EFFECT OF ESSENTIAL OILS AND THEIR MAJOR COMPOUNDS ON ENDOTHELIAL DYSFUNCTION ASSOCIATED WITH PULMONARY ARTERIAL HYPERTENSION

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Thesis conducted under the supervision of Doctor Lígia Salgueiro and Doctor Henrique Girão in fulfilment of the requirements for the Master's Degree in Industrial Pharmaceutical Chemistry and presented to the Faculty of Pharmacy of the University of Coimbra

September 2018

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Abbreviations

Ang 1	Angiopoietin 1
Atg	Autophagy-related protein
Baf	Bafilomycin A1
BSA	Bovine serum albumin
CHX	Cycloheximide
CMA	Chaperone-mediated autophagy
CR	Caloric restriction
CVDs	Cardiovascular diseases
Cx	Connexin
DMEM	Dulbecco's Modified Eagle Medium
EC	Endothelial cell
ECGF	Endothelial cell growth factor
EMA	European Medicines Agency
EO	Essential oil
EPA	Environmental Protection Agency
ESC	European Society of Cardiology
ESCOP	European Scientific Cooperative on Phytotherapy
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FEMA	Flavor and Extract Manufacturer's Association
FID	Flame ionization detector
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectroscopy
GFP	Green fluorescent protein
GJ	Gap junction
GJIC	Gap junction-mediated intercellular communication
GRAS	Generally regarded as safe
HPAEC	Human pulmonary arterial endothelial cell
HR	Heart rate
HRP	Horseradish peroxidase
IA	Intussusceptive angiogenesis
IC	Intercelular communication

IF	Immunofluorescence
ISO	International Standard Organization
LC3	Microtubule-associated protein 1 light chain 3
LIR	LC3-interacting region
LSGS	Low serum growth supplement
MCEC-1	Mouse cardiac endothelial cell line
MEP/DOXP	2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate
mPAP	Mean pulmonary arterial pressure
MVA	Mevalonic acid
p62/SQSTM1	Sequestosome 1
PA	Pulmonary artery
PAH	Pulmonary arterial hypertension
PAWP	Pulmonary arterial wedge pressure
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PH	Pulmonary hypertension
PVR	Pulmonary vascular resistance
RH	Right heart
RHC	Right heart catheterization
RV	Right ventricle
RVH	Right ventricular hypertrophy
SA	Sprouting angiogenesis
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS-T	Tris-buffered saline-Tween 20
VEGF	Vascular endothelial growth factor
WB	Western blot
WHO	World Health Organization

Resumo

A hipertensão arterial pulmonar (HAP) é uma doença rara, caraterizada por uma pressão arterial pulmonar elevada, causada por uma obliteração progressiva das pequenas artérias pulmonares que podem levar à insuficiência cardíaca direita. Esta oclusão arterial é principalmente devida à disfunção das células endoteliais e à proliferação anormal do tecido muscular liso circundante. Doentes com HAP têm uma qualidade de vida reduzida e uma baixa esperança média de vida devido à falta de tratamentos eficazes. Na verdade, as terapias existentes, além de caras, apenas proporcionam pequenas melhorias a curto prazo, justificando a procura de novos agentes terapêuticos.

Terpenos de baixo peso molecular têm sido descritos como agentes terapêuticos eficazes em patologias vasculares. Portanto, é plausível que os óleos essenciais (OEs) ricos nesses compostos também exerçam os mesmos efeitos. Além disso, é provável que sinergismos entre os terpenos presentes nesses extratos possam culminar em abordagens ainda mais eficientes. De facto, os óleos essenciais são reconhecidos como uma fonte de compostos bioativos, com muitas evidências científicas validando o seu potencial preventivo/terapêutico.

Neste contexto, o presente estudo tem como objetivo avaliar o efeito de dois OEs, *Lavandula viridis* e *Thymus zygis* subps. *sylvestris*, e dos seus principais compostos, 1,8-cineol e timol respetivamente, em várias características associadas à HAP, tais como autofagia comprometida, comunicação intercelular ineficiente e angiogénese desregulada. O presente trabalho assenta na hipótese que células endoteliais submetidas a hipóxia (de forma a mimetizar a patologia) desenvolvem características de HAP que poderão ser prevenidas ou revertidas pelo tratamento com OEs e/ou terpenos.

Utilizando diferentes abordagens complementares, o presente trabalho mostra que os OEs/terpenos estimulam a angiogénese em vários modelos *in vitro* e *ex vivo*. Demostra-se pela primeira vez que os OEs/terpenos promovem a migração e modulam a formação de tubos, e aumentam o número de sprouts angiogénicos em anéis de aorta de rato.

Por último, o efeito desses compostos foi avaliado na autofagia das células endoteliais e os resultados mostram que a presença dos OEs e do 1,8-cineol levam a um aumento dos níveis de LC3-II, sugerindo um aumento da autofagia. Além disso, em células endoteliais da artéria pulmonar humana, HPAEC, o OE de *Lavandula viridis* promove o fluxo autofágico.

Acreditamos que estes resultados abrem novos caminhos para o desenvolvimento de abordagens preventivas ou terapêuticas eficazes para a disfunção endotelial associada à HAP.

Abstract

Pulmonary arterial hypertension (PAH) is a rare disorder characterized by an elevated pulmonary arterial pressure, caused by a progressive obliteration of small pulmonary arteries, that can ultimately lead to right heart failure. This artery occlusion is primarily due to endothelial cell dysfunction and abnormal proliferation of the surrounding smooth muscle tissue. PAH patients have reduced quality of life as well as a low average life expectancy due to the lack of effective treatments. Indeed, current therapies provide small improvements with short-term benefits and are very expensive. Therefore, efficient and less costly treatments are required.

Terpenes of low molecular weight have been described as effective therapeutic agents in vascular-related disorders. Therefore, it is conceivable that essential oils (EOs) rich in these compounds may also have these beneficial effects. Moreover, it is likely that synergisms between terpenes present in these extracts, may result in more efficient approaches. Indeed, EOs are recognized as a source of bioactive compounds, with many scientific evidences validating their preventive/therapeutic potential.

In this context, the present study aims to evaluate the effect of selected EOs, namely *Lavandula viridis* and *Thymus zygis* subps. *sylvestris*, and their major compounds, 1,8-cineole (for *Lavandula viridis*) and thymol (for *Thymus zygis* subps. *sylvestris*), on several features associated with PAH, like compromised autophagy, inefficient intercellular communication and dysregulated angiogenesis. We hypothesize that endothelial cells (ECs) subjected to hypoxia (to mimic the disease) will develop PAH features that can be prevented and/or reverted by the treatment with essential oils and/or isolated terpenes.

Using different complementary approaches, we demonstrate that EOs/terpenes stimulate angiogenesis in different *in vitro* and *ex vivo* models. Indeed, we demonstrate that the treatments promote migration and modulate tube formation in ECs and increase the number of angiogenic sprouts in rat aortic rings.

Furthermore, we evaluated the effect of these compounds in the autophagic response and showed that the presence of EOs and 1,8-cineole led to an accumulation of LC3-II, suggesting an increase of autophagy. Moreover, in human pulmonary arterial endothelial cells, HPAEC, *Lavandula viridis* EO enhanced the autophagic flux.

We believe that these results will open new avenues for the development of effective preventive/therapeutic approaches for endothelial dysfunction associated with PAH.

Introduction

1. Introduction

According to the World Health Organization (WHO) cardiovascular diseases decrease the quality of life of patients and account for 31% of total deaths worldwide (WHO, 2017). These disorders are a major burden for health care systems (Stewart, Manmathan and Wilkinson, 2017) and greatly impact global economy.

Although therapeutic strategies and surgical interventions are known to improve patients' quality of life and contribute to their long-term survival, they remain insufficient, justifying novel preventive/therapeutic strategies. Indeed, alternative and/or complementary approaches have gained interest among the scientific and medical community, namely plant-based strategies (Walden and Tomlinson, 2011). Despite the development of chemically synthesized drugs, approximately 80% of the world's population still relies on herbal medicines as a source of primary health care (Thomford *et al.*, 2015). Particularly, plants represent a huge reservoir of lead compounds as many species have still not been investigated for their pharmacological potential (Hostettmann and Marston, 2003). Currently, herbal medicines are used in the treatment of chronic and acute conditions such as cardiovascular diseases (CVDs), depression, inflammation and others (Liperoti *et al.*, 2017; Wang *et al.*, 2018; Yatoo *et al.*, 2018). These effects are many times attributed to the presence of secondary metabolites, such as phenolic compounds, alkaloids and terpenes (Jenke-Kodama, Müller and Dittmann, 2008). Importantly, some of these compounds are used in drug development, directly as therapeutic agents or as starting materials and models for the synthesis of other drugs.

Bearing in mind the global social and economic impact of CVDs and the bioactive potential of plant extracts, the present study aims to highlight the effect of two essential oils, *Lavandula viridis* and *Thymus zygis* subps. *sylvestris*, obtained from Portuguese aromatic plants as well as their main isolated monoterpenes, 1,8-cineole and thymol, respectively, on the cardiovascular system.

2. Cardiovascular Diseases

Cardiovascular diseases are responsible for over 17 million annual deaths and continue to be the leading cause of mortality in Europe. It is well known that women are more affected then men (51% *vs.* 42%) and mortality rate tends to increase with age (WHO, 2017).

According to the WHO, CVDs are a general term used to describe disorders that affect the heart and blood vessels and can be classified into six groups, as summarized in Table 1.

***Table 1*. Characterization of CVDs**

CVDs	Body region affected	Pathophysiology	Mortality rate	References
Coronary Artery Disease	Coronary artery	Disease of the blood vessels supplying the heart muscle	1.2 – 2.4% *per annum*	(Montalescot, 2013)
Peripheral Arterial Disease	Carotid, mesenteric, renal, upper and lower extremities arteries	Disease of blood vessels supplying peripheral arteries	63% of patients with >50% asymptomatic carotid stenosis showed late death associated with cardiac events	(Aboyans *et al.*, 2018)
Cerebrovascular Disease	Brain	Disease of the blood vessels supplying the brain	14% (♀), 9% (♂)	(Seshadri e Debette, 2016; Townsend *et al.*, 2016)
Rheumatic Heart Disease	Muscles and valves of heart	Damage to the heart muscle and heart valves from rheumatic fever, caused by streptococcal bacteria	4.8 deaths/100000 habitants	(Watkins *et al.*, 2017)
Congenital Heart Disease	Heart	Malformations of heart structure existing at birth	77% of patients with ACHD	(Baumgartner *et al.*, 2010; Kelleher,

CVDs	Body region affected	Pathophysiology	Mortality rate	References
				2012)
Deep vein thrombosis and pulmonary embolism	Veins in the lower limbs	Venous thrombi which can dislodge and move to the heart and lungs	Short-term mortality 2 – 5%	(Mazzolai *et al.*, 2017)

Heart attacks and strokes are usually the first warning of unhealthy blood vessels. These acute events are mainly caused by a blockage of blood flow to the heart or brain, respectively, being the most common reason a build-up of fat, cholesterol, calcium, and other substances present in the blood (Scott, 2004).

The major risk factors of CVDs include age, high blood pressure (hypertension), cholesterol, obesity, elevated blood glucose, tobacco use, physical inactivity, unhealthy diet and excessive alcohol (Buttar, Li and Ravi, 2005; WHO, 2017). Hypertension is the most common disease in industrialized nations and it is the major cause of heart attack, stroke, and congestive heart failure. The European Society for Cardiology (ESC) designed specific guidelines for the prevention of CVDs that address modifiable risk factors, namely changes in lifestyles (Baumgartner *et al.*, 2017). Indeed, clinical trials have demonstrated that an healthy diet (Mediterranean-style diet, for example) in combination with physical activity, moderate alcohol consumption and no smoking decrease mortality in 10 years and improve quality of life (Esposito *et al.*, 2004). Nevertheless, when the disease is already installed, pharmacological and/or surgical approaches are needed. Although these strategies may improve patients' quality of life and contribute to their long-term survival, many pharmacological treatments are associated with several adverse effects that are dependent of the individual, the existence of comorbidities, the specificity of the disease being treated, namely the molecular mechanisms underlying its onset and development, and the interaction with other drugs prescribed concomitantly (Faulx and Francis, 2008).

2.1. Pulmonary Arterial Hypertension

Pulmonary hypertension (PH) and its effects on right heart (RH) function contribute markedly to the global burden of chronic CVDs. PH is a haemodynamic pathophysiological condition defined as an increase in mean pulmonary arterial pressure (mPAP) above 25

mmHg at rest as assessed by RH catheterisation (Galie *et al.*, 2009). In 2015, the ESC proposed a classification for different PH conditions (Table 2) (Galiè *et al.*, 2016).

Table 2. **Classification of PH clinical groups.**

Adapted from Galie et al., 2009 and Galiè et al., 2016

1. Pulmonary arterial hypertension (PAH)
1.1. Idiopathic
1.2. Heritable
1.2.1. BMPR2 mutation
1.2.2. AKL-1, endoglin
1.2.3. Other mutations
1.3. Drugs and toxins
1.4. Associated with (APAH)
1.4.1. Connective tissue disease
1.4.2. Human immunodeficiency virus infection
1.4.3. Portal hypertension
1.4.4. Congenital heart
1.4.5. Schistosomiasis
2. Pulmonary hypertension due to left heart disease
3. Pulmonary hypertension due to lung diseases and/or hypoxia
4. Chronic thromboembolic pulmonary hypertension and other pulmonary artery obstructions
5. Pulmonary hypertension with unclear and/or multifactorial mechanisms

In addition to the above classification, other haemodynamic parameters can be used to classify PH, namely pulmonary arterial wedge pressure (PAWP), as shown in table 3.

Table 3. **Haemodynamic characterization of PH.**

(Adapted from Galiè et al., 2016)

Definition	Characteristics	Clinical Group
Pre-capillary PH	PAWP $\leq$ 15 mmHg	1,3,4,5
Pos-capillary PH	PAWP > 15 mmHg	2

Pulmonary arterial hypertension (PAH), group I of table 2, is a highly malignant cardiovascular disease characterized by elevated pulmonary arterial pressure that leads to right ventricle (RV) failure and death (Chan and Loscalzo, 2008). Although the right ventricle dysfunction is a major player in PAH, this disease is primarily a lung disorder. PAH has a multifactorial pathobiology that involves different mechanisms, including vasoconstriction, proliferative and obstructive remodelling of the pulmonary vessel wall, inflammation and thrombosis (Chan and Loscalzo, 2008). The symptoms of PAH coincide with other similar conditions such as heart disease and asthma, thus making it a difficult disease to diagnose. Indeed, PAH patients are often late-diagnosed when severe symptoms are already installed and, therefore, when the disease is irreversible the patients may only have a few years to live (Liu *et al.*, 2018).

Pulmonary vascular remodelling (Figure 1) is the hallmark feature of PAH and includes alterations associated with the intima layer of vessels namely, endothelial injury, proliferation and invasion of the intima by myofibroblast-like cells, enhanced matrix deposition with consequent intimal fibrosis, thrombotic lesions and, rarely, obstruction by plexiform lesions (Tuder *et al.*, 2013). Other modifications include medial layer thickening due to abnormal pulmonary arterial smooth muscle cell proliferation and adventitia layer thickening due to perivascular inflammatory infiltrates (Stacher *et al.*, 2012). Moreover, hypoxia is a major contributor to PAH development (Stenmark, Fagan and Frid, 2006) with endothelial cells (ECs) being the direct target of hypoxic injury (Howell, Preston and McLoughlin, 2003; Helan *et al.*, 2014). Importantly, these findings are not commonly found in the majority of pulmonary hypertension forms and, therefore, define PAH as a pulmonary vascular disease (Dorfmüller and Humbert, 2012).

Current therapies for PAH aim the dilation of pulmonary vasculature to attenuate pulmonary vascular resistance (PVR), thus reducing RV pressure overload. Despite many available drugs approved by Food and Drug Administration (FDA) to attenuate the symptoms of PAH, the disease remains incurable (Ghouleh *et al.*, 2017), justifying studies to elucidate the molecular mechanisms underlying the pathophysiology of this disease, thus opening new avenues for the development of efficient therapeutic strategies.

