomer (*R*)- has the characteristic smell of oranges, while the (*S*)- smells like lemons. In oranges, essential oil comprises 95% of (*R*)-limonene, whereas lemon peel contains mostly (*S*)-limonene. This latter terpene generally can be metabolized in plants through six different pathways giving rise to several terpenes and terpenoides. (*S*)-limonene is rapidly hydroxylated at C 7 position giving rise to (*S*)-perillic alcohol, which is further transformed to (*S*)-perillyl alcohol-8,9-epoxide, (*S*)-perillaldehyde, (*S*)-perillic acid and further metabolites (figure 4.23). At the other hand, (*R*)-limonene is mainly converted in (*S*)- perillyl alcohol¹¹³. Evidences suggest that both (+)- and (-)-limonene enantiomers are oxidized at 6- and 7-positions by the cytochromes CYP2C9 and CYP2C19 in human liver microsomes and their oxygenated derivatives show potential bioactive action¹¹⁴. These compounds finally undergo glucuronidation and excretion primarily in the urine and to lesser extent in bile¹¹⁵. The two optically active forms (*S*)-(-)-limonene and (*R*)-(+)-limonene and its oxygenated derivative (*S*)-perillyl alcohol, have been extensively tested in vivo for their anticancer properties (for skin, lung, stomach, blood and nervous system cancers). For this reason, the analyses were focused on products of the S-metabolic pathway.

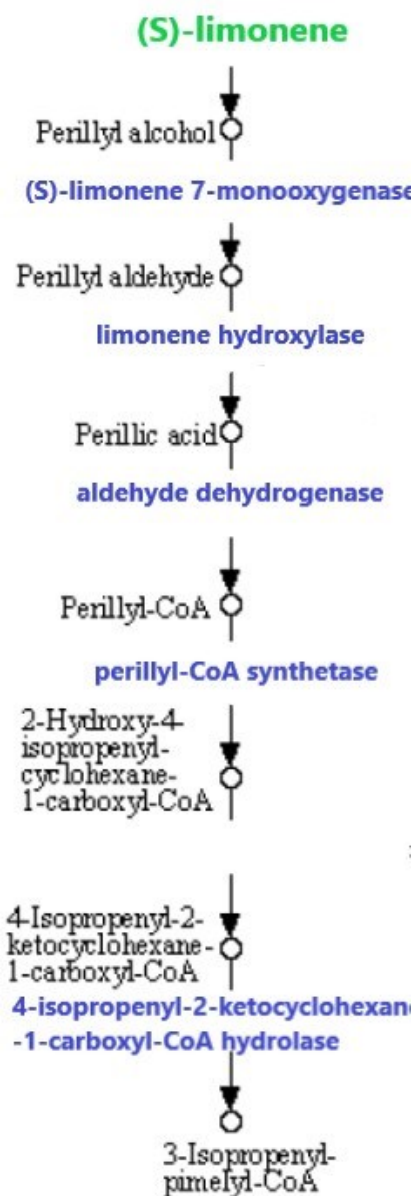


Figure 4.23 (S)-Limonene peculiar metabolic pathway adapted from Kyoto Encyclopedia of Genes and Genomes (KEGGs). <https://www.genome.jp/kegg/>

The above described results showed that the lemon essential oil produced a mild uncoupling due to activation of oxygen consumption during the basal respiration that was not paralleled by an increase in state 3 respiration. As fresh and preserved samples of the lemon essential oil similarly affected the mitochondria energetics, it was hypothesized that their main component, limonene, was responsible for the observed effect. To verify this hypothesis, the freshly isolated mitochondria were treated with increasing concentrations of both limonene enantiomers and the derivatives (S)-perillyl alcohol and (S)-perillic acid bring effects.

Basal mitochondrial respiration

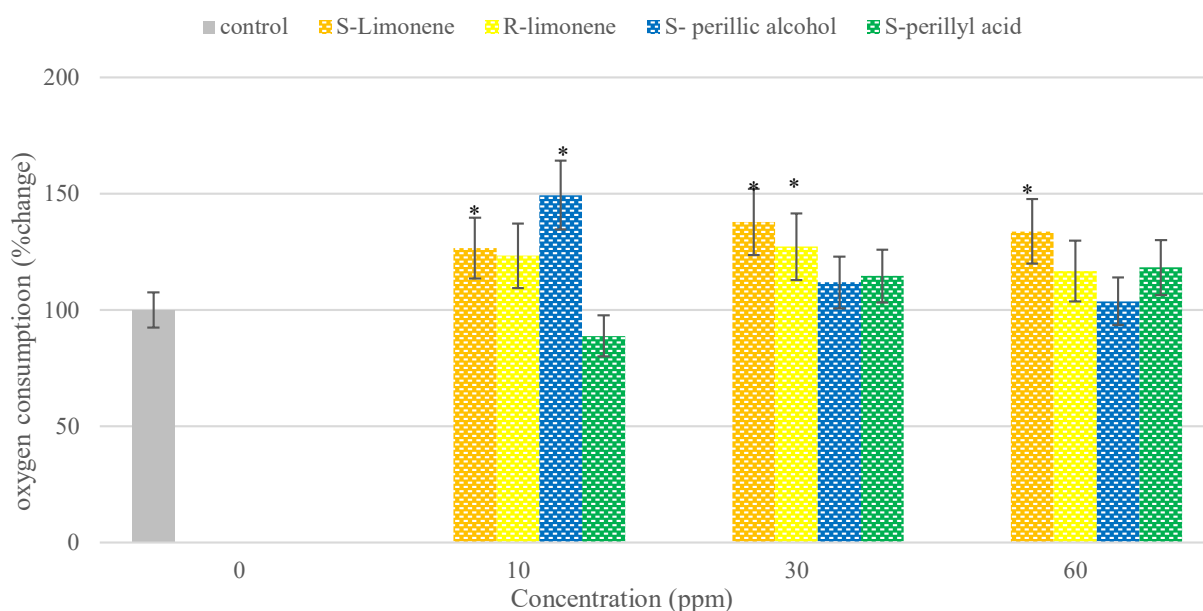


Figure 4.24 Effect of (R)-limonene, (S)-limonene, (S)-perillyl alcohol and (S)-perillic acid on basal mitochondrial respiration. 0.5 mg/mL of freshly isolated beef heart mitochondria were energized with 5 mM glutamate + 5 mM malate. Mitochondrial respiration rates were determined by O₂ consumption using a Clark-type electrode. All experiments were carried out in triplicate with three mitochondria preparations and expressed as % of the oxygen consumption of the control (taken as 100%). The mean values ± standard deviation are reported ($p \leq 0,5$, $n=3$, Test T).