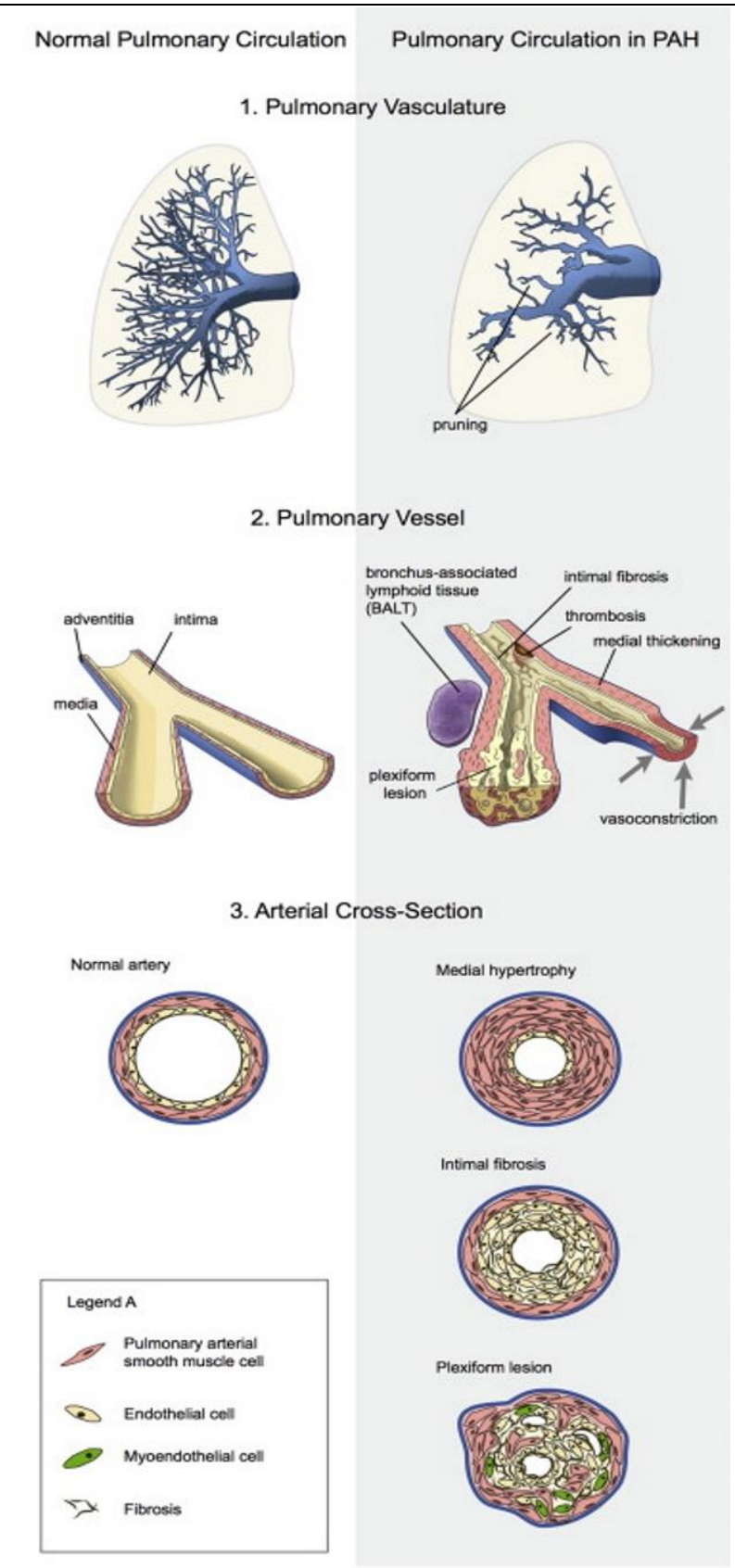

***Figure 1*. Vascular Remodelling in PAH**
Pulmonary arteries (PAs) become "pruned" in PAH. PAs develop medial hypertrophy, intimal fibrosis, vasoconstriction, and inflammation in the adventitia, which causes severe luminal obstruction in PAH, compared with controls. Adapted from Canadian Journal of Cardiology, Vol. 31, Ryan J. et al., Right Ventricular Adaptation and Failure in Pulmonary Arterial Hypertension, 391-406., Copyright 2015.

3. Intercellular communication

Intercellular communication (IC) is important to ensure the correct and efficient flow of information between cells, essential to maintain tissue and organ homeostasis (Ribeiro-Rodrigues *et al.*, 2017). It allows cells to exchange information and can occur directly, between adjacent cells, through Connexin (Cx)-containing channels, called gap junctions (GJ), or indirectly, between non-adjacent cells via nanotubes or extracellular vesicles (Sluijter *et al.*, 2014; Waldenstrom and Ronquist, 2014; Martins-Marques *et al.*, 2016; Ribeiro-Rodrigues *et al.*, 2017).

Gap junction mediated intercellular communication (GJIC) allows the transfer of small regulatory molecules and ions between neighboring cells (Goodenough, Goliger and Paul, 1996). GJs are formed by the docking of two hemichannels (one from each cell), being each hemichannel composed by the assembly of six subunits of connexin, called connexons (Ribeiro-Rodrigues *et al.*, 2015; Freund-Michel *et al.*, 2016) (Figure 2). Dysfunctional GJIC occurs in a number of human diseases, such as cancer (Naus and Laird, 2010), heart diseases (Martin, 2004; Severs *et al.*, 2008) diabetes (Watts *et al.*, 2005), Alzheimer's (Haughey and Mattson, 2003), Parkinson's (Schwab *et al.*, 2014), and atherosclerosis (Ebong and DePaola, 2013).

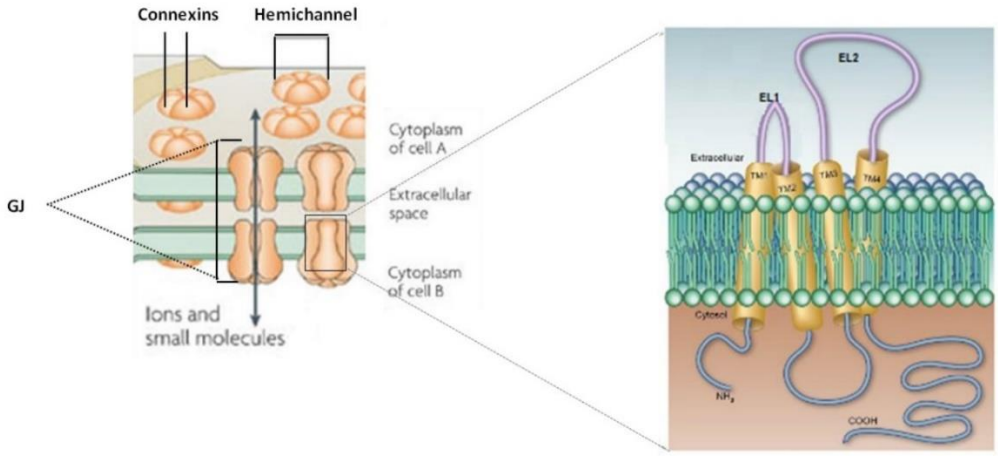

Figure 2. **Structure of Cx43 and assemble of GJ**
Cx43 is an integral transmembrane protein. Cxs are assembled in groups of six to form hemichannels and two hemichannels can dock to form a GJ. GJ allow the passage of ions and small molecules, mediators of intercellular communication, between neighboring cells. Adapted from Bloomfield SA, et al. 2009 and from Chun-hong X, et al. 2012.

Pulmonary arteries are composed of a single layer of ECs and several layers of smooth muscle cells depending on the diameter of the vessel. GJs can be found between endothelial cells, smooth muscle cells and endothelial cells or between smooth muscle cells. Several connexins are expressed in the pulmonary vasculature, namely connexin 37 (Cx37),

connexin 40 (Cx40), connexin 43 (Cx43) and connexin 45 (Cx45) (Johnstone, Isakson and Locke, 2009) being Cx43 the most ubiquitously expressed and commonly studied (Su and Lau, 2014). Cell-to-cell communication seems to play an important role in both, pulmonary vasculature constriction and remodelling, by mediating vascular tone, cell growth, angiogenesis, lipid metabolism, cell differentiation and development (Lamping, 2001; Kemp-Harper and Schmidt, 2009; Patel *et al.*, 2012; Dempsie, Martin and Upton, 2015).

Bearing in mind that pulmonary vascular remodelling is an hallmark of PAH, it seems reasonable that dysfunctional connexin-mediated signalling may be involved in the pathophysiology of the disease. Indeed, blood outgrowth endothelial cells from patients with PAH showed abnormal gap junctional communication (Dempsie, Martin and Upton, 2015; (McNair *et al.*, 2017) and restoring the function of connexins was required for normal pulmonary vascular signalling (Dempsie, Martin and Upton, 2015). Since the regulation of connexins in vascular cells remains largely unexplored, these studies can bring new insights to overcome PAH therapeutic limitations.

4. Autophagy

Autophagy represents a homeostatic mechanism essential for cell survival. This process is involved in protein quality control, by selectively degrading in the lysosome unwanted damaged or obsolete cellular components (Klionsky, 2010; Klionsky, 2013). Three major types of autophagy have been described, based on the route by which a substrate enters the lysosome: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) (Boya, Reggiori and Codogno, 2013). During macroautophagy, the most studied pathway, a double-membrane vesicle called autophagosome sequesters proteins, cytosolic components or organelles, and then expands and fuses with the lysosome, where degradation of the sequestered material takes place. The core macroautophagy machinery is composed primarily by autophagy related proteins (Atg) that control the induction of autophagosome formation, membrane delivery to the expanding phagophore, vesicle nucleation and expansion (Figure 3) (Klionsky *et al.*, 2016).

During phagophore elongation, the ubiquitin-like protein Atg8, better known as microtubule associated protein light chain 3 (LC3), plays a vital role. LC3 is initially synthesized in an unprocessed form, proLC3, that is immediately cleaved to produce an active cytosolic form, LC3-I that interacts with phosphatidylethanolamine to form LC3-II (Martinet and Meyer, De, 2009). The conversion of LC3-I into LC3-II and the recruitment of LC3-II to the autophagosome constitute a reliable marker of autophagic activity (Wu *et al.*,

2006; Liu *et al.*, 2010). Among the several autophagy adaptors identified, the best studied one is p62, an adaptor that recognizes ubiquitinated substrates and directs them to the autophagosome, binding to Atg8/LC3 through the Interacting Region (LIR) of this protein (Lippai and Lőw, 2014; Martins-Marques *et al.*, 2015).

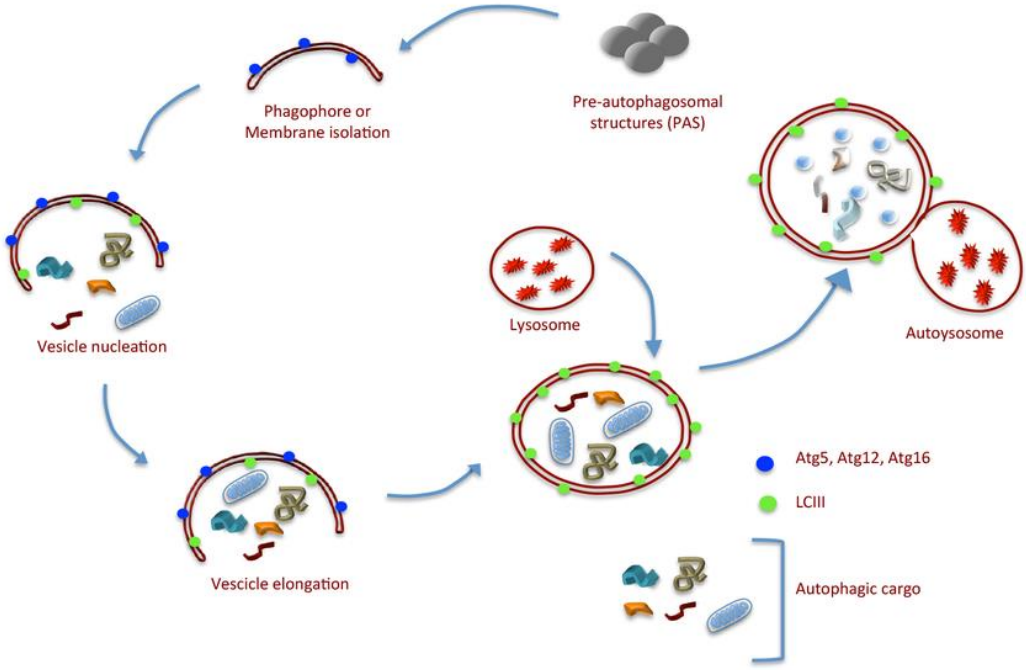

Figure 3. **The macroautophagy pathway**
The steps of autophagy include induction, vesicle nucleation, membrane elongation, autophagosome formation and subsequent fusion with the lysosome for degradation and recycle of its cargo. Adapted from Orozco-García and Gallego-Gómez, 2016.

Autophagy has been shown to be both protective and detrimental in a variety of different models, suggesting that its role in human diseases is complex. Some studies demonstrate that an imbalance in autophagosome production *vs* clearance is implicated in multiple disease processes including malignancy, neurodegeneration and vascular remodelling (Cherra and Chu, 2008; Choi, Ryter and Levine, 2013; Fraidenburg and Yuan, 2013). Autophagy can be induced in the cardiovascular system by hypoxia, hemodynamic stress, proatherogenic agents, and other noxious stimuli, suggesting a role in vascular adaptation. Moreover, it is know that LC3-I/LC3-II conversion promotes EC survival and confers angiogenic and antiapoptotic effects (Jin and Choi, 2012), and that LC3 exacerbates a protective function during the pathogenesis of PH, through the regulation of hypoxic cell proliferation (Lee *et al.*, 2011). Although, in PAH the role of autophagy remains inconclusive, it is conceivable that

disruption of autophagy activity plays an important role in vascular remodelling of ECs associated with the progression of the disease (Jin and Choi, 2012).

5. Angiogenesis

Angiogenesis is a vital process for organ growth and repair that consists in the formation of new capillaries from the pre-existing vasculature. Capillaries are essential in all tissues for diffusion of nutrients and metabolites (Adair and Montani, 2010). In the process of angiogenesis, the vascular plexus progressively expands by means of vessel sprouting and remodels into a highly organized and stereotyped vascular network of larger vessels ramifying into smaller ones (Adair and Montani, 2010). During this process ECs migrate, proliferate, establish junctions and apical-basal polarity, and deposit a stabilizing basement membrane which provides strength and allows regulation of vessel perfusion (Carmeliet, 2003; Carmeliet, 2005). This complex mechanism is regulated by growth factors and a myriad of signalling pathways that contribute to maintain the balance between pro and anti-angiogenic factors (Carmeliet and Jain, 2011).

The formation of new vessels during angiogenesis can occur by two specific mechanisms, namely sprouting angiogenesis (SA) or intussusceptive angiogenesis (IA) (Figure 4).

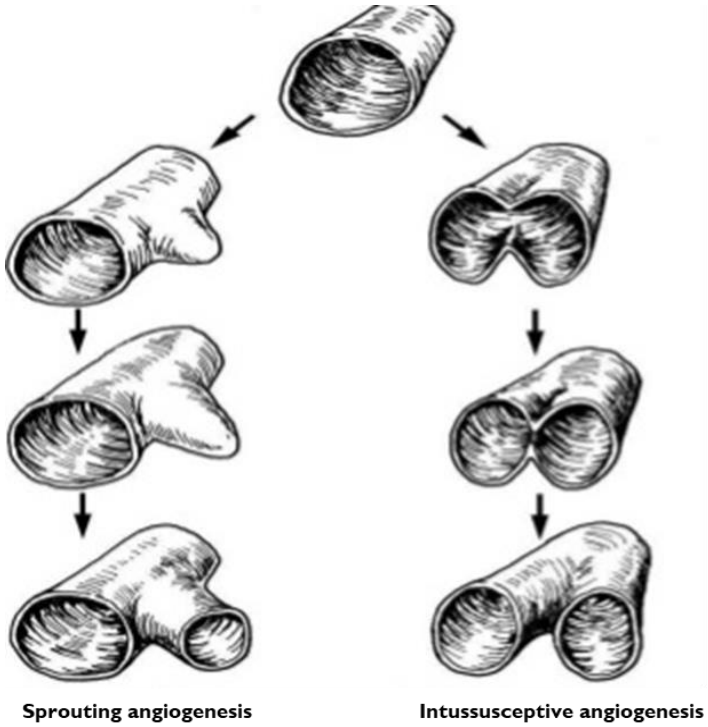

Figure 4. **The two specific mechanisms of angiogenesis, sprouting angiogenesis (SA) or intussusceptive angiogenesis (IA)**
IA occurs by dividing pre-existing capillaries and SA can occur by the formation of new capillaries from the existing vessels. Adapted from https://www.slideshare.net/ssuser4145a1/angiogenisis-in-tumer

In SA, also called tubular morphogenesis, activated ECs branch out from an existing capillary (or venule), extending through the surrounding matrix to form a new vessel. The steps of SA include enzymatic degradation of capillary basement membrane, EC proliferation, directed migration of ECs, tubulogenesis, vessel fusion, vessel pruning, and pericyte stabilization (Gerhardt *et al.*, 2003; Adams and Alitalo, 2007). In IA, also named splitting angiogenesis, the formation of new vessels occurs by a process characterized by the formation of intraluminal tissue pillars that arise from the invagination of the capillary walls into the vascular lumen. This type of angiogenesis is thought to be fast and efficient compared with sprouting angiogenesis because, initially, it only requires reorganization of existing ECs and does not rely on immediate endothelial proliferation or migration (Burri and Djonov, 2002; Spiegelaere *et al.*, 2012).

Angiogenesis is a central process during fetal growth that alters the initial capillary network into a mature and vascular structure. During adulthood, most blood vessels remain quiescent and angiogenesis plays a pivotal role in specific processes, such as ovulation, pregnancy and wound healing (Oellerich and Potente, 2012). Despite its importance in normal physiological processes, this mechanism has also been indirectly involved in a different number of pathological conditions. Indeed, the excessive outgrowth of blood vessels is implicated in cancer, psoriasis, arthritis, blindness, obesity, asthma, atherosclerosis and infectious diseases (Harada *et al.*, 2000; Luttun *et al.*, 2002; Cerimele *et al.*, 2003; Ozerdem and Stallcup, 2004; Abdou *et al.*, 2018; Lin *et al.*, 2018). On the other hand, insufficient vessel growth and abnormal vessel regression not only cause heart and brain ischaemia, but can also lead to neurodegeneration, hypertension, pre-eclampsia, respiratory distress and osteoporosis (Carmeliet, 2003; Ribeiro-Rodrigues *et al.*, 2017).

To maintain vascular homeostasis in the lung, an ideal number of capillaries per unit of lung volume is required. In sustained chronic hypoxic conditions, similar to what occurs in PAH, vascular homeostasis is compromised due to dysregulated angiogenesis, that leads to more pressure on the proximal pulmonary artery and consequent lung vessel obliteration (Voelkel, Douglas and Nicolls, 2007; Ma *et al.*, 2011; Voelkel and Gomez-Arroyo, 2014). This suggests that angiogenesis is a crucial player in the pathogenesis of PAH.

6. Essential oils

Essential oils are complex mixtures of volatile compounds produced by aromatic plants. The European Pharmacopeia 7.0 (Council of Europe, 2010) defines essential oil as a "Odorous product, usually of complex composition, obtained from a botanically defined

plant raw material, by steam distillation, dry distillation, or a suitable mechanical method without heating". Essential oils are a complex mixture of different compounds, being the most common, terpenes of low molecular weight (monoterpenes and sesquiterpenes) and/or phenylpropanoids (Başer and Buchbauer, 2010). In nature, EOs plays very important roles in plants, like defence against insects, herbivores and microorganisms, attraction to pollinating insects and fruit-dispersing animals, water regulation and allelopathic interactions (Goossens *et al.*, 2003). Also, EOs are relevant in many industries like cosmetic, pharmaceutical, agronomic, food, perfume and sanitary ones (Burt, 2004; Edris, 2007).