ADP-stimulated mitochondrial respiration

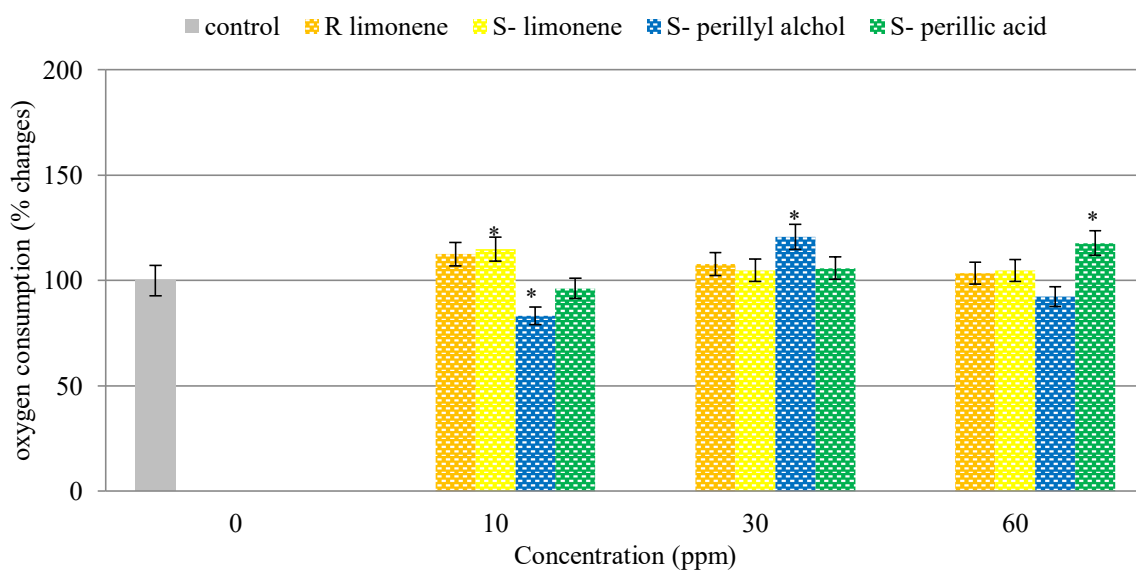


Figure 4.25 Effect of (R)-limonene, (S)-limonene, (S)-perillyl alcohol and (S)-perillic acid on mitochondrial in the state 3. 0.5 mg/mL mitochondria energized with 0.25 mM of ADP. Mitochondrial respiration rates were determined by O₂ consumption using a Clark-type electrode. All experiments were carried out in triplicate of three separate experiments. Results are expressed as percentage change of the mean ± standard deviation. * indicates statistical significant difference with respect to control ($p \leq 0,5$, $n=3$, Test T).

Figures 4.24 and 4.25 show that (*R*)-limonene and (*S*)-limonene similarly slightly activated the basal mitochondrial respiration, without affecting the oxygen consumption in state 3, causing a mild uncoupling. These effects are consistent with those observed with the lemon essential oil. Interestingly, perillic acid did not affect the basal and state 3 respiration, maintaining the RCR value unaltered at any concentration tested. Conversely, perillyl alcohol reduced the RCR value already at 10 ppm, as activation of the basal respiration was paralleled by inhibition of the ADP-stimulated respiration. On the other hand, at 30 and 60 ppm the RCR values were comparable to that of the untreated mitochondria. Such behaviour might suggest that perillyl alcohol has more than one target able to mediate opposite effects on the oxidative phosphorylation system (figure 4.26).

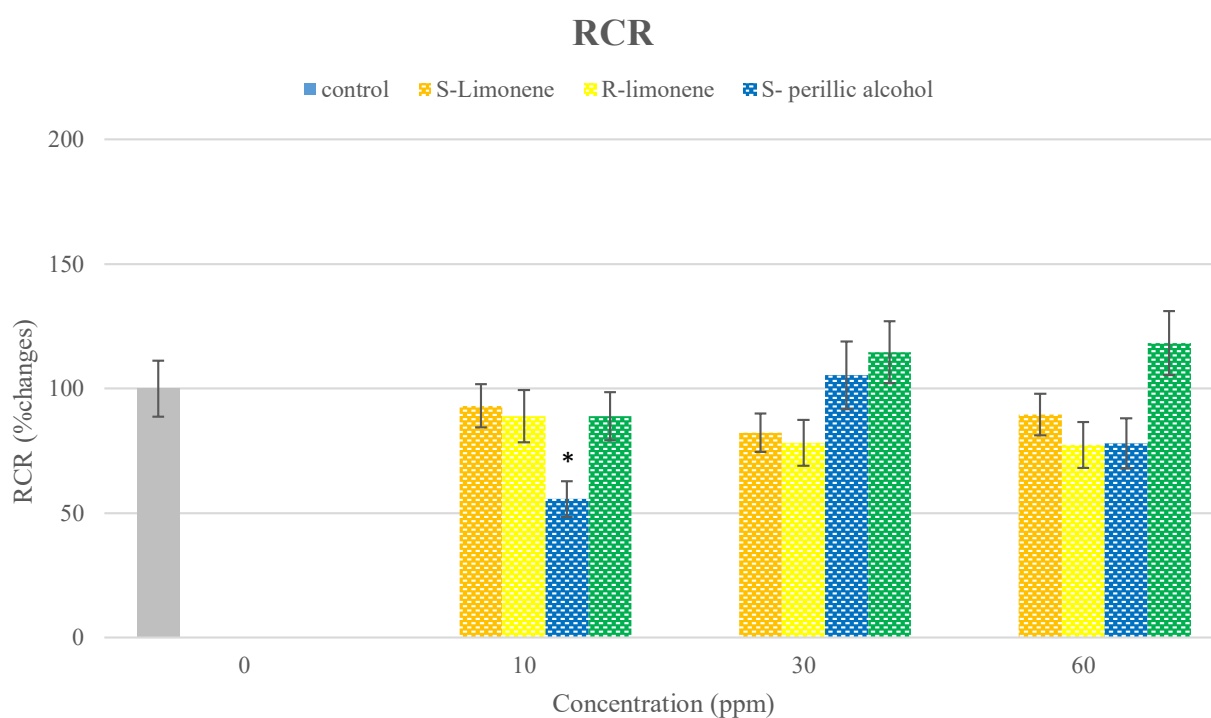


Figure 4.26 Effects of R-limonene, S-limonene, S-perillyl alcohol and S-perillic acid on the mitochondrial respiratory control ratio RCR. Results are expressed as percentage change of control value taken as 100%. * indicates statistical significant difference with respect to the control ($p \leq 0,5$, $n=3$, test T).