The composition of essential oils usually varies considerably due to both intrinsic (sexual, seasonal, ontogenetic and genetic variations) and extrinsic (ecological and environmental aspects) factors (Figueiredo *et al.*, 2008; Taiz *et al.*, 2015). Genetic variations may result in the expression of different metabolic pathways and consequently, quantitative and qualitative variations in essential oil composition may occur. When significant differences are found, an intraspecific category (chemotype) is defined. Essential oil quality and yield strongly depends on all these factors. Therefore, analytical guidelines published by several institutions such as the European Pharmacopoeia, International Standard Organization (ISO) and WHO are available and must be followed to assure the good quality of commercialized essential oils and of the plants from which they are obtained.

Terpenes are one of the main groups of compounds found in essential oils. In aromatic plants, terpenes are biosynthesized via two pathways, the mevalonate dependent (MVA) pathway (occurs in the cytosol) and the mevalonate independent or 2C-metil-D-erythritol 4-phosphate/1-deoxi-D-xilulose-5-phosphate (MEP/DOXP) pathway (occurs in the chloroplasts) (Figure 5) (Aharoni *et al.*, 2006).

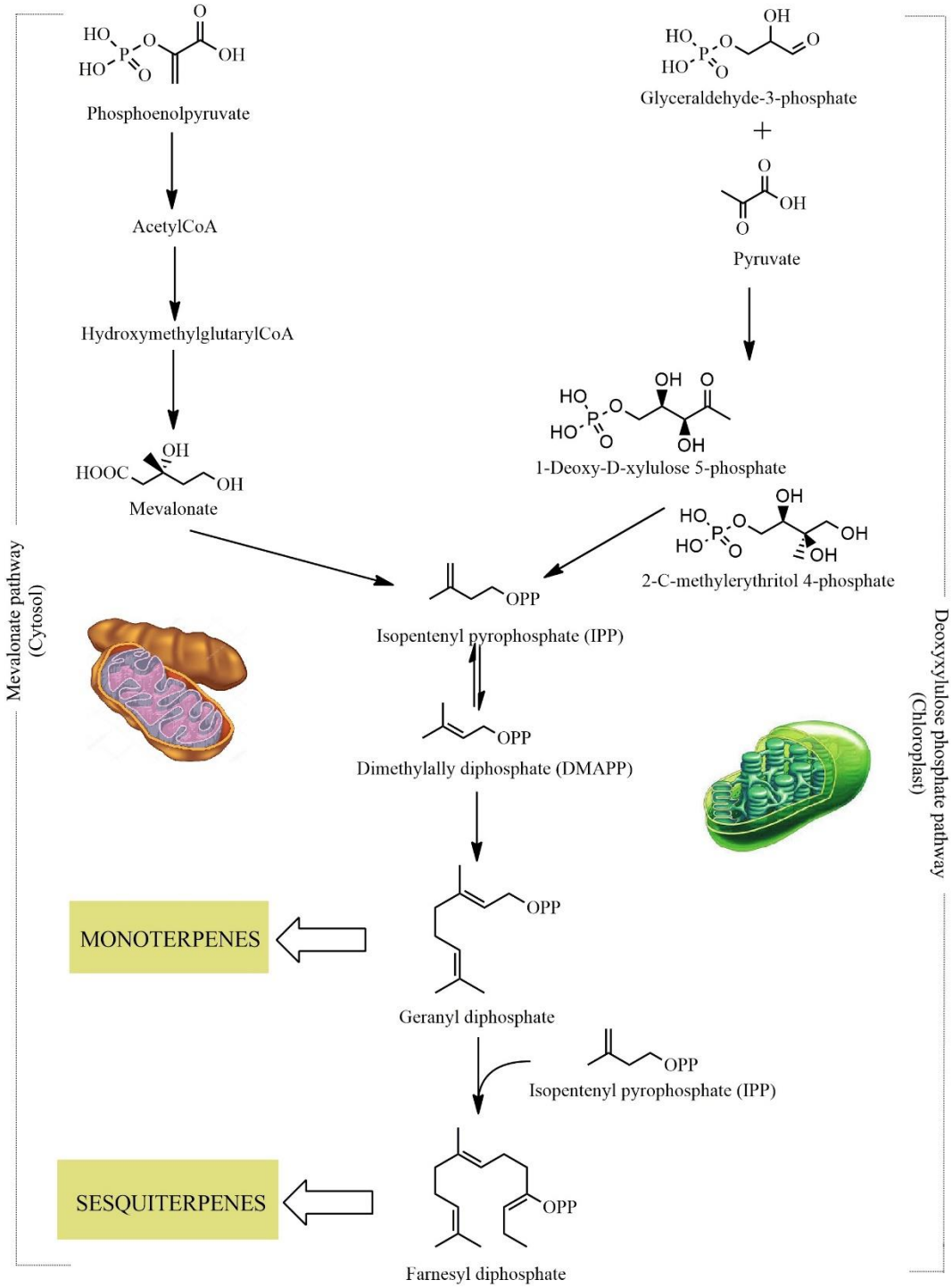

Figure 5. **Biosynthetic pathway of mono and sesquiterpenes**
Adapted from Zuzarte and Salgueiro, 2015

Terpenes result from the condensation of a pentacarbonate unit (isoprene) being classified according to the number of isoprene units in their structure, as hemiterpenes (I unit), monoterpenes (2 units), sesquiterpenes (3 units), diterpenes (4 units) and so on. Terpenes of low molecular weight, like monoterpenes ($C_{10}H_{16}$) and sesquiterpenes ($C_{15}H_{24}$) are the most predominant in essential oils. These compounds have many isomeric cyclic or

linear structures, various degrees of unsaturations, substitutions, and oxygenated derivatives (Baser and Demirci, 2007).

The major essential oil bearing plant families include: Lamiaceae, Apiaceae, Lauraceae, Asteraceae, Rutaceae, Myrtaceae, Poaceae, Cupressaceae and Piperaceae. Most of them are included in the GRAS (Generally Regarded As Safe) list approved by the FDA and EPA (Environmental Protection Agency). Essential oils have several beneficial activities, such as, antioxidant, anti-inflammatory, antimicrobial, amongst several others (Raut and Karuppayil, 2014).

6.1. The genus *Lavandula*

The genus *Lavandula L.*, belongs to the Lamiaceae family. This family consists of approximately 3500 species that are native mainly to the Mediterranean region, although some occur in Australia, Southwest Asia and South America (Mariutti and Bragagnolo, 2007). *Lavandula* genus is subdivided into 3 subgenera: Lavandula, Fabricia and Sabaudia. Each subgenus is further subdivided into eight sections, that include 39 species (Upson and Andrews, 2004; Boeckelmann, 2008). The genus *Lavandula* is cultivated, to a large extent, due to its oil content which is extracted by steam distillation from the flowering branches (Başer and Buchbauer, 2010). In Portugal alone, five species are represented: *L. latifolia, L. luisieri, L. multifida, L. pedunculata* and *L. viridis.*

Essential oils from the genus *Lavandula* have extensive applications in cosmetics, perfumery, hygiene products, food industry (as a flavouring agents), medicinal products and are also very popular in aromatherapy (Upson and Andrews, 2004; Zuzarte, 2012) with consumptions around 1000 tons for some species (Başer and Buchbauer, 2010). Lavanders are generally known for their multiple pharmacological effects such as anticonvulsant, sedative, antispasmodic, analgesic, antimicrobial, antioxidant, local anaesthetic activity and anti-inflammatory properties which might explain their uses in traditional medicine (Büyükokuroğlu *et al.*, 2003; Pavela, 2005; Moon, Cavanagh and Wilkinson, 2007; Matos *et al.*, 2009). Furthermore, EOs from *L. angustifolia* (Saeki and Shiohara, 2001; Salamati, Mashouf and Mojab, 2017) are able to decrease systolic and diastolic blood pressures and heart rate (HR). In addition, some of the major compounds, such as linalool, 1,8-cineole and terpinen-4-ol have also demonstrated several activities, including cardioprotective effects (Alves-Silva *et al.*, 2016; Andrade *et al.*, 2017).

6.1.1. *Lavandula viridis*

The first reference of *L. viridis* L'Hér dates to 1651 (Upson and Andrews, 2004). This plant (Figure 6A) is a xerophytic aromatic shrub endemic to the south west Iberian Peninsula, which grows in dry conditions and in nutrient poor and degraded soils. In Portugal it occurs in Algarve and Alentejo regions (Figure 6B) (Nogueira and Romano, 2002).

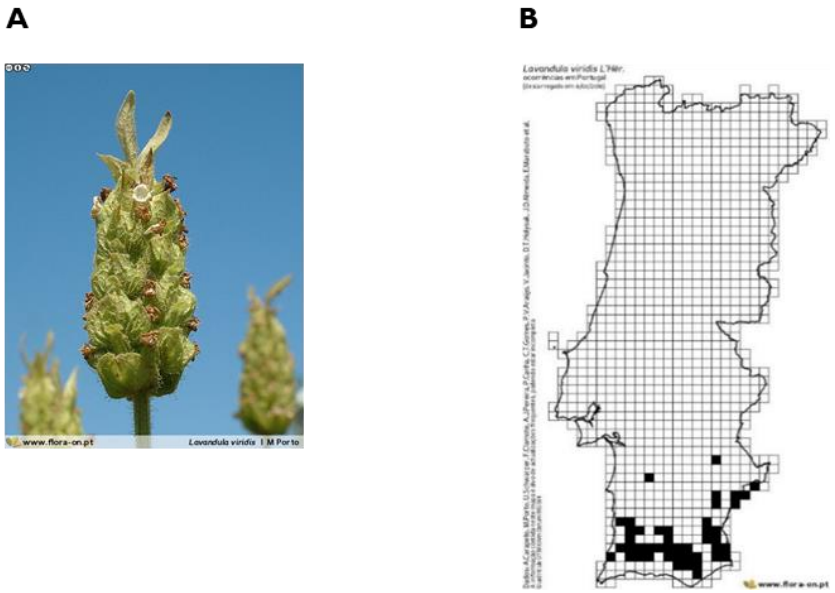

***Figure 6.* (A) *Lavandula viridis*; (B) Distribution of *Lavandula viridis* in Portugal**
http://www.flora-on.pt/#wLavandula+viridis.

In Portugal, *L. viridis* is used for food flavouring (Upson and Andrews, 2004) and in folk medicine due to its antiseptic, anti-inflammatory and antimicrobial properties (Gonçalves et al., 2013). Indeed, several biological effects, namely the antioxidant, anti-cholinesterase (Costa *et al.*, 2012), antifungal (Zuzarte *et al.*, 2011) and anti-inflammatory properties (Zuzarte *et al.*, 2012) of its EOs, have been scientifically validated.

L. viridis EOs from Portugal and Spain have a high chemical homogeneity, being 1,8-cineole the major compound. 1,8-Cineole (1,8-epoxy-p-menthane) (Figure 7) is present in high amounts in *Eucalyptus* spp. oils, and therefore is also known as eucalyptol. It is a cyclic monoterpene with a GRAS status by the Flavor and Extract Manufacturer's Association (FEMA) in 1965 and approved by the FDA for food use (Vincenzi, De *et al.*, 2002). This compound has a characteristic camphoraceous odour and a pungent, cooling, spicy taste. It is used in a wide variety of products such as nasal inhalers and sprays, external analgesics, and mouthwashes (Tyler, Brady and Robbers, 1988). This monoterpene has been described as having several bioactive properties, such as antioxidant and anti-inflammatory effects (Sá, Andrade and Sousa, 2013; Sá *et al.*, 2014), and might be responsible for the pharmacologic

activity attributed to *L. viridis* EOs (Zuzarte, 2012; Benabdelkader *et al.*, 2015). Regarding the cardiovascular system, several studies have demonstrated a protective potential, as reviewed elsewhere (Alves-Silva *et al.*, 2016). The main cardiovascular effects include direct effects on the vascular system, such as vasorelaxant (Lahlou *et al.*, 2002; Soares *et al.*, 2005) and hypotensive properties (Moon *et al.*, 2014) as well as effects on HR (Lahlou *et al.*, 2002).

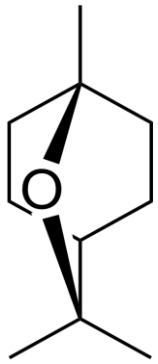

Figure 7. **1,8-Cineole**

6.2. The genus *Thymus*

The genus *Thymus* includes most of the medicinal and aromatic plants used in the world, primarily due to its EOs (Zarzuelo and Crespo, 2002; Salgueiro L., 2007; Rodrigues *et al.*, 2015). This genus belongs to the Lamiaceae family and is distributed in seven sections that comprises 150 species (Morales Valverde, 1997). *Thymus* is widely distributed in the Iberian Peninsula with several endemic species. In Portugal alone, eleven species, totalizing fourteen taxa are represented namely, *T. mastichina* subsp. *mastichina*, *T. mastichina* subsp. *donyanae*, *T. albicans*, *T. caespititius*, *T. lotocephalus, T. villosus* subsp. *villosus*, *T. villosus* subsp. *lusitanicus, T. carnosus*, *T. zygis* subsp. *zygis, T. zygis* subsp. *sylvestris, T. capitellatus, T. camphoratus, T. pulegioides* and *T. praecox* subsp. *ligusticus* (Franco, 1984; Morales Valverde, 1997).

Thymes are used for flavouring and food conservation (Nguefack *et al.*, 2009). Also, these plants are widely used in traditional medicine for the treatment of several ailments, such as coughs associated with cold, laryngitis, bronchitis, catarrh and inflammations of the mouth as well as diseases of the digestive system (Heinrich and Jager, 2015; Hosseinzadeh *et al.*, 2015; Jarić, Mitrović and Pavlović, 2015).

Some species are of economic importance, with several monographs published by different entities: Pharmacopeias, European Scientific Cooperative on Phytotherapy (ESCOP), European Medicines Agency (EMA) and WHO. For instance, thymes have two monographs approved (Thymi aetheroleum-*Thymus vulgaris*.; *Thymus zygis* and Thymi herba-

Thymus vulgaris; *Thymus zygis)* by the Committee on Herbal Medicinal Products of EMA (HMPC/EMA, 2010, 2013).

Several studies have pointed out the high chemical variability for *Thymus* spp. EOs (Stahl-Biskup, 2002). Indeed, in Portugal several chemotypes have been identified for some species (Salgueiro, 1994; Figueiredo *et al.*, 2008). *Thymus* spp. EOs are generally recognized for their multiple pharmacological effects such as antispasmodic, expectorant, anti-inflammatory and antiseptic properties (Zarzuelo and Crespo, 2002; Pinto *et al.*, 2006; Gonçalves *et al.*, 2010; Machado *et al.*, 2010).

6.2.1. *Thymus zygis*

T. zygis grows throughout the Iberian Peninsula and it is also found in the south western of Europe. In Portugal two subspecies can be identified which are further classified into two highly polymorphic subspecies, *Thymus zygis.* Loefl. ex L. subsp. *sylvestris* (Hoffmans. & Link) Cout. and *Thymus zygis* Loefl. ex L. subsp. *zygis* (Castroviejo *et al.*, 2010).

T. zygis. subsp. *sylvestris* (Figure 8A) is an aromatic shrub endemic to the central and south of the Iberian Peninsula and it is found in cold and wet environments. In Portugal it occurs in the central region (Figure 8B). On the other hand, *T. zygis*. subsp. *zygis* occurs in the north of Portugal. *T. zygis*. subsp. *sylvestris* is very difficult to distinguish morphologically from the subspecies *zygis* (Proença da Cunha and Salgueiro, 1991).

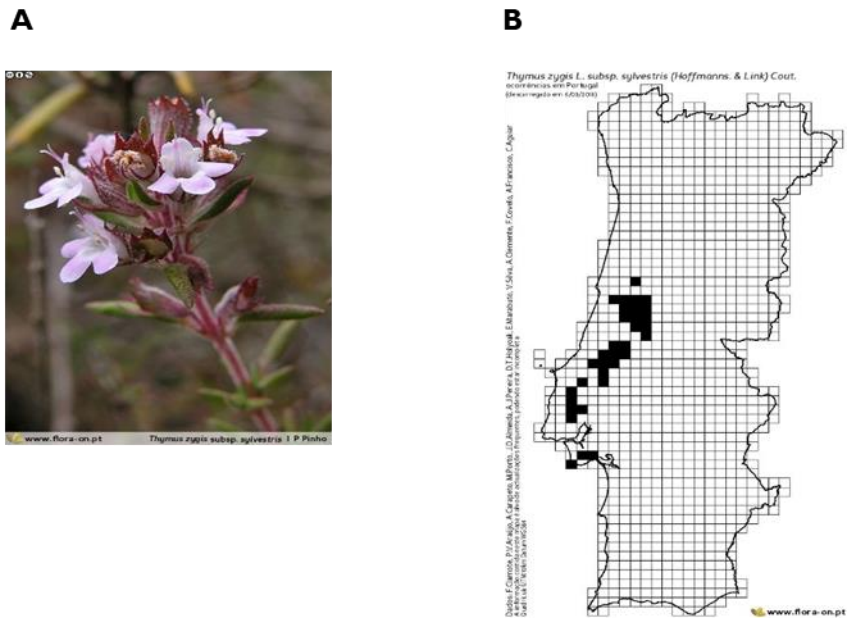

***Figure 8.* (A) *Thymus zygis* subsp. *sylvestris*; (B) Distribution of *Thymus zygis* subsp. *sylvestris* in Portugal** http://www.flora-on.pt/#wThymus+zygis+subsp.+sylvestris.

In Portugal, *T. zygis* is used as a culinary herb, a fragrance agent in soaps and perfumes (Santos, 2004; Camejo Rodrigues, 2007; Figueiredo *et al.*, 2008; Fonseca, 2015) and in traditional medicine for the treatment of inflammatory problems, mainly inflammation of the mouth, pharynx and skin, as well as rheumatism (Rivera and Obón, 1995; Carapeto, 2006; Proença da Cunha, Ribeiro and Roque, 2007; Silva *et al.*, 2011).