FCCP-induced mitochondrial respiration

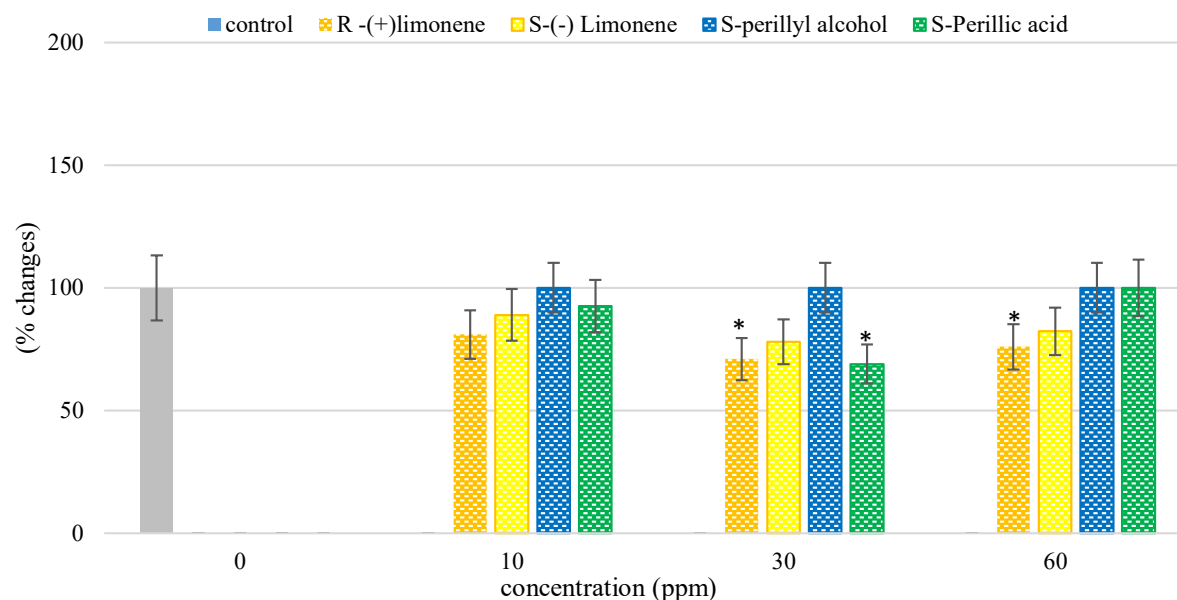


Figure 4.27 Dose-dependent effects of (R)-limonene, (S)-limonene, (S)-perillyl alcohol and (S)-perillic acid on the FCCP-stimulated respiration. Mitochondria were incubated as in Figure 24, except that 2 μ M FCCP was added. All experiments were carried out in triplicate using three mitochondria preparations and expressed as % of the activity of the control taken as 100%. The mean values \pm standard deviation are reported. * indicates statistical significant difference with respect to the control ($p \leq 0,5$, $n=3$, Test T).

After addition of FCCP, the oxygen consumption decreased in mitochondria treated with R and S-limonene suggesting that, in addition to a mild uncoupling, these compounds slightly inhibited the mitochondrial respiratory chain when maximally stimulated. Conversely, perillyl alcohol and perillic acid did not affect the uncoupled respiration.

Mg²⁺-ATPase activity of SMP

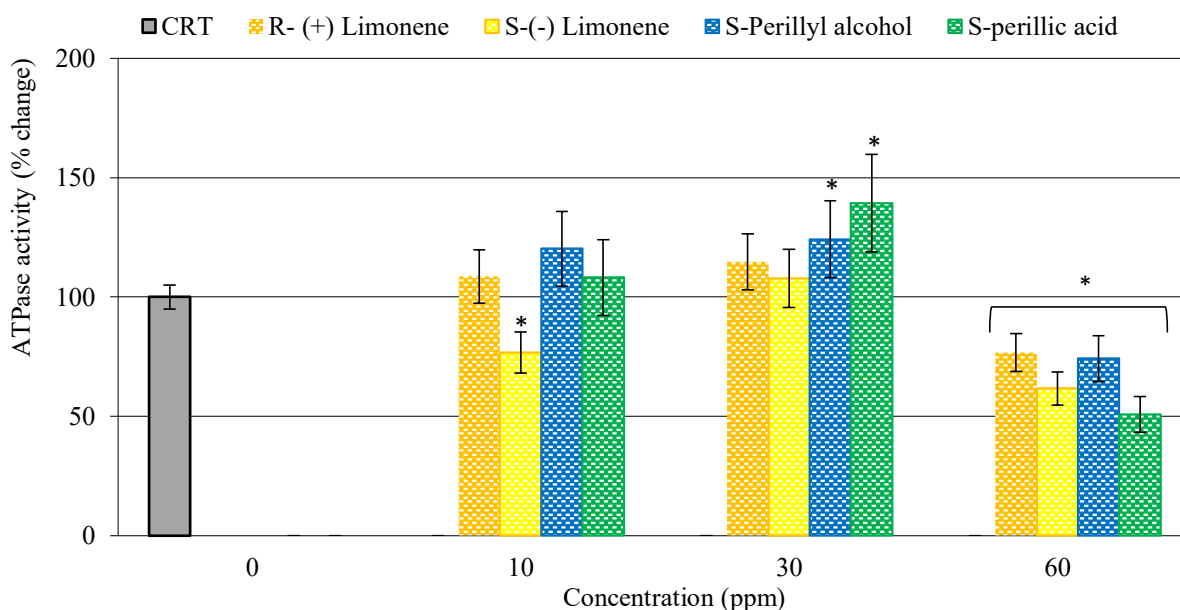


Figure 4.28 Dose-dependent effects of (*R*)- and (*S*)-limonene, (*S*)-perillyl alcohol and (*S*)-Perillic acid on Mg²⁺-ATPase activity of SMP. 0.25 mg/mL of SMP were treated with increasing concentrations of the different solutions. The activity was determined spectrophotometrically using an ATP regenerating system. All experiments were carried out in triplicate and expressed as % of the activity of the Control taken as 100%. The mean values \pm standard deviation are reported. * indicates statistical significant difference with respect to the control ($p \leq 0,5$, $n=3$, test T).

When (*R*)-limonene, (*S*)-limonene and its derivatives were tested in submitochondrial particles, the ATP hydrolysis activity was not affected at 10 and 30 ppm, while at 60 ppm was similarly inhibited, possibly as a consequence of the direct exposition to the solvent of the F1 sector that unmasked a common inhibitory site to multiple compounds.

4.5 Effects of 1,8-cineole of the rosemary essential oil on the mitochondrial function.

1,8-Cineole is traditionally used as a food flavouring agent, although for years it was believed to possess various pharmacological properties, including anti-microbial, anticancer, anti-inflammatory, antioxidation etc. activities^{47,116,117,118}. This terpenoid is the main components of the rosemary essential oil. When tested in isolated mitochondria, 1,8-cineol caused effects similar to those induced by the rosemary essential oil, increasing the basal respiration (figure 4.29) and inhibiting the ADP-stimulated oxygen consumption already at low concentrations (figure 4.30), thus acting as an uncoupler of the oxidative phosphorylation system, as evidenced by the low RCR with respect to the untreated mitochondria (figure 4.31).

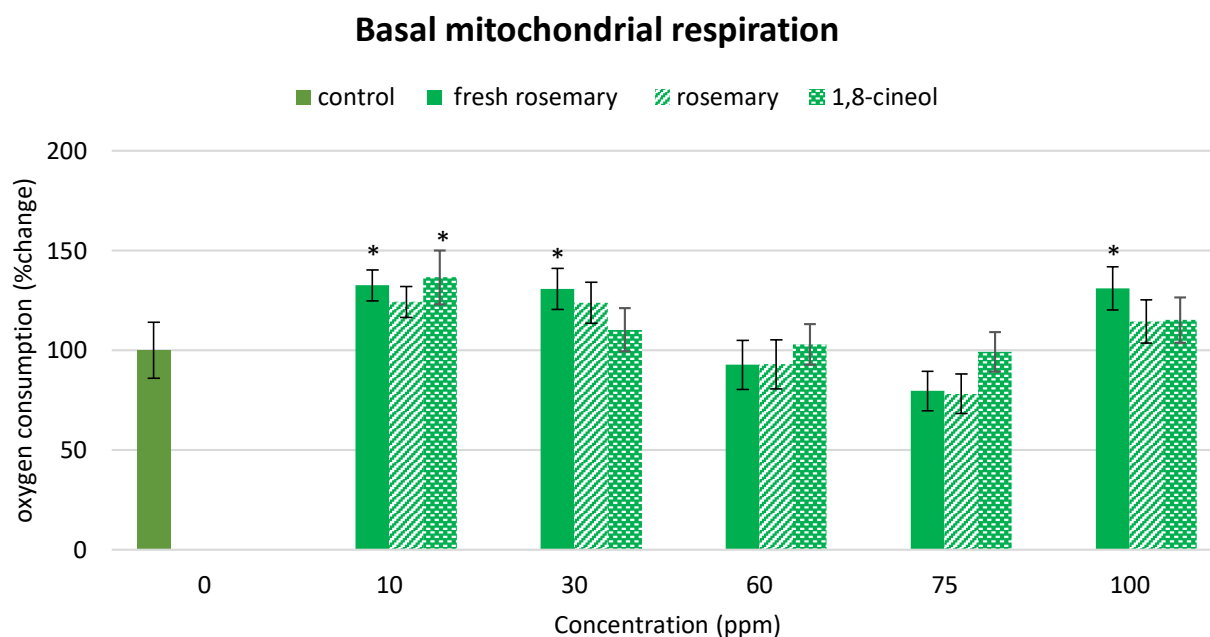


Figure 4.29 Effect of 1,8-cineol and of the rosemary essential oil on the basal mitochondrial respiration. 0.5 mg/mL of fresh beef heart mitochondria were added with 5 mM glutamate + 5 mM malate in the presence or absence of increasing concentrations of the different DMSO-essential oils or 1,8-cineol solutions. Mitochondrial respiration rates were determined by O₂ consumption using a Clark-type electrode. All experiments were carried out in triplicate using three mitochondrial preparations and expressed as % of the activity of the untreated mitochondria (Control) taken as 100%. The mean values \pm standard deviation are reported. * indicates statistical significant difference with respect to the control ($p \leq 0,5$, $n=3$, Test T).

These results suggest that the uncoupling effect due to the rosemary essential oil might be mediated by its main component 1,8-cineol, which possesses a marked uncoupling activity, affecting both the electron transport chain and the ATP synthase.

Mitochondrial ADP-stimulated respiration

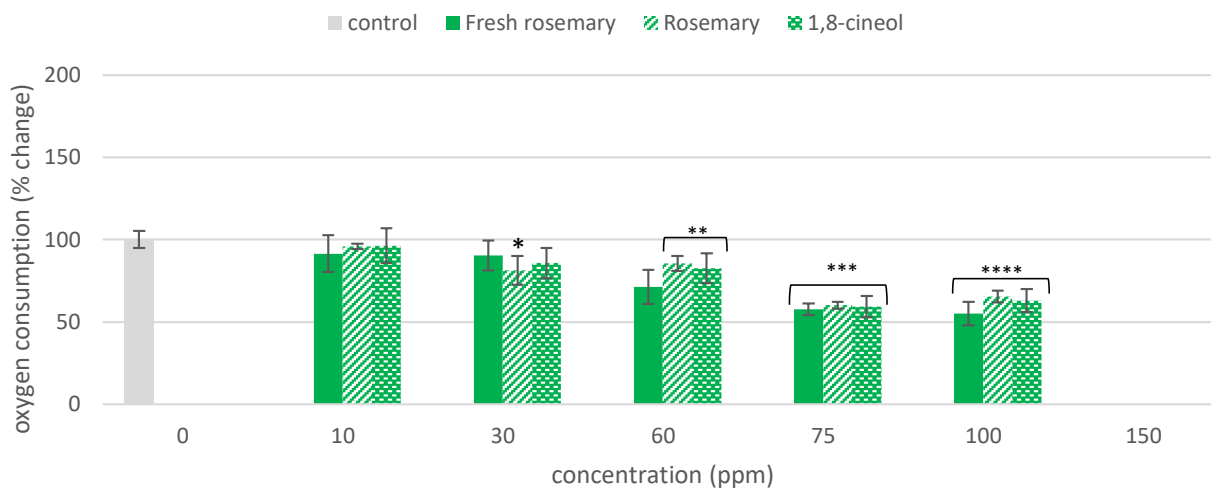
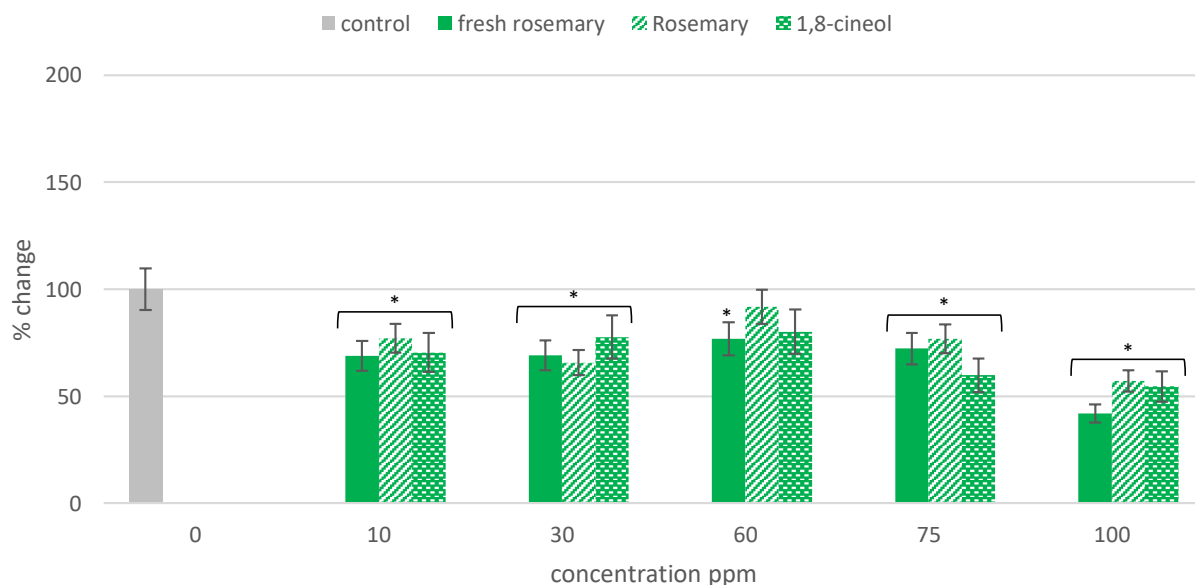


Figure 4.30 Effect of 1,8-cineol and of the rosemary essential oil on the ADP-stimulated mitochondrial respiration. 0.5 mg/mL of fresh beef heart mitochondria were added with 5 mM glutamate + 5 mM malate and 0.25 mM of Mg²⁺-ADP in the presence or absence of increasing concentrations of the different DMSO-essential oils or 1,8-cineol solutions. Mitochondrial respiration rates were determined by O₂ consumption using a Clark-type electrode. All experiments were carried out in triplicate using three mitochondrial preparations and expressed as % of the activity of the untreated mitochondria taken as 100%. The mean values ± standard deviation are reported. * indicates statistical significant difference with respect to the control (p≤0,5, n=3, Test T).