The essential oil from *T. zygis* subsp. *sylvestris* has demonstrated anti-inflammatory, antioxidant (Rodrigues *et al.*, 2015) and antifungal (Gonçalves *et al.*, 2010) activities. Although, several chemotypes have been identified, *T. zygis* subsp. *sylvestris* and *T. zygis* subsp. *zygis* are usually characterized by high amounts of phenolic monoterpenes, namely thymol and carvacrol (Rodrigues *et al.*, 2015). These compounds, in particular thymol are highly valued by the industry (Council of Europe, 2010).

Thymol (2-isopropyl-5-methylphenol) (figure 9) is a monoterpene phenol with low water solubility and low palatability due to its unpleasant taste and smell. Nevertheless, this monoterpene is used as a flavour additive in food and beverages, as a disinfectant in mouth care products, and as a fungicide in some medicinal products and cosmetics (Zuzarte and Salgueiro, 2015). Moreover, several studies have pointed out its potential as antioxidant, anti-inflammatory, local anaesthetic, antinociceptive, cicatrizing and antiseptic agent (Marchese *et al.*, 2016). Interestingly, beneficial effects on the cardiovascular system have also been described (Alves-Silva *et al.*, 2016). Indeed, thymol is able to induce hypotension (Saravanan and Pari, 2015; Alves-Silva *et al.*, 2016), modulate ion channels (Magyar *et al.*, 2002; Magyar *et al.*, 2004; Szentandrássy *et al.*, 2004) and display vasorelaxant effects (Peixoto-Neves *et al.*, 2010; Alves-Silva *et al.*, 2016).

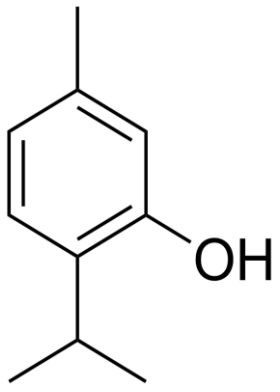

Figure 9. **Thymol**

7. Objectives

Bearing in mind that terpenes of low molecular weight have been previously described as effective therapeutic agents in vascular-related disorders, it is plausible that EOs rich in these compounds may also have these effects. Moreover, it is likely that synergisms between terpenes present in these extracts, will lead to even more efficient therapeutic effects.

In this study, we hypothesize that EOs and/or terpenes may modulate angiogenesis, intercellular communication and autophagy, processes dysregulated in many cardiovascular disorders, including PAH. For that, *in vitro* models, namely, a cell culture and a primary culture of endothelial cells, and an *ex vivo* model of rat aortic rings were selected, and a hypoxic stimulus used to mimic the pathological effects of PAH.

To achieve this main aim the following specific tasks were performed:

- Evaluation of the angiogenic potential of EOs/terpenes, through migration, tubulation, sprouting and proliferation assays;
- Evaluation of effects on gap junctional intercellular communication, trough Cx43 distribution and expression;
- Assessment of EOs/terpenes effects on autophagic flux and autophagy-related proteins (LC3 and p62) distribution and expression.

Materials and methods

8. Materials and methods

8.1. Essential oils

8.1.1. Plant material

Aerial parts of *L. viridis* and *T. zygis* subsp. *sylvestris* were collected in the flowering stage at Porto Nobre (South Portugal) and at Serra de Aire and Candeeiros (Central Portugal), respectively. Vouchers specimens were deposited at the Herbarium of the Faculty of Pharmacy of the University of Coimbra.

8.1.2. Pure compounds

The following synthetic compounds (terpenes) were purchased: 1,8-cineole (Merck; 99.5 % purity) and thymol (BDH; 99.5% purity).

8.1.3. Essential oil isolation and composition

Essential oils were obtained by hydrodistillation, performed in a Clevenger-type apparatus according to the procedure described in the European Pharmacopoeia during 3 h (Council of Europe, 2010). The chemical characterization of the essential oils was conducted using both gas chromatography (GC) and gas chromatography-mass spectroscopy (GC-MS). The volatile compounds were identified by their retention indices (using SPB-1 and SupelcoWax-10 columns) and their mass spectra. Relative amounts of individual components were calculated based on GC peak areas without Flame Ionization Detector (FID) response factor correction, according to Cavaleiro *et al.*, 2004.

8.2. Cell cultures

8.2.1. Mouse cardiac endothelial cell line

The mouse cardiac endothelial cell line (MCEC-1) was developed by the Faculty of Medicine, National Heart & Lung Institute in London, United Kingdom and nobly provided by Professor Justin Mason. The cell line was cultured in 1% gelatin (Sigma-Aldrich, St. Louis, MO) coated vessels and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA), supplemented with 10% fetal bovine Serum (FBS), 1% Penicillin/Streptomycin (100 U/mL:100 µg/mL) with 10 U/ml heparin (Invitrogen) and 75 ug/ml endothelial cell growth factor (ECGF) Sigma at 37°C under 5% CO_2.

8.2.2. Human pulmonary artery endothelial cell

The human pulmonary arterial endothelial cells (HPAECs) were obtained from GIBCO (Life Technologies, Carlsbad, CA, USA). The cells were cultured in gelatin/fibronectin (0.02%

gelatin/0.1% fibronectin) (Sigma-Aldrich, St. Louis, MO) coated vessels and maintained with Medium 200 (Life Technologies, Carlsbad, CA, USA) supplemented with 2% Low Serum Growth Supplement (LSGS) (Life Technologies, Carlsbad, CA, USA) and 1% Penicillin/Streptomycin (100 U/mL:100 µg/mL) (Life Technologies, Carlsbad, CA, USA), at 37°C with 5% CO_2.

8.3. Cells treatments

When indicated, cells were treated with 100 nM Bafilomycin A1 (Baf) (Millipore, Bedford, MA, USA) to prevent lysosomal protein degradation and 50 µg/mL Cycloheximide (CHX) (Sigma-Aldrich, St. Louis, MO) to inhibit protein synthesis.

8.4. Evaluation of cell viability

8.4.1. Alamar Blue assay

To assess the cytotoxicity of the essential oils and terpenes, the Alamar Blue (Resazurin) assay was performed in both types of cells. Briefly, MCEC-1 and HPAEC were cultured in 48-well microplates for 24 h with different concentrations of the essential oils and terpenes. The cells were further incubated with resazurin (final concentration 50µM) for 2 h, at 37°C, after which the absorbance was recorded at 570 nm with a reference filter at 620 nm using a Biotek Synergy HT spectrophotometer (Biotek, Winooski, VT, USA). A cell-free control was performed to exclude unspecific effects of the essential oils/terpenes on resazurin (data not shown). Cell viability (% control) was calculated according to the equation:

$$\frac{(\mathrm{A570} - \mathrm{A620})\ \text{treated cells}}{(\mathrm{A570} - \mathrm{A620})\ \text{control cells}} x\ 100$$

8.5. Characterization of functional endothelial parameters

8.5.1. Scratch assay

To assess the migration of endothelial cells a wound healing assay was performed. MCEC-I and HPAEC were cultured in 12-well or 24-well microplates, respectively. Then, the confluent layer of cells was wounded by a linear scratch performed with a 20-200 µl sterile pipette tip to detach cells at the centre of the well. Detached cells were discarded by washing the wells with phosphate buffered saline 1x, pH=7.4 (PBS) and the attached ECs were maintained in 1 mL (MCEC-1) or 0.5 mL (HPAEC) culture medium alone or supplemented with EOs/terpenes, at 37 °C in a 5% CO_2 atmosphere. The progress of cell migration was observed by phase contrast using a Zeiss Axio HXP IRE 2 microscope (Carl

Zeiss AG, Jena, 46 Germany) and images were taken at 0, 2, 4, 6, 8, 10 and 12 h for MCEC-1 or 0, 2, 4 and 6 h for HPAEC, following scratch injury. The area of wound reduction was calculated using ImageJ software and results were expressed according to the formula:

$$\frac{Wound\ area\ (oh) - Wound\ area\ (xh)}{Wound\ area\ (oh)}$$

8.5.2. Matrigel angiogenesis assay

The tube formation assay was used to evaluate the angiogenic potential of the EOs/terpenes on HPAEC. The day before experimentation, growth factor reduced Matrigel was thawed at 4 °C overnight. Then, 10 µL of Matrigel was filled into the lower well of the µ-Slide Angiogenesis (ibidi GmbH, Martinsried, Germany) and a humidity chamber was prepared in a petri-dish with water-soaked paper towels where the setup was inserted. Following Matrigel polymerization at 37 °C, cell suspension (12 000 cells) was applied to the upper well in a total volume of 50 µL of medium with or without EOs/terpenes. The images of tube formation were taken at 2, 4 and 6 h after cell seeding, by phase-contrast using a Zeiss Axio HXP IRE 2 microscope (Carl Zeiss AG, Jena, Germany) and analysed using angiogenesis' plugin for ImageJ.

8.5.3. Aortic ring assay

Wistar rats were obtained from our local breeding colony (Faculty of Medicine of University of Coimbra, Coimbra, Portugal). All animals received care in accordance with the Portuguese Law on Experimentation with Laboratory Animals (last amendment, 2004), which is based on the principles of laboratory animal care as adopted by the EC Directive 86/609/EEC for animal experiments. The aortic ring assay was performed according to Baker *et al.*, 2012 with slight modifications. The Wistar rats (3 months old) were anesthetized and then sacrificed by cervical dislocation. The thoracic cavity was opened, and the thoracic aorta was removed and placed in 60 mm cell culture dish containing 2 ml Opti-MEM. After dissecting, exterior fat and extravenous brachings were removed and the aorta was cleaned and cut in equal rings ~0.5 mm in length (total of 20-25 rings were obtained). Aortic rings were placed into a 96-well plate coated with Matrigel (Corning® Matrigel® Matrix Growth Factor Reduced) and then covered with another layer of Matrigel. Aortic rings were cultured overnight with Opti-MEM (supplemented with 2.5% (vol/vol) FBS and Penicillin/Streptomycin (100 U/mL:100 µg/mL). Afterwards, the medium was replaced with

Opti-MEM (supplemented with 2.5% (vol/vol) FBS and Penicillin/Streptomycin (100 U/mL:100 µg/mL) and treated with EOs or compounds. Aorta preparations were cultured for 7 days and growth was assessed after this period. Phase contrast images were taken with a Zeiss Axio HXP IRE 2 microscope (Carl Zeiss AG, Jena, Germany) and the number of sprouts was counted in a blind fashion manner.

8.6. Evaluation of protein expression

8.6.1. Western blot analysis

To subject HPAEC and MCEC-1 to hypoxia, cells were maintained in hypoxic pouches (GasPak™ EZ, BD Biosciences) either in the presence or absence of EOs/terpenes, for 1 hour. Cells cultured in normoxic conditions, at 37°C with 5% CO_2 were used as controls.

Total cell extracts were obtained by harvesting the cells in Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH≈6.8). Then, cell lysates were sonicated (3x40 µm peak to peak) in a Vibra Cell Sonicator (Sonica and Material INC) and denaturated at 95 °C for 5 min. Proteins were separated by electrophoresis on a 10% (for Cx43) or 15% (for LC3 and p62) Sodium Dodecyl Sulphate polyacrylamide gel (SDS-PAGE), at 120-190 V for 1 h and transferred to nitrocellulose membranes, using a wet transfer system at 150 mA (per gel) for 2 h. The membranes were blocked with 5% non-fat milk in Tris-buffered saline-Tween 20 (TBS-T) (20 mM Tris, 150 mM NaCl, 0.2% Tween 20, pH=7.6), and then probed overnight at 4 °C with primary antibodies against Cx43, LC3 and p62 (Table 1). The membranes were washed with TBS-T three times (10 min each) and further incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h, at room temperature. After washing, immunoreactive proteins were detected on a LAS 500 system (ImageQuant™ LAS 500). Calnexin and GAPDH antibodies (Table 4) were used as protein loading controls.

8.6.2. Immunofluorescence

Mouse cardiac endothelial cells and human pulmonary arterial endothelial cells grown on glass coverslips coated with gelatin (MCEC-1) or gelatin/fibronectin (HPAEC) were incubated either in the absence or presence of EOs/terpenes for 1 h, at 37°C with 5% CO_2. Cells were fixed with 4% paraformaldehyde (PFA) in PBS, for 10 min, washed three times with PBS, permeabilized with 0.2% v/v Triton X-100 in PBS, for 5 min, and blocked with 2% bovine serum albumin (BSA) for 30 min. Then, cells were probed with primary antibodies

against Cx43 and LC3, for 2 h at room temperature (Table 4). Before incubation with fluorescent-coupled secondary antibodies (1 h), the cells were washed three times with PBS. Nucleus were stained with DAPI. The cells were rinsed in PBS and mounted on microscope slides with MOWIOL 4-88 Reagent (Calbiochem). All solutions were prepared in 2% w/v BSA in PBS.

Actin was stained using Phalloidin-Tetramethylrhodamine B isothiocyanate (TRITC-Phalloidin).

For cell proliferation assessment, cells were incubated with antibody against Ki-67 (Table 4) for 2 h at room temperature, followed by incubation with the anti-mouse fluorescent-coupled secondary antibody, for 1 h at room temperature.

Fluorescence images were obtained using a Zeiss Axio HXP IRE 2 microscope (Carl Zeiss AG, Jena, Germany).

8.7. Statistical analysis

Data presented in this work is representative of at least 3 independent experiments. Data were analysed with GraphPad Prism 6 for Windows, version 6.01 (GraphPad Software, Inc.). Results are expressed as mean ± S.E.M. and statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test. Differences were considered significant at $p<0.05$.

Table 4. **List of primary and secondary antibodies used for WB and immunofluorescence.**

Antibody	Host/Clonality	Clone/Cat#	Application	Dilution	Company
anti-Cx43	goat polyclonal	AB0016-500	WB	1:5000	SICGEN (Cantanhede, Portugal)
anti-LC3	rabbit polyclonal	pa116931	WB	1:1000	Thermo Fisher Scientific (Waltham, MA, USA
anti-p62	rabbit polyclonal	51145	WB	1:1000	Cell Signaling Technology

Antibody	Host/Clonality	Clone/Cat#	Application	Dilution	Company
					(MA, USA)
anti-Cx43	goat polyclonal	AB0016-500	IF	1:200	SICGEN (Cantanhede, Portugal)
anti-LC3	rabbit polyclonal	pa116931	IF	1:100	Thermo Fisher Scientific (Waltham, MA, USA
anti-calnexin	goat polyclonal	AB0041	WB	1:5000	SICGEN (Cantanhede, Portugal)
anti-GAPDH	goat polyclonal	AB0049	WB	1:5000	SICGEN (Cantanhede, Portugal)
anti-Ki67	mouse monoclonal	sc-23900	IF	1:100	Santa Cruz Biotechnology (Heidelberg, Germany)
anti-goat-HRP	rabbit	61-1620	WB	1:10000	BioRad (Hercules, CA, USA)
anti-rabbit-HRP	goat	656120#	WB	1:10000	BioRad (Hercules, CA, USA)
Alexa Fluor 594 anti-mouse	donkey/ IgG	R37115	IF	1:500	Molecular Probes, Life Technologies (Carlsbad, CA)
Alexa Fluor 647 anti-rabbit	donkey/ IgG	A-31573	IF	1:500	Molecular Probes, Life Technologies (Carlsbad, CA)

Antibody	Host/Clonality	Clone/Cat#	Application	Dilution	Company
Alexa Fluor 488 anti-goat	donkey/IgG	A-11055	IF	1:500	Molecular Probes, Life Technologies (Carlsbad, CA)

Results

9. Results

9.1. Essential oils composition

The EOs of *L. viridis* and *T. zygis* subsp. *sylvestris* were obtained with a yield of 1.7% (v/w) and 1.2% (v/w), respectively. In Table 5 the major compounds identified in these two EOs are presented. Both EOs were characterized by high amounts of oxygenated monoterpenes, namely 1,8-cineole (42.0%) and camphor (13.0%) for *L. viridis*, and thymol (20.5%) and carvacrol (16%) for *T. zygis* subsp. *sylvestris*. The latter also showed high amounts of *p*-cymene (20.0%), a monoterpene hydrocarbon.

Table 5. **Major compounds identified in *Lavandula viridis* and *Thymus zygis* subsp. *sylvestris* essential oils**

Lavandula viridis	***Thymus zygis* subsp. *sylvestris***
3.7% - camphene	3.5% - borneol
6.5% - linalool	7.4% - γ-terpinene
9.5% - α-pinene	16.0% - carvacrol
13.0% - camphor	20.0% - *p*-cymene
42.0% - 1,8-cineole	20.5% - thymol

9.2. Effect of essential oils and terpenes on cell viability

Although medicinal and aromatic plants have long been used in traditional medicine, very often their use is based on empirical knowledge, without efficacy and safety validations. Therefore, the initial step of this work aimed to assess the safe concentrations of EOs/terpenes. For this purpose, we performed an Alamar Blue (resazurin) assay, to assess the effect of EOs/terpenes on metabolic activity (cell viability), in an endothelial cell line of cardiac origin, MCEC-1, and in primary pulmonary arterial endothelial cells, HPAEC.

The two types of cells were incubated for 24 hours with different concentrations of EOs/terpenes. Figures 10 and 11 show the results obtained in MCEC-1 and HPAEC, respectively. Overall, for MCEC-1, 1,8-cineole presented no toxicity in the experimental conditions tested, while thymol was highly toxic at concentrations higher that 12.5 μg/mL. Regarding the EOs, *L. viridis* and *T. zygis* subps. *sylvestris* showed a similar safety profile, with toxicity observed ≥ 200 μg/mL. Importantly, for HPAEC the EOs and thymol seemed to be more toxic, while 1,8-cineole showed a similar profile.

Based on the data obtained from the Alamar Blue assay, the following experiments were performed using selected safe concentrations of *L. viridis* EO (50 μg/mL for MCEC-1 and 25

µg/mL for HPAEC), 1,8-cineole (600 µg/mL for MCEC-1 and 400 µg/mL for HPAEC), *T. zygis* subps. *sylvestris* EO (50 µg/mL for MCEC-1 and 6.25 µg/mL for HPAEC) and thymol (12.5 µg/mL for MCEC-1 and 3.125 µg/mL for HPAEC).