RCR



v

Figure 4.31 Effect of 1,8-cineol and of the rosemary essential oil on the mitochondrial respiratory control ratio RCR Results are expressed as percentage change of control value taken as 100%. * indicates statistical significant difference with respect to the control (p≤0,5, n=3, test T)

1-8 cineol, similarly to the rosemary essential oil, also inhibited the ATPase activity of the SMP, which maintained, at least partially, the oligomycin sensitivity (figure 4.32). These results suggest that the main component of the rosemary essential oil 1,8-cineol is mainly involved in the mitochondria damage, although the contribution of other components could not be excluded, such as alpha-pinene, camphor and p-cymene.

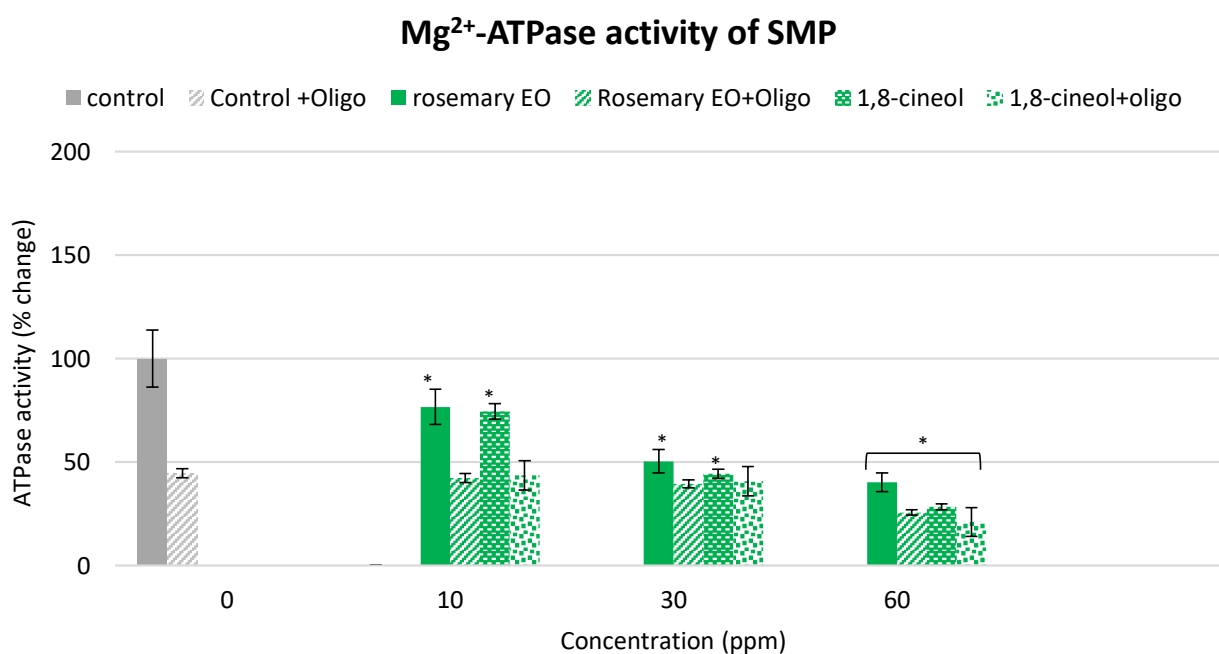


Figure 4.32 Effect of 1,8-cineol and of the rosemary essential oil on Mg²⁺ ATPase activity and oligomycin-sensitive Mg²⁺ ATPase activity of SMP. 0.25 mg/mL of SMP, with ant without 2 μ M of oligomycin were treated with increasing concentrations of the different DMSO-essential oils or 1,8-cineol solutions. The activity was determined spectrophotometrically using an ATP regenerating system. All experiments were carried out in triplicate using three SMP preparations and expressed as % of the activity of the control taken as 100%. The mean values \pm standard deviation are reported ($p \leq 0,5$, $n=3$, Test T).

4.6 Effect of the lavender essential oil and linalool on mitochondrial respiration and ATP synthase activity

According to the literature linalool has anticancer activity and *in vitro* and *in vivo* studies suggest that the induction of oxidative stress could be involve in this activity¹¹⁹.

The figures 4.33, 4.34 and 4.35 report the effects of different concentrations of linalool, in comparison with the lavender essential oils on the state 2, state 3 oxygen consumption and the RCR values, respectively. Differently from the lavender essential oil, linalool caused a decrease of the state 2 oxygen consumption, which was dependent on the concentration. In state 3 linalool induced a drastic inhibition of the oxygen consumption already at the lower concentrations, generating a marked uncoupling evidenced by the low RCR. These results suggest that lavender essential oils with high content of linalool might damage the mitochondrial oxidative phosphorylation system.