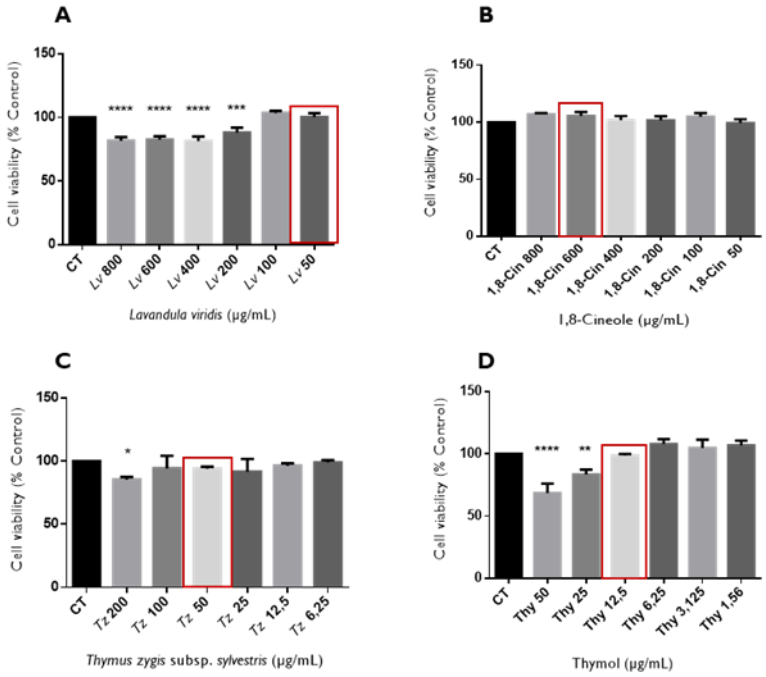

Figure 10. **MCEC-1 cell viability**
Cell viability was evaluated by Alamar Blue assay. Cells were treated for 24 hours with *L. viridis* EO (Figure 10A), 1,8-cineole (Figure 10B), *T. zygis* subps. *sylvestris* EO (Figure 10C) and thymol (Figure 10D). Representative graphs show normalized values as a percentage of resazurin reduction compared to control cells. Each value represents the mean ± SEM of at least three independent experiments performed in duplicate. Red rectangles represent selected safe concentrations used in the following experiments. Statistical differences were assessed using one-way ANOVA followed by Dunnett's multiple comparison test ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$, compared to control cells).

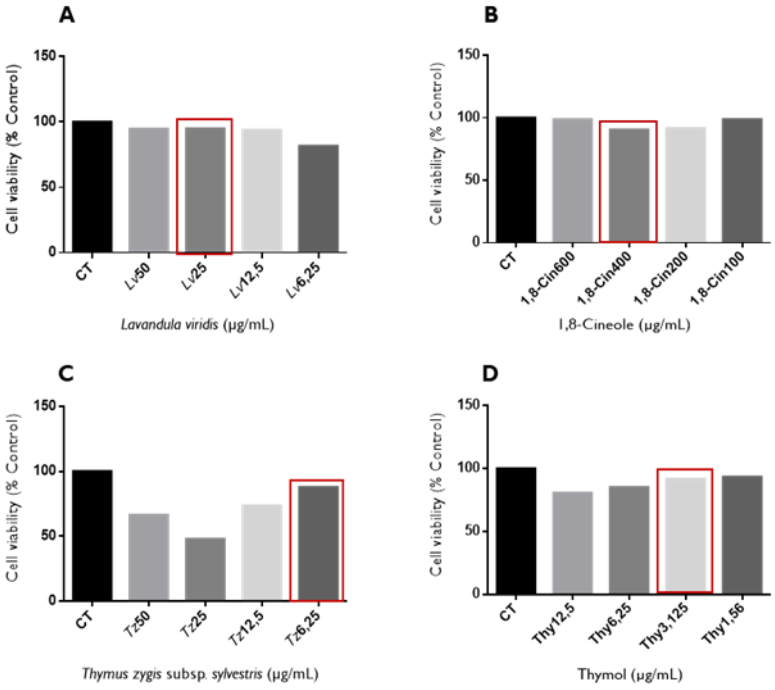

***Figure 11*. HPAEC cell viability**
Cell viability was evaluated by Alamar Blue assay. Cells were treated for 24 hours with *L. viridis* EO (Figure 11A), 1,8-cineole (Figure 11B), *T. zygis* subps. *sylvestris* EO (Figure 11C) and thymol (Figure 11D). Representative graphs show normalized values as a percentage of resazurin reduction compared to control cells. Red rectangles represent selected safe concentrations used in the following experiments. (n=1)

9.3. Effect of the essential oils and terpenes on endothelial cell migration

The formation of blood vessels through angiogenesis, critical not only for normal vascular development but also in pathological conditions, requires an orchestrated myriad of cell processes including proliferation, reorganization into capillary-like structures and migration (Risau, 1997). Not surprisingly, a disruption of these processes is often associated with defects in the formation of new vessels. For example, an impairment of EC migration has been associated with endothelial dysfunction and a diminished capacity of ECs to form new vascular structures (Favero *et al.*, 2014). To evaluate the effect of EOs/terpenes on ECs migratory phenotype, a wound healing assay (scratch assay) was performed.

9.3.1. 1,8-Cineole promotes cardiac endothelial cell migration

Representative phase-contrast images obtained immediately after the scratch (0), and 4, 8 and 12 hours after are shown in Figure 12A. The results obtained and depicted in a graph (Figure 12B) show that MCEC-1 incubated with EOs/terpenes present a tendency to migrate more than control cells, with 1,8-cineole showing a significant effect, after 10h of incubation.

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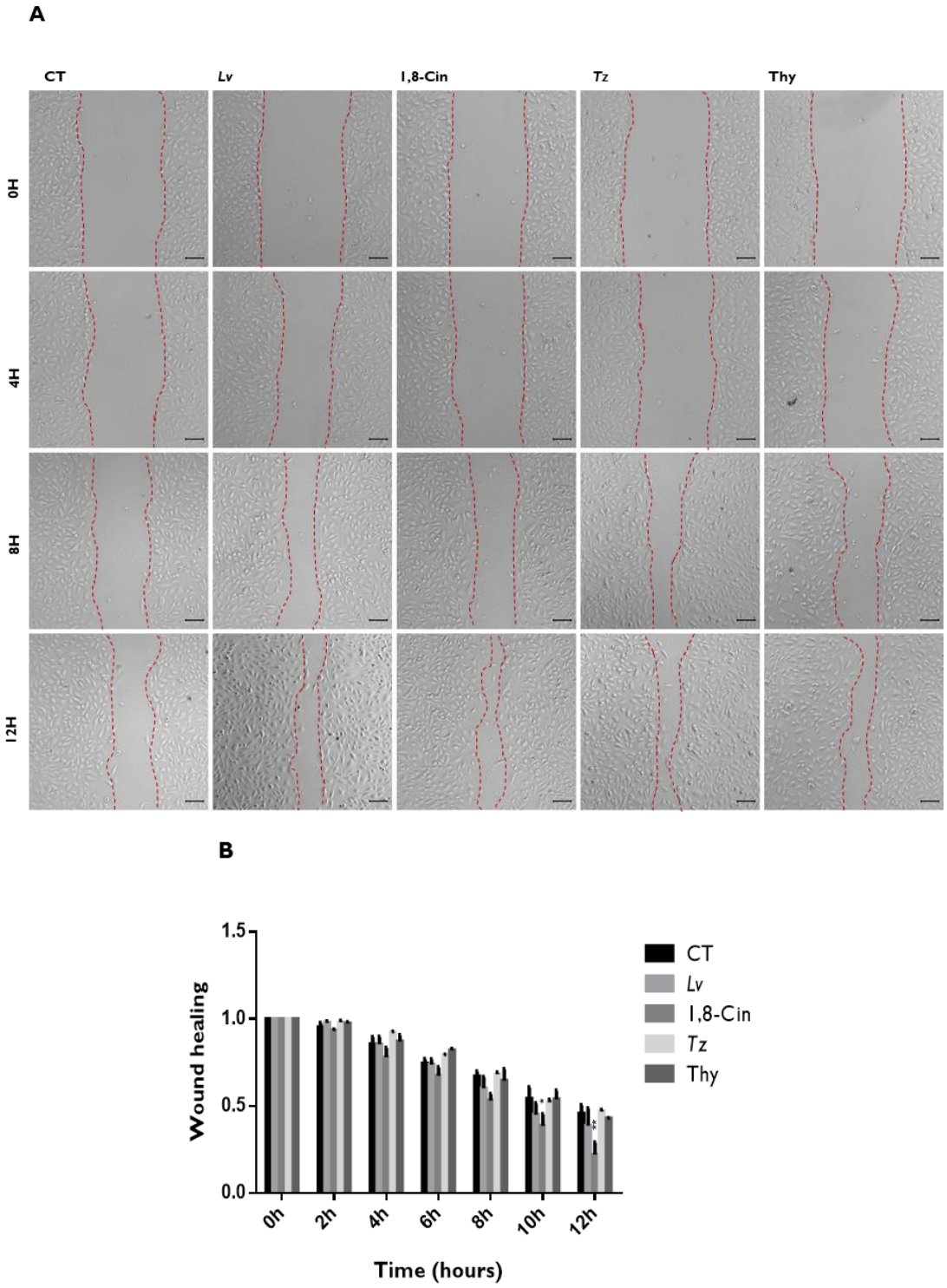
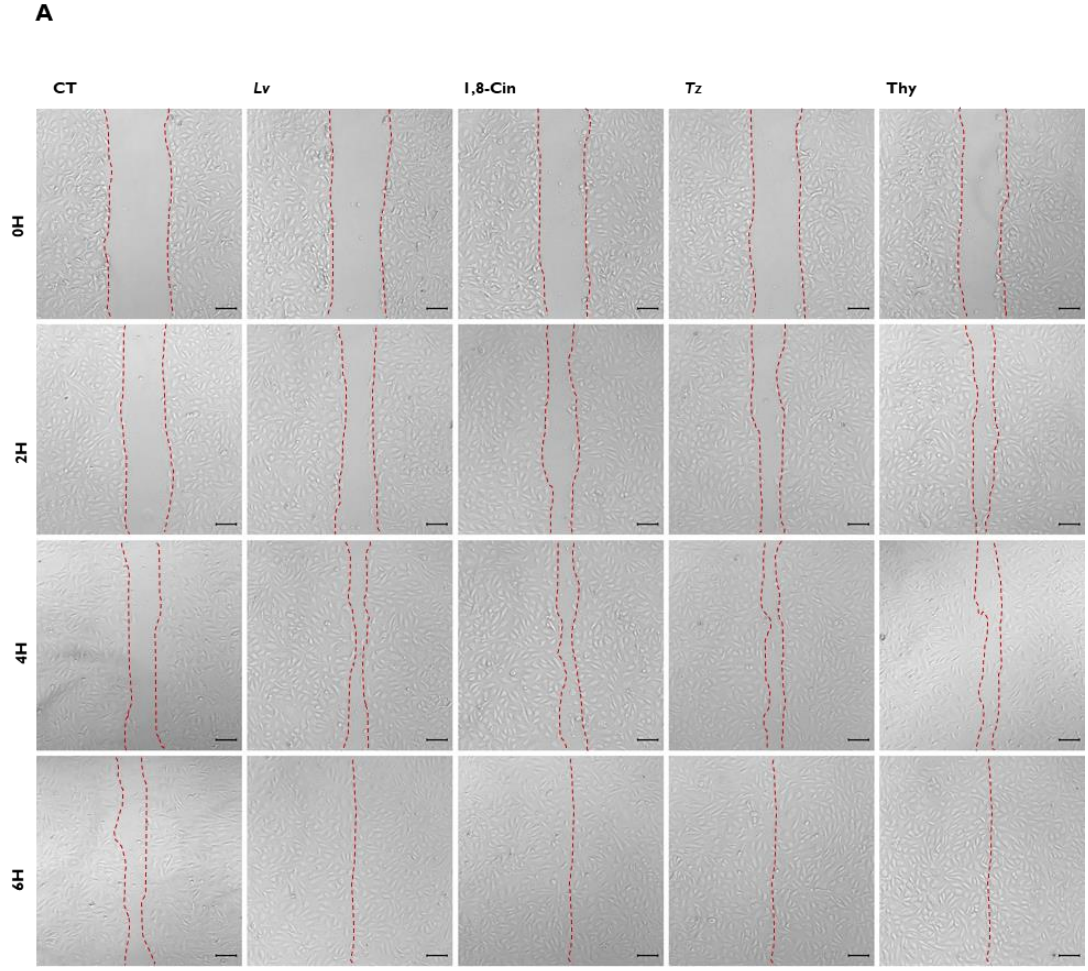


Figure 12. **1,8-Cineole promotes cardiac endothelial cell migration**
Migration capacity of MCEC-1 was analysed by the wound healing assay. ECs confluent monolayer was scratched, and medium was replaced by EOs/terpenes-depleted medium (CT) or selected EOs/terpenes concentrations: 50 µg/mL *L. viridis* EO (*Lv*), 600 µg/mL 1,8-cineole (1,8-Cin), 50 µg/mL *T. zygis* subps. *sylvestris* EO (*Tz)* and 12.5 µg/mL thymol (Thy). Cell migration was monitored for a 12-hour period after the scratch. (A) Representative phase-contrast images at time 0 (immediately after the scratch) and 4, 8, and 12 hours after the scratch are shown. The lines indicate the wound edge. Scale bar 100 µm. (B) Quantification was performed in images taken at the beginning of the assay and at the indicated times points after the scratch. Cell-free area was measured, using ImageJ. Results represent the mean ± S.E.M wound size area of triplicate samples from three independent experiments ($*p < 0.05$, $**p < 0.01$, compared to 1,8-cineole treatment at 0-hours).

9.3.2. Essential oils/terpenes promote pulmonary arterial endothelial cell migration

The effect of EOs/terpenes on the migratory capacity of HPAEC was also assessed, since it is a primary endothelial cell line and, therefore, constitutes a model that better represents and resembles the natural *in vivo* conditions.

Representative phase-contrast images in Figure 13A were obtained at 0, 2, 4 and 6 hours after the scratch. The results show that the primary cells have a higher migratory capacity than MCEC-1, with total wound closure observed much earlier, at 6 hours after the scratch (*vs* more than 12 hours in MCEC-1). Interestingly, all the EOs/terpenes tested were able to significantly increase the migratory capacity of HPAEC, at 4 hours following the scratch. Importantly, 1,8-cineole, *T. zygis* supsb. *sylvestris* EO and thymol were more effective showing a significative effect at early stages (2 hours following the scratch).



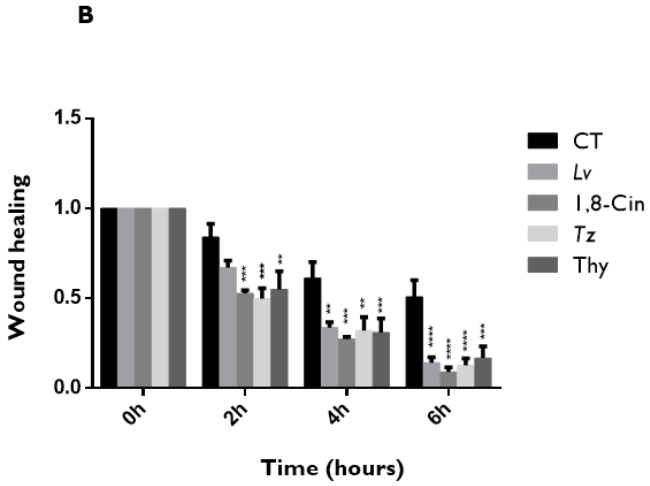


Figure 13. **Essential oils/terpenes promote pulmonary arterial endothelial cell migration**
Migration capacity of HPAEC was analysed by the wound healing assay. ECs confluent monolayer was scratched, and medium was replaced by EOs/terpenes-depleted medium (CT) or selected EOs/terpenes concentrations: 25 µg/mL *L. viridis* EO (*Lv*), 400 µg/mL 1,8-cineole (1,8-Cin), 6.25 µg/mL *T. zygis* subps. *sylvestris* EO (*Tz)* and 3.125 µg/mL thymol (Thy). Cell migration was monitored for a 6-hour period after the scratch. (A) Representative phase-contrast images at time 0 (immediately after the scratch) and 2, 4, and 6 hours after the scratch are shown. The lines indicate the wound edge. Scale bar 100 µm. (B) Quantification was performed in images taken at the beginning of the assay and at the indicated times points after the scratch. Cell-free area was measured, using ImageJ. Results represent the mean ± S.E.M wound size area of triplicate samples from three independent experiments ($**p$ < 0.01, $***p$ < 0.001, and $****p$ < 0.0001, compared to respective treatments at 0-hours).

9.3.3. Essential oils/terpenes induce the formation of lamellipodia on pulmonary arterial endothelial cells

Cell migration is a complex process that involves coordinated changes in the F-actin cytoskeleton and focal adhesions. The formation of lamellipodia, actin-enriched cytoskeletal projections on the leading edge of the cell, is known to act as a propulsive force that drives the cell forward movement, during the process of cell migration (Ballestrem *et al.*, 2000). Consequently, to corroborate the results obtained in migration assays we stained the cells with TRITC- Phalloidin, that binds to F-actin, to evaluate the effect of EOs/terpenes in the formation of lamellipodia in HPAEC.

The results presented in Figure 14 show that the EOs/terpenes induce the formation of lamellipodia, which is consistent with the results obtained for the migration assay (section 6.3.2).

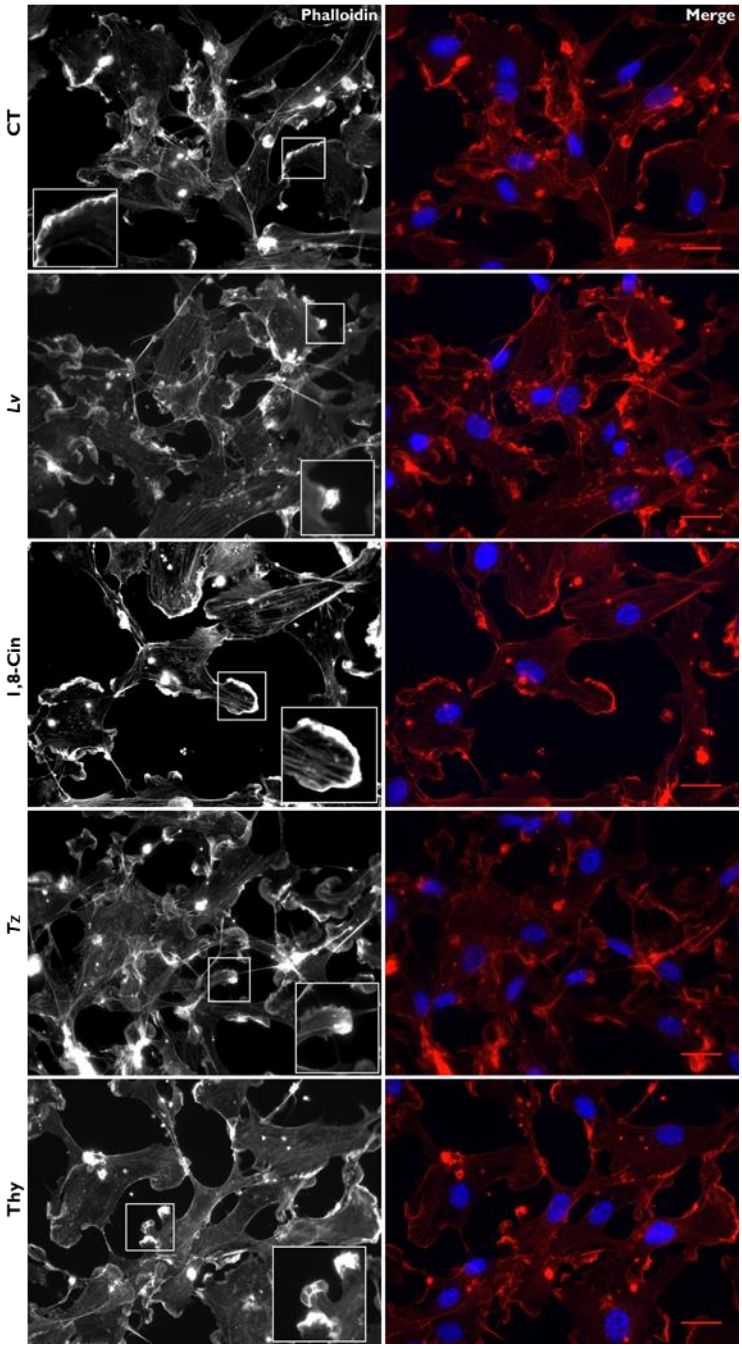


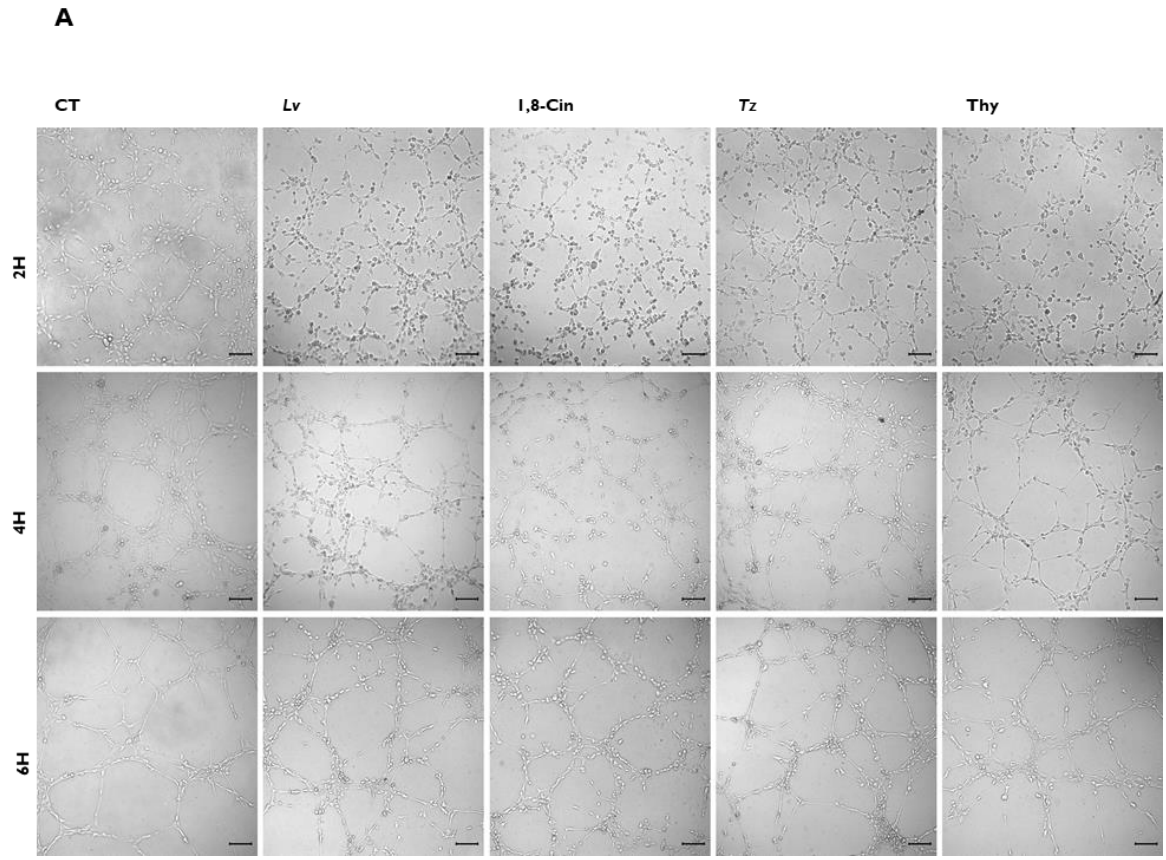
Figure 14. **Essential oils/terpenes induce the formation of lamellipodia on pulmonary arterial endothelial cells**
The lamellipodia formation were assessed by immunofluorescence in HPAEC cultured in EOs/terpenes-depleted medium (CT) or selected EOs/terpenes concentrations: 25 µg/mL *L. viridis* EO (*Lv*), 400 µg/mL 1,8-cineole (1,8-Cin), 6.25 µg/mL *T. zygis* subps. *sylvestris* EO (*Tz)* and 3.125 µg/mL thymol (Thy), for 1 hour. Representative immunofluorescence images show lamellipodia protruding from the cell body (zoom in). F-actin was stained with TRITC-Phalloidin and nuclei were stained with DAPI. Scale bar: 20 µm. (n=1)

9.4. Essential oils/terpenes modulate the formation of capillary-like structures in pulmonary arterial endothelial cells

After cell migration, we proceeded to assess the effect of EOs/terpenes on the capacity of cells to form capillary-like structures. To address this question, we performed tube formation assays on HPAEC seeded on matrigel and incubated with EOs/terpenes. Several

parameters, namely the number of meshes and nodes and the length of master segments, were quantified.

Figure 15A illustrates representative phase-contrast images following 2, 4 and 6 hours of HPAEC seeding. Although no statistical differences could be observed (Figure 15B) there is a trend towards an increase in the total number of meshes and nodes as well as master segments length formation at early stages (2 hours), in cells incubated in the presence of *L. viridis* EO. Contrarily, 1,8-cineole and thymol show a tendency to impair tube formation. Nevertheless, from 4 hours of treatment onwards, the majority of these effects are lost, and the cells present a tubular morphology similar to control (Figure 15A).



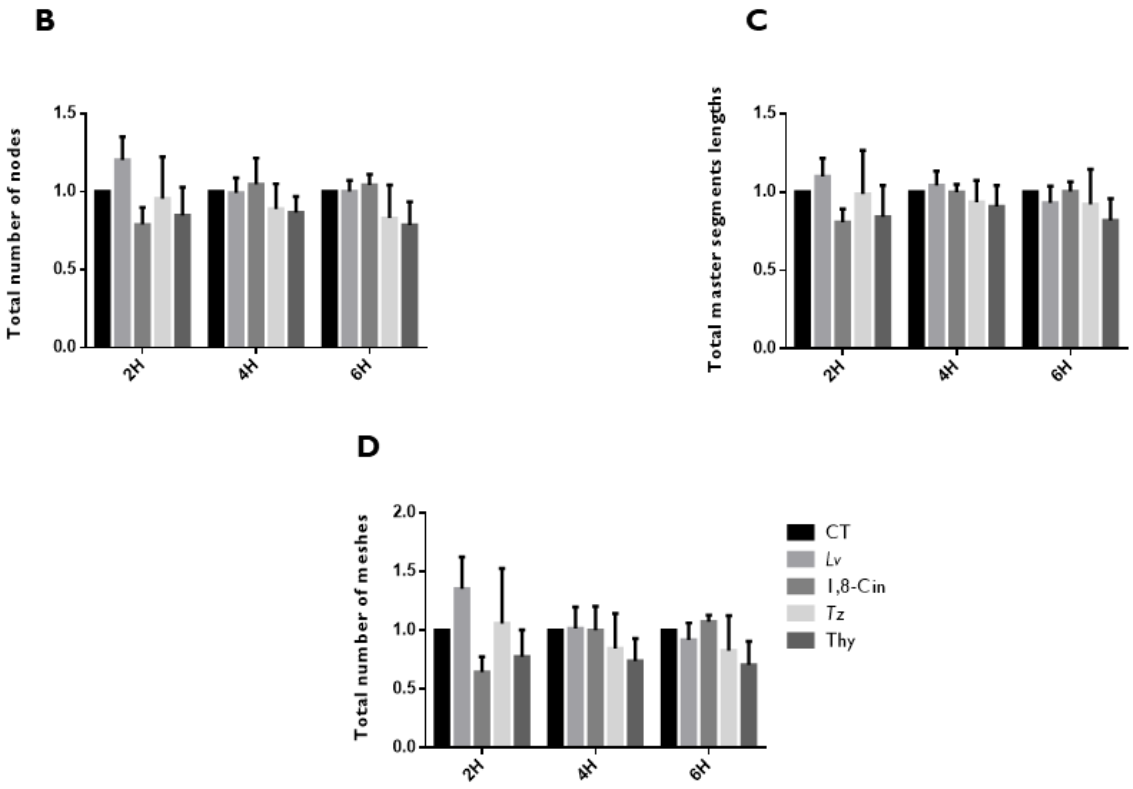


***Figure 15*. Essential oils/terpenes modulate the formation of capillary-like structures in pulmonary arterial endothelial cells**

Capillary tube formation assay was assessed 2,4 and 6 hours after ECs seeding with EOs/terpenes-depleted medium (CT) or selected EOs/terpenes concentrations: 25 µg/mL *L. viridis* EO (*Lv*), 400 µg/mL 1,8-cineole (1,8-Cin), 6.25 µg/mL *T. zygis* subps. *sylvestris* EO (*Tz)* and 3.125 µg/mL thymol (Thy). (A) Representative phase-contrast images are shown. Scale bar 100 µm. Quantitative assessment of (B) total number of nodes, (C) master segments length and (D) total number of meshes was performed by analytical software Image Pro Plus 5.1 and results were normalized to the CT. Results represent the mean ± S.E.M. of duplicate samples from three independent experiments.

9.5. Essential oils/terpenes promote endothelial cell sprouting

The most widely accepted mode of angiogenesis is sprouting, that encompasses the migration of endothelial tip cells and the proliferation of stalk cells to sustain the formation of new vessels in the avascular tissue. To unveil the effect of EOs/terpenes on cell sprouting we used the three-dimensional *ex vivo* aortic ring assay in which, isolated rat aortic rings embedded in matrigel were incubated with EOs/terpenes for 7 to assess endothelial sprout formation (Figure 16A).

The results show that most EOs/terpenes increase the number of sprouts in the aortic rings, with thymol showing a better performance (Figure 16B). Interestingly, the EOs show a lower angiogenic potential than their isolated terpenes (1,8-cineole for *L. viridis* EO and thymol for *T. zygis* subps. *sylvestris* EO).

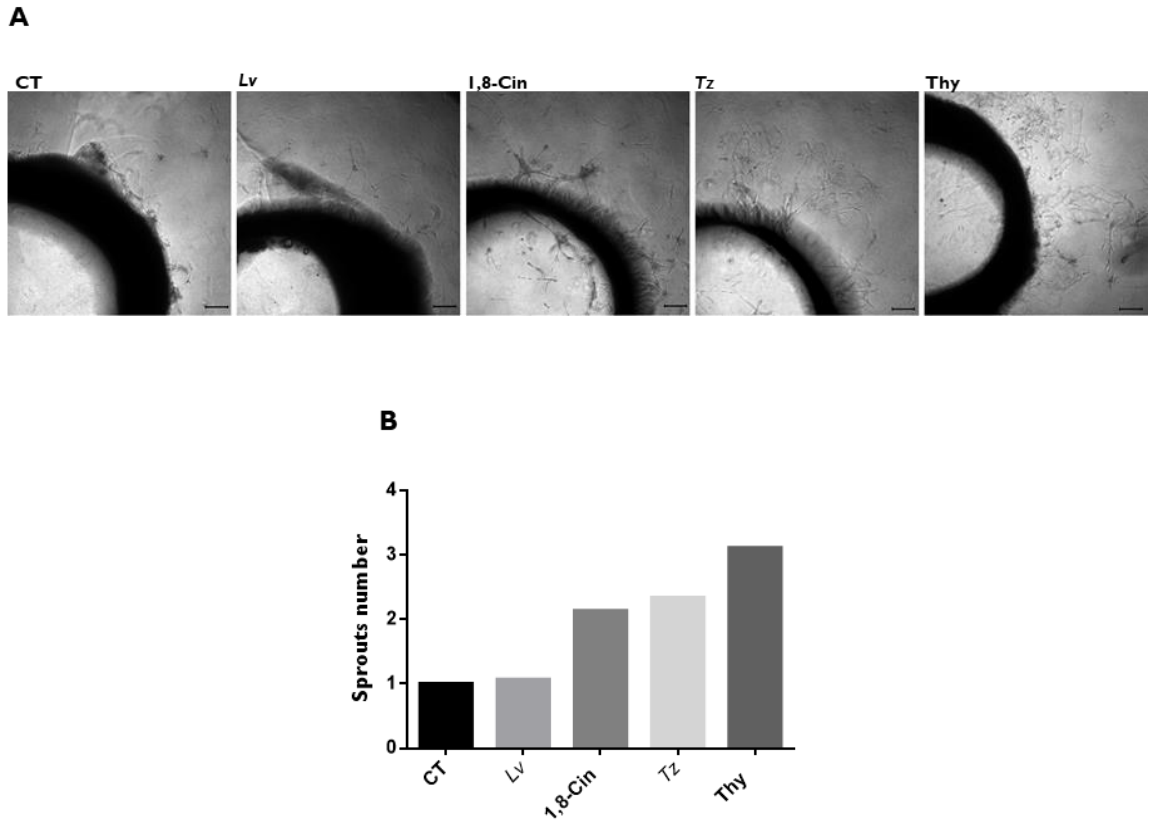


***Figure 16*. Essential oils/terpenes promote endothelial cell sprouting**
Cultures of rat aortic rings were treated with EOs/terpenes-depleted medium (CT) or selected EOs/terpenes concentrations: 25 µg/mL *L. viridis* EO (*Lv*), 400 µg/mL 1,8-cineole (1,8-Cin), 6.25 µg/mL *T. zygis* subps. *sylvestris* EO (*Tz)* and 3.125 µg/mL thymol (Thy), for 7 days. (A) Representative phase-contrast images are shown. Scale bar 200 µm. (B) Quantitative assessment of the number of sprouts per aortic ring. Results were normalized to the CT. (n=1)

9.6. Essential oils/terpenes decrease proliferation of pulmonary arterial endothelial cells

As mentioned before, cell proliferation is a key issue in the angiogenic process, implicated either in physiological or pathological conditions. Besides the formation of new vessels, proliferation of ECs has also been associated with PAH, where ECs acquire an hyperproliferative and apoptosis-resistant phenotype. Indeed, actively proliferating endothelial cells have been observed in PAH vascular lesions (Shimoda and Laurie, 2013). Therefore, the effect of the EOs/terpenes was assessed in HPAEC, by immunofluorescence using the proliferation marker, Ki67. In this assay, only active proliferating cells are positive for Ki-67 staining, since this nuclear protein is present at all stages of the cell cycle, except G0-phase.

The results presented in Figure 17 demonstrate that HPAEC treated with EOs/ terpenes show a lower number of Ki67-positive cells in comparison to control cells. Overall,

these data suggest that EOs/terpenes decrease endothelial cell proliferation, being 1,8-cineole and *T. zygis* subps. *sylvestris* slightly more active.