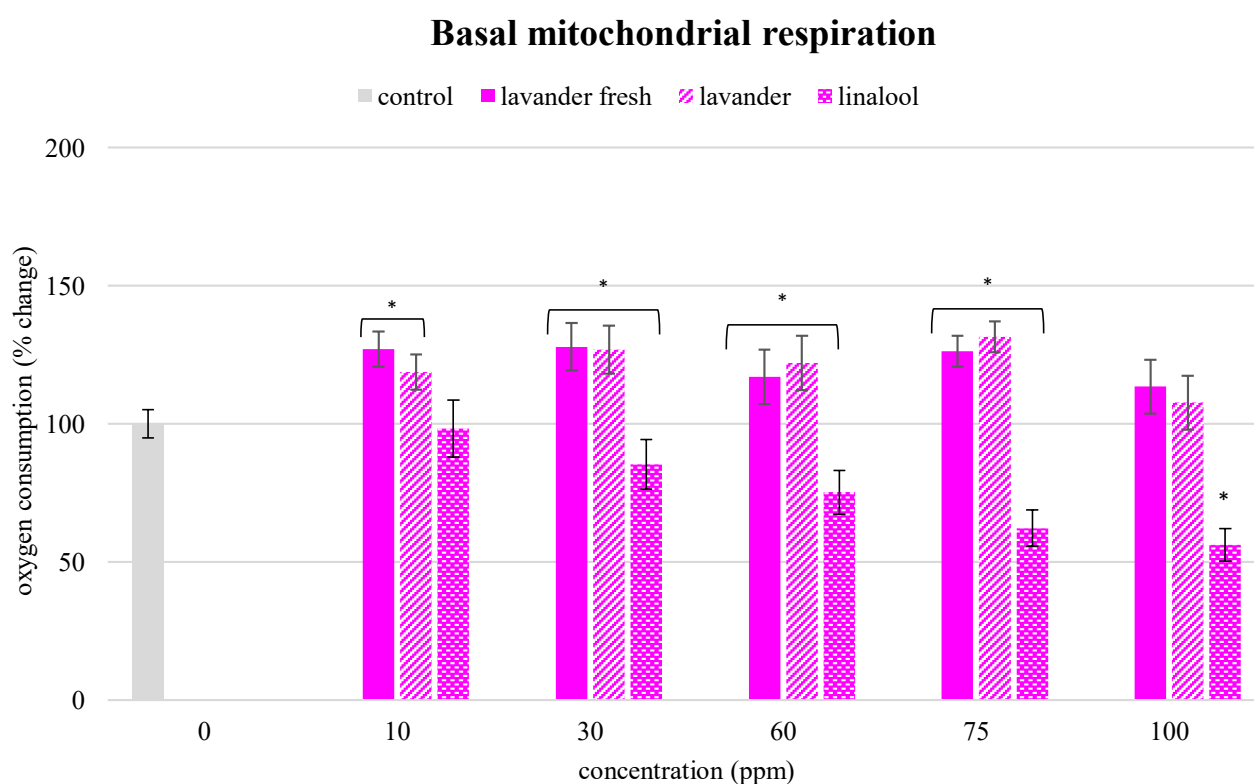


Figure 4.33 Effect of the lavender essential oils and linalool on the basal mitochondrial respiration. 0.5 mg/mL of fresh beef heart mitochondria were added with 5 mM glutamate + 5 mM malate in the presence or absence of increasing concentrations of the different DMSO-essential oils or linalool solutions. Mitochondrial respiration rates were determined by O₂ consumption using a Clark-type electrode. All experiments were carried out in triplicate using three mitochondrial preparations and expressed as % of the activity of the untreated mitochondria taken as 100%. The mean values ± standard deviation are reported. * indicates statistical significant difference with respect to the control ($p \leq 0,05$, $n=3$, Test T).

ADP-stimulated mitochondrial respiration

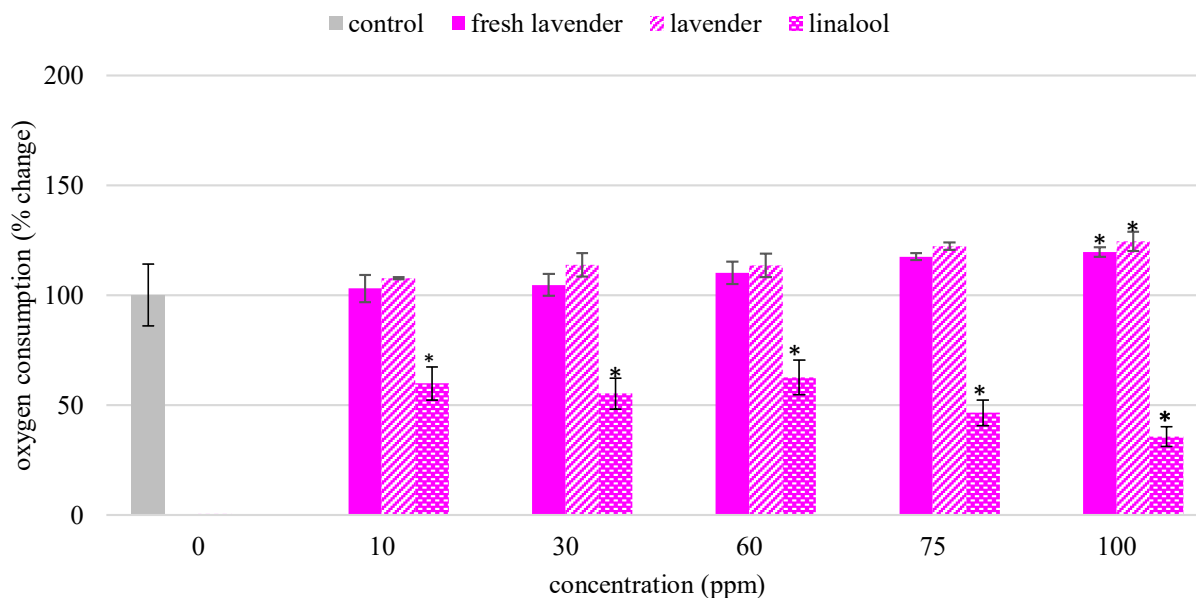


Figure 4.34 Effect of the lavender essential oils and linalool on the ADP-stimulated mitochondrial respiration. 0.5 mg/mL of fresh beef heart mitochondria were added with 5 mM glutamate + 5 mM malate and 0.25 mM of ADP in the presence or absence of increasing concentrations of the different DMSO-essential oils or linalool solutions. Mitochondrial respiration rates were determined by O₂ consumption using a Clark-type electrode. All experiments were carried out in triplicate using three mitochondrial preparations and expressed as % of the activity of the untreated mitochondria taken as 100%. The mean values ± standard deviation are reported. * indicates statistical significant difference with respect to the control (p ≤ 0,5, n=3, Test T).

RCR

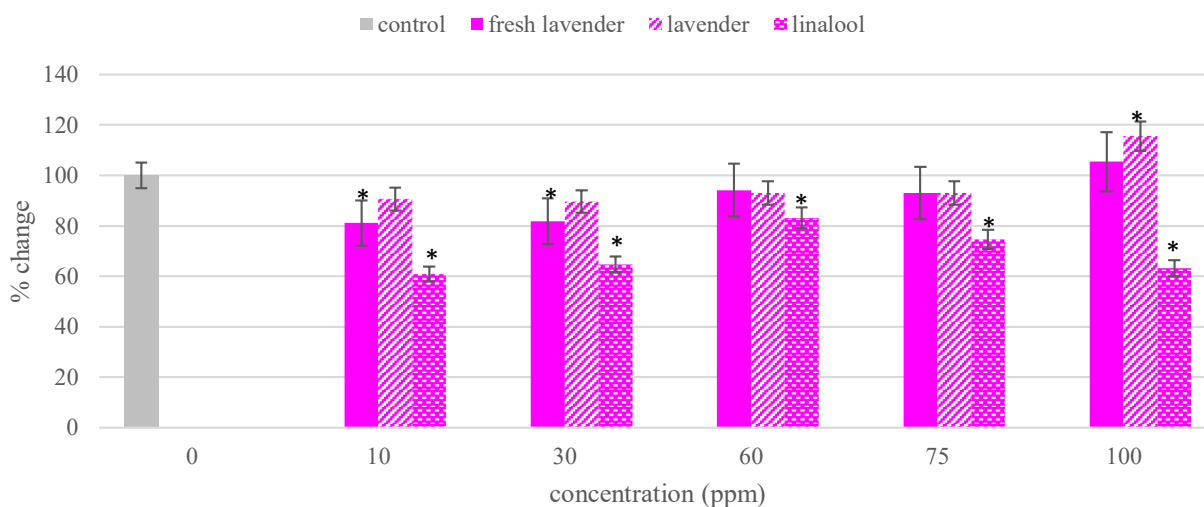


Figure 4.35 Effect of the lavender essential oils and linalool on the mitochondrial respiratory control ratio RCR. Results were expressed as % of the activity of the untreated mitochondria taken as 100%. * indicates statistical significant difference with respect to the control (p ≤ 0,5, n=3, test T)

Consistently with the effects on the ADP-stimulated oxygen consumption, linalool strongly inhibited the FOF1ATPase activity in SMP (figure 4.35), suggesting that linalool could have also this complex as target. Interestingly, the ATPase activity remained sensitive to oligomycin, suggesting that linalool did not interact with the FO sector of the complex. Differently from linalool, the lavender essential oil at high concentrations affected the oligomycin sensitivity of the SMP, thus excluding that this effect was mediated by linalool.