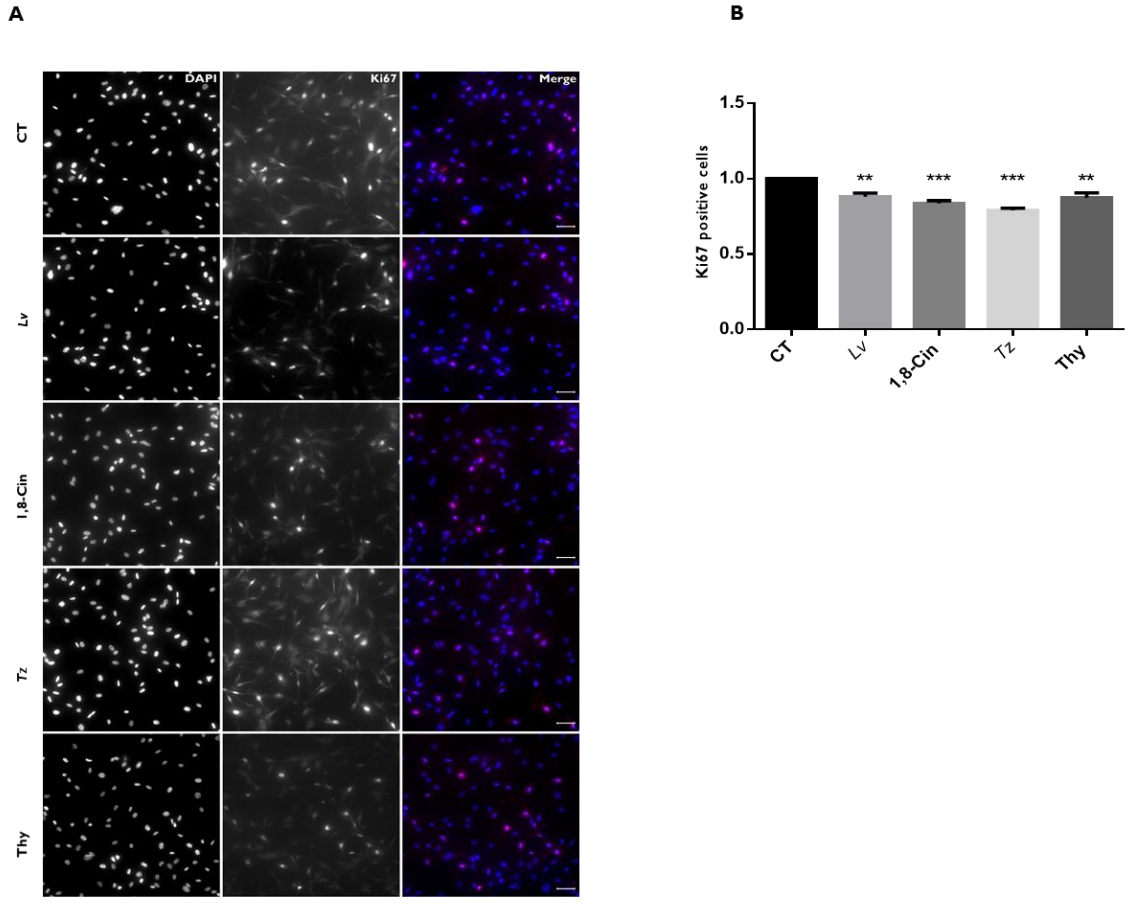


Figure 17. **Essential oils/terpenes decrease proliferation of pulmonary arterial endothelial cells**
HPAECs were cultured in EOs/terpenes-depleted medium (CT) or selected EOs/terpenes concentrations: 25 µg/mL *L. viridis* EO (*Lv*), 400 µg/mL 1,8-cineole (1,8-Cin), 6.25 µg/mL *T. zygis* subps. *sylvestris* EO (*Tz)* and 3.125 µg/mL thymol (Thy), for 6 hours. A) Representative immunofluorescence images show cells positively stained for Ki67. Nuclei were stained with DAPI. Scale bar 100 µm. (B) Quantitative assessment of proliferation rates was calculated as the ratio between the number of cells positively stained for Ki67 and the number of cells stained for DAPI. Nuclei stained with DAPI were quantified using ImageJ and cells positively stained for Ki67 were manually counted. Results represent the mean ± S.E.M of 15 images per condition from three independent experiments ($**p < 0.01$, $***p < 0.001$, compared to control).

9.7. Essential oils/terpenes modulate the expression of proteins associated with autophagy and intercellular communication

Bearing in mind that autophagy and intercellular communication are dysregulated in PAH, we proceeded to investigate the effect of EOs/terpenes on these biological processes. To mimic the environment stress conditions to which endothelial cells are exposed in the disease we subjected cells to hypoxia. The effect of EOs/terpenes on the levels of different proteins, like Cx43, associated with intercellular communication, as well as LC3 and p62, associated with autophagy, was assessed by WB analysis in both MCEC-1 and HPAEC. Moreover, the intercellular localization of Cx43 and LC3 was assessed by immunofluorescence in both cell lines.

9.7.1. Essential oils/terpenes modulate the levels and distribution of Cx43 and LC3-II in cardiac endothelial cells

Regarding the effect of *L. viridis* EO, and its major compound 1,8-cineole, and hypoxia on p62 (Figure 18) the results show that the levels of this protein are not significantly affected (Figure 18B). Nevertheless, although no statistical differences were observed, it seems that these stimuli tend to stabilize Cx43 (Figure 18C) and increase LC3-II levels, suggesting that autophagy activity is compromised (Figure 18D-E).

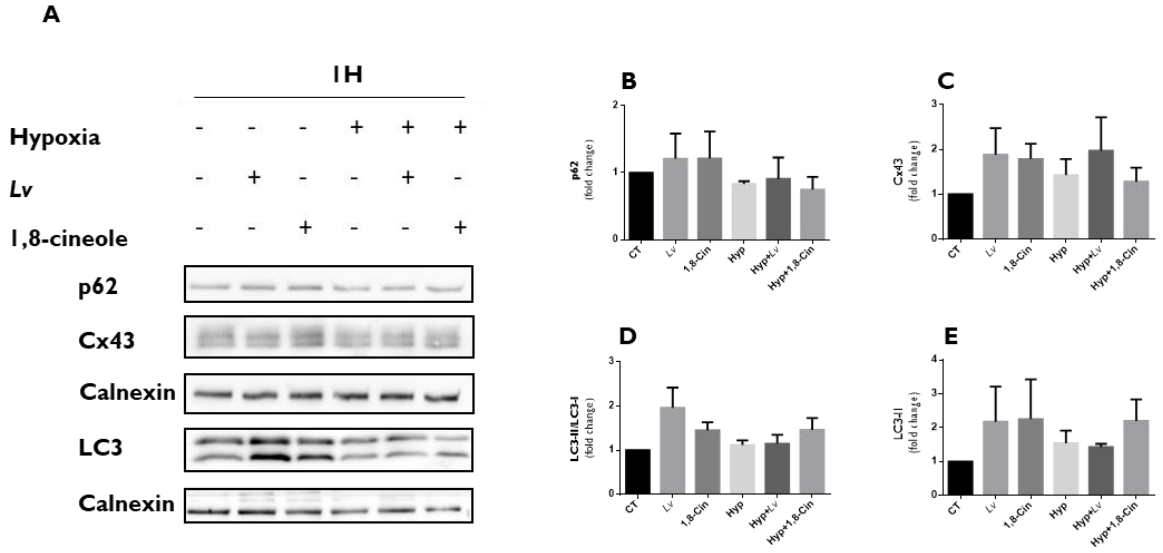


***Figure 18. Lavandula viridis* essential oil and 1,8-cineole modulate the levels of Cx43 and LC3-II in cardiac endothelial cells**
MCEC-1 in hypoxic or normoxic conditions were cultured in EOs/terpenes-depleted medium (CT) or selected EOs/terpenes concentrations: 50 µg/mL *L. viridis* EO (*Lv*) and 600 µg/mL 1,8-cineole (1,8-Cin), for 1 hour. The levels of proteins associated with autophagy and intercellular communication were assessed by WB analysis. (A) Representative WB of protein levels of p62, Cx43, LC3 (LC3-I and LC3-II) and Calnexin. Quantification of p62 (B), Cx43 (C), LC3-II/LC3-1 (D) and LC3-II (E) protein levels. Quantification of p62, Cx43 and LC3-II was normalized to Calnexin levels. Results represent the mean ± S.E.M. of three independent experiments.

Concerning the effect of *T. zygis* subps. *sylvestris* EO and thymol the results presented in Figure 19 show that the levels of p62 are not affected (Figure 19B), suggesting that *the* treatments with these compounds are also ineffective. Again, although no statistical differences were observed, there is a tendency towards a decrease of Cx43 in cells incubated with *T. zygis* subps. *sylvestris* EO, thymol and hypoxic stimuli (Figure 19C), contrarily to what was observed with *L. viridis* EO and 1,8-cineole (Figure 18C). Interestingly, although not statistically different, a slight increase in the levels of LC3-II could be observed in the presence of *T. zygis* subsp. *sylvestris* EO (Figure 19D-E), suggesting that autophagy is impaired.

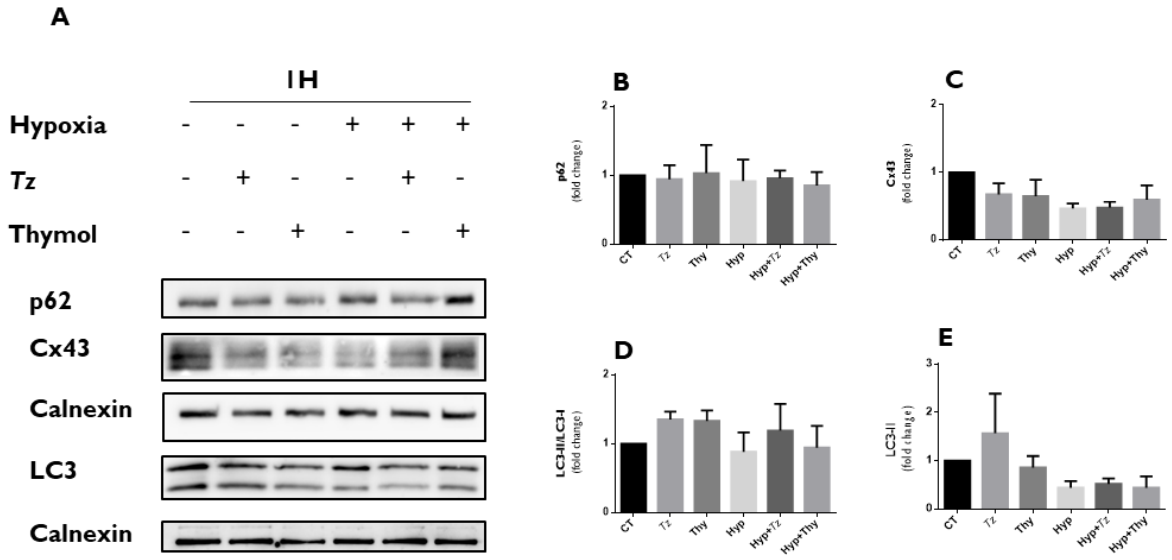


***Figure 19. Thymus zygis* subps. *sylvestris* essential oil and thymol modulate the levels of Cx43 and *Thymus zygis* subps. *sylvestris* modulates LC3-II levels in cardiac endothelial cells**
MCEC-1 in hypoxic or normoxic conditions were cultured in EOs/terpenes-depleted medium (CT) or selected EOs/terpenes concentrations: 50 µg/mL *T. zygis* subsp. *sylvestris (Tz)* EO and 12.5 µg/mL thymol (thy), for 1 hour. The levels of proteins associated with autophagy and intercellular communication were assessed by WB analysis. (A) Representative WB of protein levels of p62, Cx43, LC3 (LC3-I and LC3-II) and Calnexin. Quantification of p62 (B), Cx43 (C), LC3-II/LC3-I (D) and LC3-II (E) protein levels. Quantification of p62, Cx43 and LC3-II was normalized to Calnexin levels. Results represent the mean ± S.E.M. of three independent experiments.

Grounded on results presented above suggesting that the EOs/terpenes modulate Cx43 and LC3 levels, we next evaluated their effect on the subcellular distribution of these proteins by immunofluorescence microscopy in MCEC-1 incubated with *L. viridis* EO, 1,8-cineole, *T. zygis* subsp. *sylvestris* EO and thymol for 1 hour. The autophagy activity was evaluated by the presence of LC3 puncta, which is associated with the conversion of LC3-I into LC3-II and the formation of autophagosomes. The images presented in Figure 20 show that Cx43 distribution has similar pattern in all the tested conditions, with the protein being localized at both, the membrane and perinuclear region. Interestingly, it was observed a co-localization of Cx43 and LC3 in cells treated with EOs/terpenes, suggesting that Cx43 is being recruited to autophagic vesicles. Regarding LC3 distribution, all treatments resulted in an increase of LC3 puncta in the cytoplasm, which is likely associated with the formation of autophagic vesicles, in the sequence of autophagy activation. These images are in accordance with previous WB analysis, and show that *L. viridis* EO, 1,8-cineole, *T. zygis* subsp. *sylvestris* EO, have a greater autophagic effect.

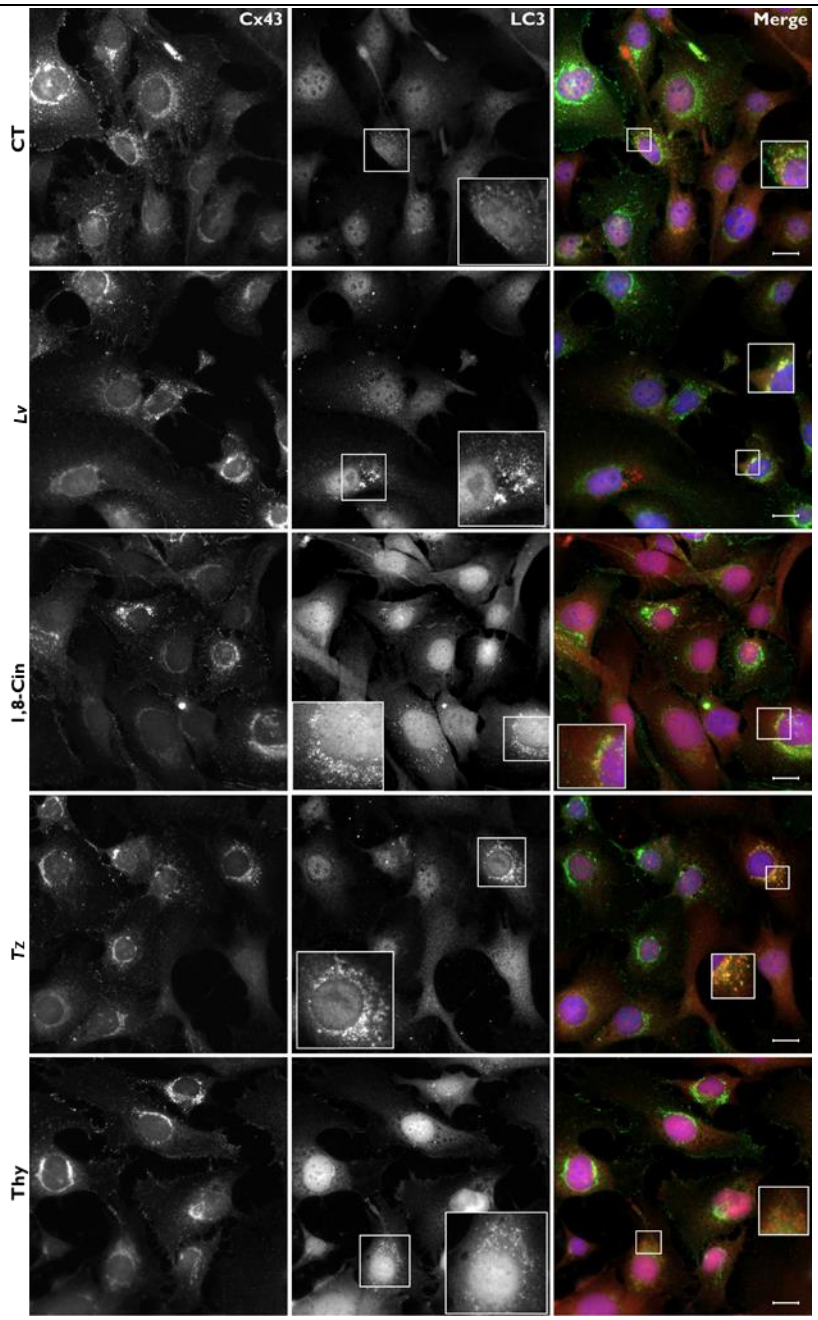


Figure 20. **Essential oils/terpenes increase autophagic vesicles in cardiac endothelial cells**
The presence of LC3 was assessed by immunofluorescence assay in MCEC-1 cultured in EOs/terpenes-depleted medium (CT) or selected EOs/terpenes concentrations: 50 µg/mL *L. viridis* EO (*Lv*), 600 µg/mL 1,8-cineole (1,8-Cin), 50 µg/mL *T. zygis* subps. *sylvestris* EO (*Tz)* and 12.5 µg/mL thymol (Thy), for 1 hour. Representative immunofluorescence images show an increase in LC3 presence and puncta positively stained for LC3. Nuclei were stained with DAPI. Scale bar: 20 µm. (n=1).

9.7.2. *Lavandula viridis* essential oil and 1,8-cineole modulate the levels and distribution of Cx43 and LC3-II in pulmonary arterial endothelial cells

After assessing how EOs/terpenes can modulate the levels of proteins associated with intercellular communication and autophagy in MCEC-1, we replicated the experiments in HPAEC.

Results presented in Figure 21 show that hypoxia, *L. viridis* EO and 1,8-cineole stimuli tend to increase the levels of Cx43, although no statistical significance was observed.

Nevertheless, a significant increase in Cx43 levels occurred during hypoxia in the presence of *L. viridis* EO (Figure 21B), suggesting that hypoxia and *L. viridis* EO have an addictive effect. The levels of p62 remain unaltered in cells incubated with *L. viridis* EO and 1,8-cineole, whereas in cells subjected to hypoxia the levels of this protein decrease, indicating that p62 is being degraded (Figure 21C). The levels of LC3 are significantly increased in the presence of *L. viridis* EO, which may indicate that this extract can upregulate autophagosome formation or block autophagic degradation (Figure 21D-E).