Mg²⁺-ATPase activity of SMP

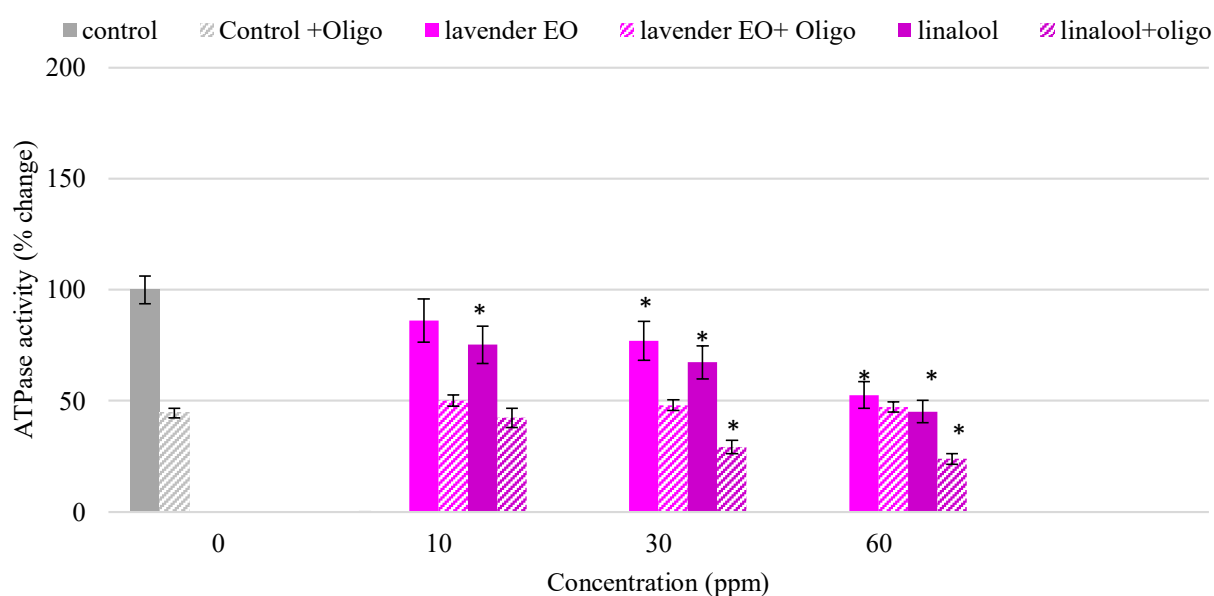


Figure 4.36 Effect of the lavender essential oils and linalool on Mg²⁺ ATPase activity and oligomycin-sensitive Mg²⁺ ATPase activity of SMP. 0.25 mg/mL of SMP, with ant without 2 μ M of oligomycin, were treated with increasing concentrations of the different DMSO-essential oils or linalool solutions. The activity was determined spectrophotometrically using an ATP regenerating system. All experiments were carried out in triplicate using three SMP preparations and expressed as % of the activity of the control taken as 100%. The mean \pm standard deviation is reported ($p \leq 0,5$, $n=3$, Test T).

4.7 Effects on the mitochondrial calcium retention capacity (CRC) of (R)-limonene and (S)-limonene of the lemon essential oil.

Ca²⁺ homeostasis supports the cell ability to integrate stimuli, trigger Ca²⁺ signals and ultimately control molecular pathways essential for cellular physiology. Mitochondrial dysfunction may be the result of a sudden increase in permeability of the inner mitochondrial membrane, via persistent Ca²⁺-dependent opening of a multi-protein channel known as the mitochondrial permeability transition pore (PTP), whose main component has been recently proposed to be the FOF1-ATP synthase⁵⁷. This is followed by unregulated flux of water, ions and small solutes into and out of the mitochondrial matrix, resulting in rupture of the outer mitochondrial membrane and eventually cell death by necrosis/apoptosis. The calcium retention capacity (CRC) assay challenges isolated mitochondria with spikes of calcium ions. Upon opening of the PTP, Ca²⁺ leaks into the assay buffer and increases fluorescence of the membrane-impermeable CalciumGreen™ dye. As (R)- and (S)-limonene affected both basal and ADP-stimulated respiration suggesting a possible effect on the F-ATP synthase, their effect on the CRC in digitonin-permeabilized cells has been tested. The results of figure 4.37 show that R-limonene and its enantiomer didn't affect propensity of the PTP to open, making their use safe.

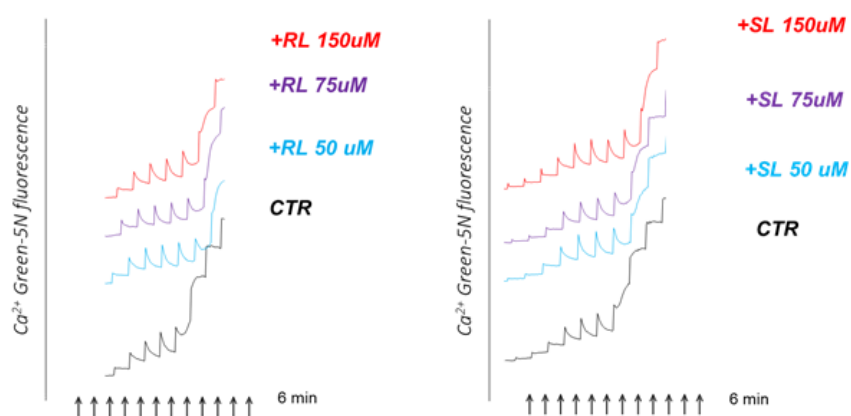


Figure 4.37 Ca^{2+} retention capacity (CRC) of HeLa cells in the presence of (S)-limonene or (R)-limonene. Extra-mitochondrial Ca^{2+} in digitonin-permeabilized HeLa cells was measured fluorometrically with the Ca^{2+} -binding green fluorescent dye Calcium-Green® 5 N in the presence of 50, 75, 150 μM of (R)-limonene or (S)-limonene, respectively as specified in methods.

CHAPTER 6

DISCUSSION

Essential oils are liquid mixtures of volatile compounds obtained from aromatic plants. Several species of medicinal plants have long been used for the complementary treatment of obesity and diabetes in various systems of medicine²⁹. Later studies aimed at defining the molecular mechanisms underlying the biological effects of essential oils have proposed that the lemon essential oil causes activation of the sympathetic nerve activity innervating the white adipose tissue, increasing lipolysis and resulting in the suppression of body weight gain³¹. Moreover, lemon essential oil significantly reduces lipid peroxidation levels and nitrile content, by increasing the reduced glutathione levels, as well as the superoxide dismutase, catalase, and glutathione peroxidase activities in mouse hippocampus¹²⁰. Consistently, (R)-limonene, a main component of the lemon essential oil, was reported to protect against the development of dyslipidaemia and hyperglycaemia in high fat diet (HFD)-fed mice and in obese mice, ameliorating the insulin resistance and lipid profiles. These effects appeared to be mediated through activation of the PPAR α and inhibition of the LXR β signalling pathways⁴⁰. On the other hand, as the lemon essential oil, similarly to all essential oils, is a rich source of volatile organic compounds, it is commonly used in aromatherapy for weight loss program³¹.

The rosemary essential oil was also reported to have hypolipidemic and anti-hyperglycaemic activities in (HFD)-fed mice¹²¹, but the underlying mechanisms remain to be defined.

Many essential oils have antioxidant properties, as tested *in vitro*, that could play an important role *in vivo* by protecting from oxidative stress-induced damage. This is indeed at the base of various disorders including diabetes and obesity. On the other hands, the lipophilic nature of the EOs might suggest their ability to enter the mitochondria affecting the oxidative phosphorylation system, which is crucial for ATP production from nutrient oxidation. Indeed, the excess of nutrients (commonly associated with obesity) can overwhelm the Krebs cycle and the mitochondrial respiratory chain leading to mitochondrial dysfunction. For these reasons, mild mitochondrial uncoupling has been proposed as a therapeutic strategy for increasing nutrient oxidation and reducing ROS production by the respiratory chain. As such, the field of “mitochondrial medicine” is emerging in which disease states are being targeted therapeutically at the level of the mitochondrion, including specific antioxidants, bioenergetic substrate additions, and membrane uncoupling agents¹²².

When the mitochondrial uncoupling is mild, there can still be a build-up and maintenance of membrane potential across the inner mitochondrial membrane, thus allowing the synthesis of ATP and avoiding the opening of the permeability transition pore, a high conductance channel that is causally involved in cell death in several diseases⁵⁶. On the other hands, mild uncouplers increase the oxygen consumption and keep low the mitochondrial electrochemical gradient, decreasing ROS production. Conversely, inhibition of the ATP synthase capacity leads to accumulation of the

electrons in the upstream complexes of the respiratory chain, promoting oxidative stress (review). Mild uncoupling was obtained by different ways, i.e. by activation of the endogenous Uncoupling Proteins (UCPs), such as in obese diabetic mice where UCP2 overexpression restored the impaired endothelial function¹²³ or by the use of chemical uncouplers, such as niclosamide ethanolamine, which improved diabetic symptoms in mice¹²⁴ or the novel mitochondrial uncoupler BAM15, which has no off-target activity at other cellular membranes¹²⁵. Nevertheless, caution is always required when targeting mitochondrial uncoupling via lipophilic weak acids, even when selective, as, differently from the UCPs, their activity lacks of auto-regulation.

In order to compare the direct effects of *Citrus limon*, *Rosmarinus officinalis* and *Lavandula angustifolia* essential oils on mitochondrial oxidative phosphorylation system, the mitochondrial respiration rates in various metabolic states were measured. Before such analyses, the chemical characterization of fresh samples and of samples maintained in the dark at 4 °C of each essential oil was performed by GC-MS and NMR. The identification of the main components, i.e. limonene, 1.8-cineol and linalool in the *Citrus limon*, *Rosmarinus officinalis* and *Lavender angustifolia* essential oil respectively, allowed to confirm the plant chemotypes. Interestingly, these analyses highlighted that only slight changes of the oils chemical profiles occurred during storage that do not seem to influence their scavenging activity, at least that measured *in vitro* by the DPPH and ABTS assays.

When tested in freshly isolated beef heart mitochondria, all essential oils affected the oxygen consumption, although to different extent. Both lemon and lavender essential oils slightly increased the basal oxygen consumption. In state 3, i.e. when the redox reactions of the respiratory chain were coupled to the synthesis of ATP, the lemon essential oil caused a gradual inhibition of the oxygen consumption at concentrations higher than 75 ppm, while the lavender oil did not affect the respiration even at 150 ppm. Conversely, the rosemary essential oil, after an initial activation of the basal oxygen consumption, induced a marked inhibition at concentrations between 60 to 75 ppm that was followed by an activation at higher ppm. In state 3 this oil inhibited the oxygen consumption in a concentration-dependent manner, markedly decreasing the respiratory control ratio (RCR) value and resulting in a strong mitochondrial uncoupling. On the contrary, the lemon essential oil caused a mild uncoupling, while the lavender essential oil even slightly increased the RCR value due to an activation of the state 3 respiration proportionally greater than that obtained in state 4.

As the addition of the FOF1-ATP synthase inhibitor oligomycin during the basal respiration reverted the oxygen consumption stimulated by the three essential oils, the uncoupling effects induced by the lemon and rosemary essential oils are probably due to formation of proton leaks through the FO sector of the FOF1-ATP synthase complex that are reverted by oligomycin. Consistently, the FCCP-stimulated maximal capacity of the mitochondrial electron transport chain was not affected by the

three essential oils, suggesting a mechanism different from that of a protonophore that increases the oligomycin-insensitive proton leak of the inner mitochondrial membrane.

When the effects of the three essential oils on the FOF1-ATP synthase activity were tested in bovine heart sub-mitochondrial particles (SMP), in which the substrate binding sites are on the outside, the results confirmed that the rosemary essential oil did not affect the oligomycin sensitivity. On the other hand, the lemon and lavender essential oils affected the oligomycin sensitivity, in contrast with the effects observed on the oxygen consumption in whole mitochondria, suggesting that in SMP additional targets for essential oils components have been exposed.

The experiments using the main components of the three essential oils, i.e. limonene and derivatives, 1,8-cineol, and linalool, provided interesting results. While (S)-limonene and (R)-limonene and 1,8-cineol appeared to be directly involved in the peculiar uncoupling effects induced by the respective essential oils, linalool, differently from the lavender essential oil, induced a drastic mitochondrial uncoupling, suggesting caution in the use of this essential oil with high content of linalool.

S-limonene can be transformed through the C7 oxidation in perillyl alcohol and perillic acid. These compounds elicited dose-dependent cytotoxicity, inducing cell cycle arrest and apoptosis by increasing the expression of the mitochondrial protein BAX, the cyclin-dependent kinase inhibitor p21 and the caspase-3¹²⁶. Several studies in preclinical animal tumor models have characterized perillyl alcohol as a powerful chemotherapeutic agent against different cancer types, including pancreatic, breast, liver, and brain cancers¹²⁷.

The experiments reported in this thesis on isolated mitochondria highlighted the marked uncoupling effect of the perillyl alcohol that, differently from the perillic acid, reduced the RCR value already at 10 ppm. Such effect may be consistent with the pro-apoptotic effect of perillyl alcohol¹²⁶. On the other hand, at 30 and 60 ppm this compound did not affect the RCR values, which were comparable to that of untreated mitochondria. Such behaviour might suggest that perillyl alcohol has more than one target able to mediate opposite effects on the oxidative phosphorylation system, thus suggesting caution in its therapeutic use.

CHAPTER 6

CONCLUSION

The heterogeneity of essential oil chemical composition requires particular attention in their use for human health. Although all three essential oils tested in this study are reported to be potentially efficient in preventing and treating obesity and its metabolic complications, the chemistry, bioavailability, and efficacy of their constituents for human health are still only partially defined. Such lack limits understanding the mechanisms underlying their beneficial effects. Lemon and rosemary essential oils are both known to have an anti-obesity action but, the results presented in this thesis show that they act through diverse mechanisms that could differently impact on human health. Based on its effects on the mitochondrial bioenergetics, only the lemon essential oil caused a mild uncoupling of the oxidative phosphorylation system, leading to propose its therapeutic use for increasing nutrient oxidation and reducing ROS production by the respiratory chain. However, the still limited data on the essential oils efficacy in human, as well as the lack of information regarding their mechanisms of action lead to the conclusion that further studies are required to reach a definitive recommendation for their use in human healthcare.

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