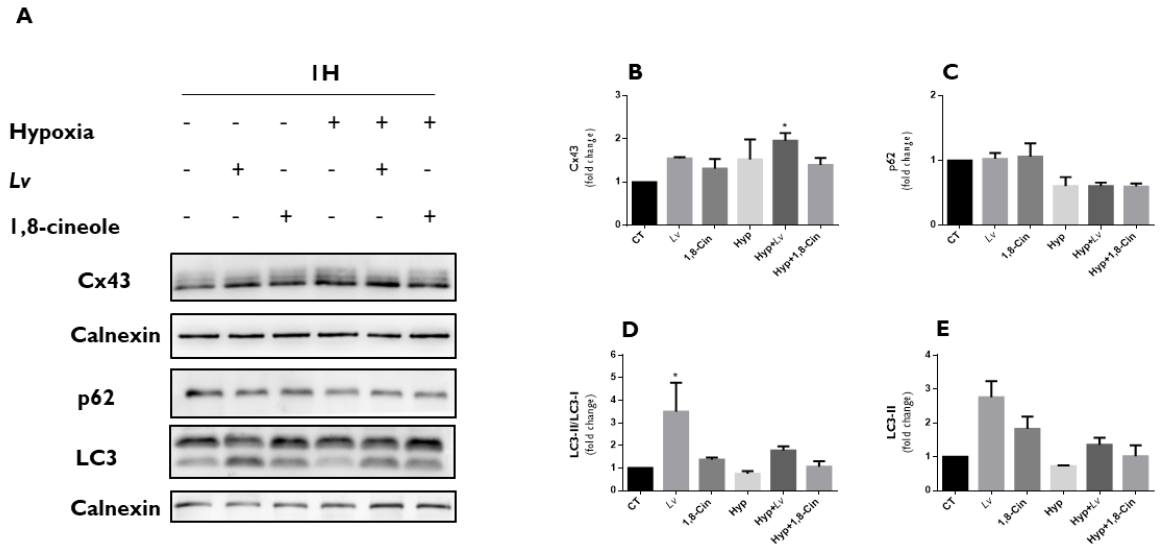


***Figure 21. Lavandula viridis* essential oil and 1,8-cineole modulate the levels of Cx43 and LC3-II in pulmonary arterial endothelial cells**
HPAEC in hypoxic or normoxic conditions were cultured in EOs/terpenes-depleted medium (CT) or selected EOs/terpenes concentrations: 25 µg/mL *L. viridis* EO (*Lv*) and 400 µg/mL 1,8-cineole (1,8-Cin), for 1 hour. The levels of proteins associated with autophagy and intercellular communication were assessed by WB analysis. (A) Representative WB of protein levels of Cx43, p62, LC3 (LC3-I and LC3-II) and Calnexin. Quantification of Cx43 (B), p62 (C), LC3-II/LC3-I (D) and LC3-II (E) protein levels. Quantification of p62, Cx43 and LC3-II was normalized to Calnexin levels. Results represent the mean ± S.E.M. of three independent experiments ($*p < 0.05$, compared to control).

On the other hand, on a different set of experiments, very slight differences in Cx43 (Figure 22B), p62 (Figure 22C) and LC3-II levels were observed (Figure 22D-E) in cells subjected to hypoxia, *T. zygis* subps. *sylvestris* EO and thymol treatments, thus suggesting no effect of these stimuli.

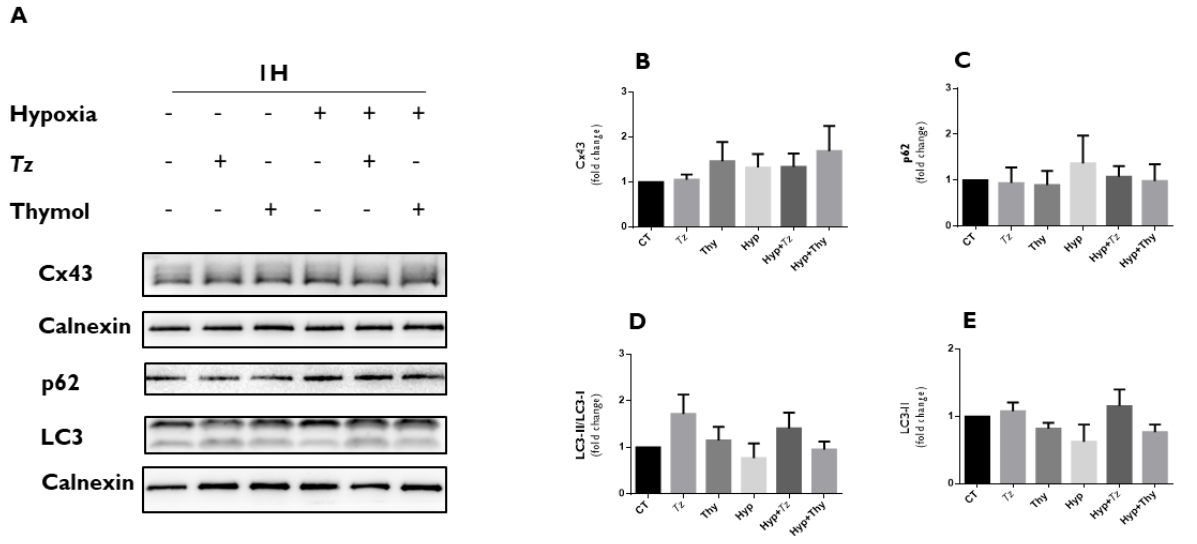


***Figure 22. Thymus zygis* subps. *sylvestris* essential oil and thymol show no effect on p62, Cx43 and LC3-II levels in pulmonary arterial endothelial cells**

HPAEC in hypoxic or normoxic conditions were cultured in EOs/terpenes-depleted medium (CT) or selected EOs/terpenes concentrations: 6.25 µg/mL *T. zygis* subsp. *sylvestris* EO (*Tz*), 3.125 µg/mL thymol (Thy), for 1 hour. The levels of proteins associated with autophagy and intercellular communication were assessed by WB analysis. (A) Representative WB of protein levels of Cx43, p62, LC3 (LC3-I and LC3-II) and Calnexin. Quantification of Cx43 (B), p62 (C), LC3-II/LC3-I (D) and LC3-II (E) protein levels. Quantification of p62, Cx43 and LC3-II was normalized to Calnexin levels. Results represent the mean ± S.E.M. of three independent experiments.

Like in MCEC-1, an immunofluorescence assay for detection of Cx43 and LC3 puncta in HPAEC was performed, following 1 hour of EOs/terpenes treatments. Figure 23 shows that Cx43 distribution has a similar pattern in all the experimental conditions tested, with the protein being localized at both the membrane and perinuclear region. Once again, the simultaneous staining with antibodies against Cx43 and LC3 shows a co-localization of these two proteins in cells treated with EOs/terpenes, suggesting that Cx43 is being engulfed in autophagic vesicles. Moreover, LC3 puncta in the cell cytoplasm increased with EOs/terpenes treatment, similarly to what was observed in MCEC-1. This indicates an increase in autophagic vesicle formation, suggesting that autophagy is being activated, which corroborates WB analysis.

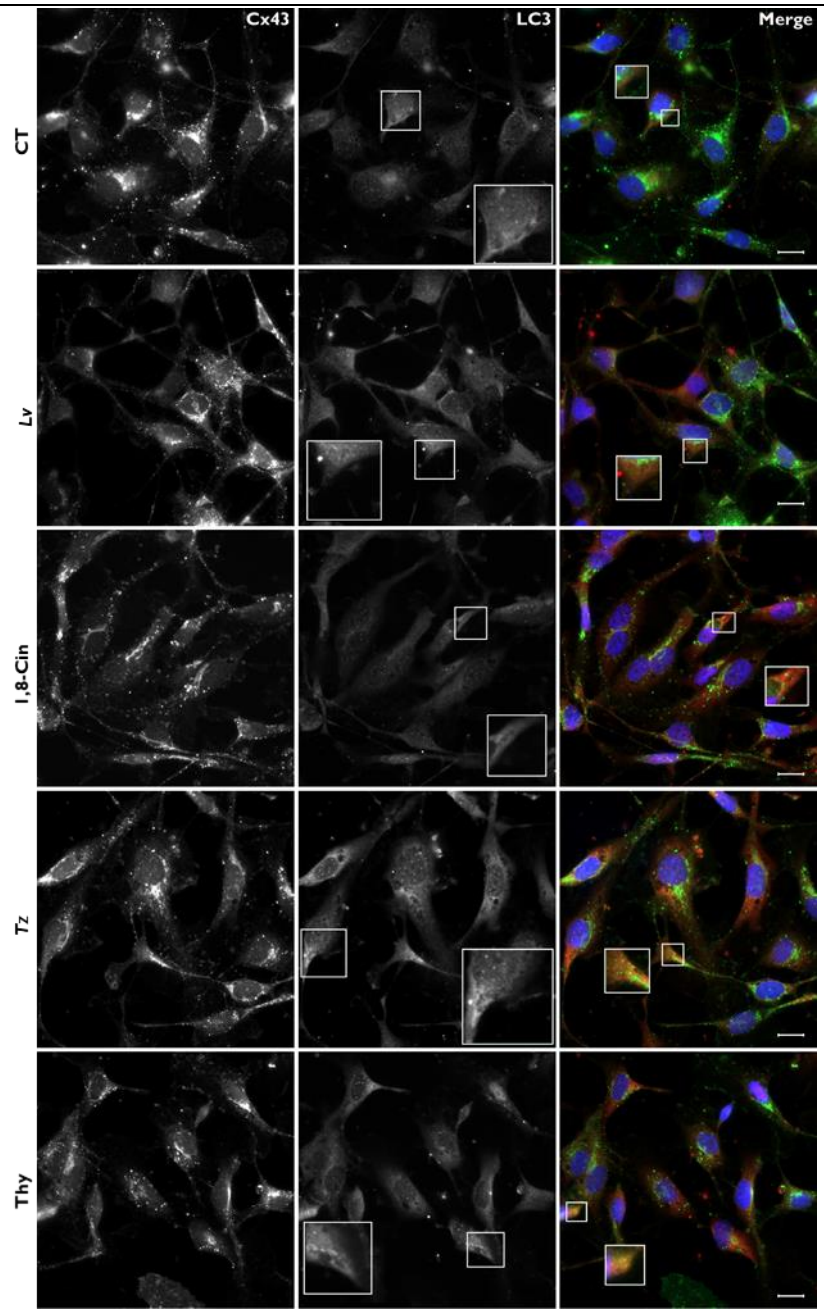


Figure 23. **Essential oils/terpenes increase autophagic vesicles in pulmonary arterial endothelial cells**
The presence of LC3 was assessed by immunofluorescence in HPAEC cultured in EOs/terpenes-depleted medium (CT) or selected EOs/terpenes concentrations: 25 µg/mL *L. viridis* EO (*Lv*), 400 µg/mL 1,8-cineole (1,8-Cin), 6.25 µg/mL *T. zygis* subps. *sylvestris* EO (*Tz)* and 3.125 µg/mL thymol (Thy), for 1 hour. Representative immunofluorescence images show an increase in LC3 presence and puncta positively stained for LC3. Nuclei were stained with DAPI. Scale bar: 20 µm. (n=1).

9.7.3. *Lavandula viridis* essential oil enhances the autophagic flux in pulmonary arterial endothelial cells

The previous results with *L. viridis* EO treatment suggest that this EO may be either promoting autophagic flux or inhibiting lysosomal degradation in HAPEC. To distinguish between these two possibilities an inhibitor of autophagosome-lysosome fusion, bafilomycin A1, was used.

Following 1-hour incubation with *L viridis* EO and bafilomycin A1, a WB analysis was performed to assess the levels of Cx43, p62 and LC3. As expected, bafilomycin A1 led to an increase in Cx43, p62 and LC3-II protein levels (Figure 24B-E). Moreover, the treatment with EO alone also leads to an accumulation of these proteins. Importantly, the results presented in Figure 24 C-E, reveal that cells concomitantly treated with *L. viridis* EO and bafilomycin A1, have a greater accumulation of p62 and LC3-II, suggesting that the EO enhances autophagic flux in HPAEC.

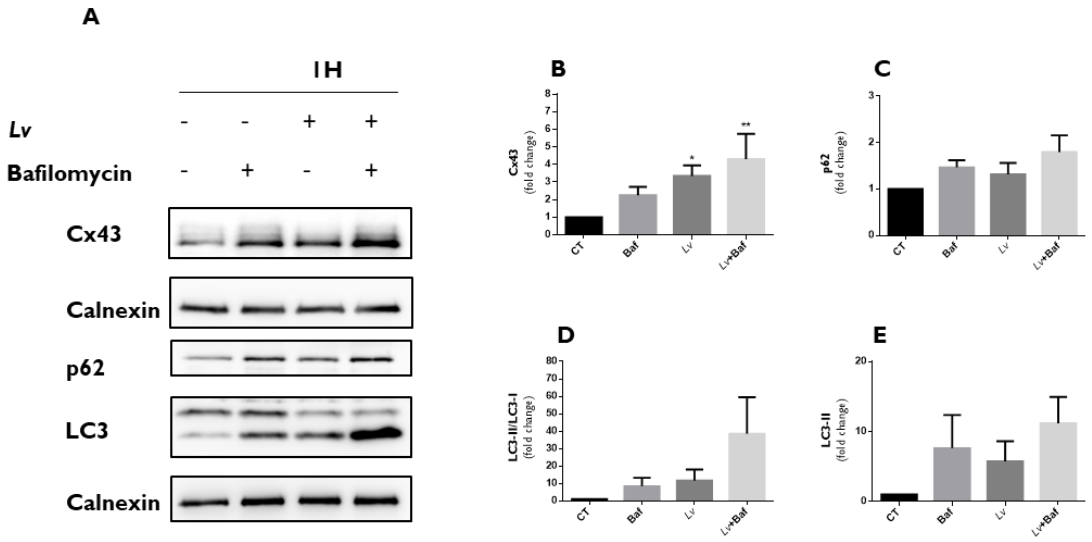


***Figure 24. Lavandula viridis* essential oil enhances the autophagic flux in pulmonary arterial endothelial cells**
HPAEC were incubated with EO-depleted medium (CT), selected EO concentration: 25 µg/mL L. viridis EO (Lv) and 100 nM of bafilomycin A1 (Baf), for 1 hour. Differences in proteins associated with autophagy were assessed by WB analysis. (A) Representative WB of protein levels of Cx43, p62, LC3-I and LC3-II. Quantification of Cx43(B), p62 (C), LC3-II/LC3-1 (D) and LC3-II (E) protein levels. Quantification of Cx43, p62 and LC3-II was normalized to Calnexin. Results represent mean ± S.E.M. of three independent experiments ($*p$ < 0.05, $**p$ < 0.01, compared to control).

Interestingly, immunofluorescence images corroborate WB analysis, by showing an increase in Cx43 levels and LC3 vesicles. *L. viridis* EO seems to increase the amount of Cx43 in the plasma membrane, this suggesting that *L. viridis* EO may influence Cx43 synthesis. Also, a clear accumulation of LC3 puncta was observed with *L. viridis* treatment and an even higher accumulation occurred in the presence of bafilomycin A1, thus suggesting an obvious increase in the autophagic flux.

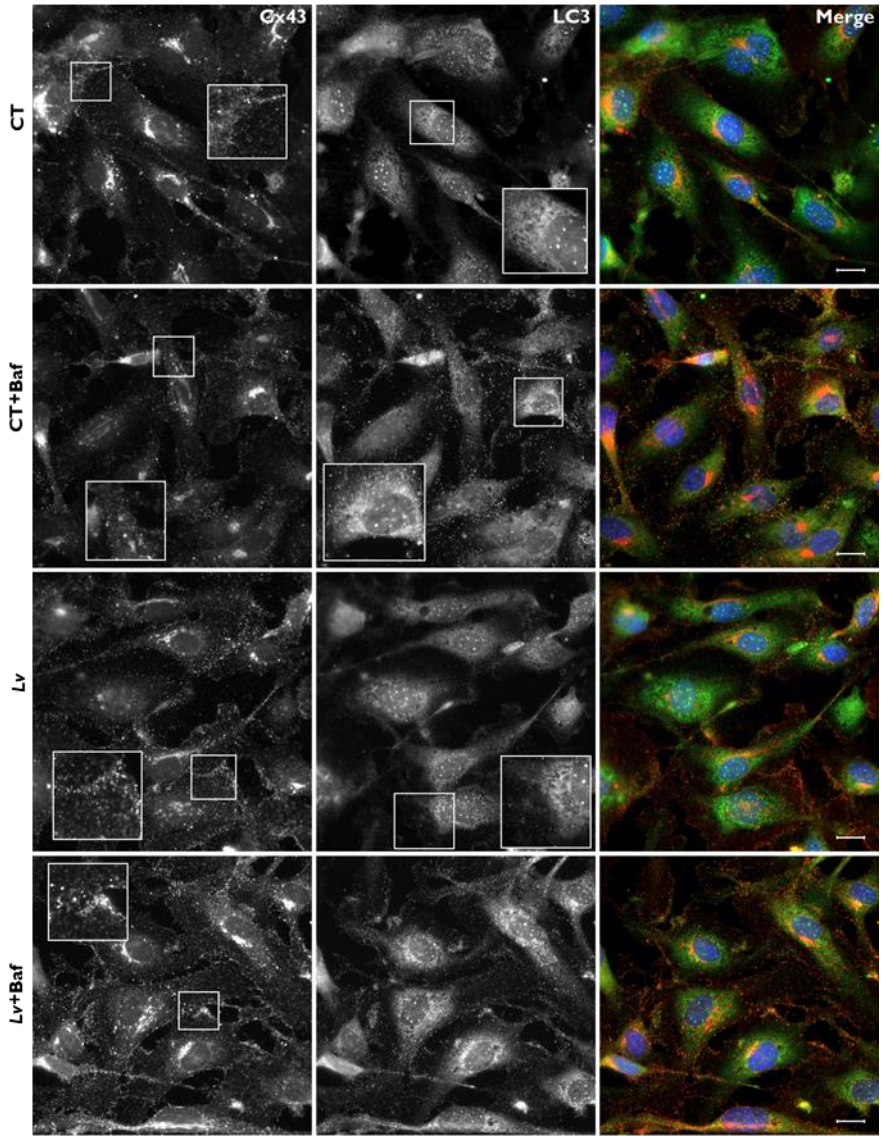


***Figure 25. Lavandula viridis* essential oil increases the formation of autophagic vesicles in pulmonary arterial endothelial cells**
The presence of LC3 was assessed by immunofluorescence in HPAEC cultured in EO-depleted medium (CT), selected EO concentration: 25 µg/mL *L. viridis* EO (*Lv*) and 100 nM of bafilomycin A1 (Baf), for 1 hour. Representative immunofluorescence images show an increase in LC3 presence and puncta positively stained for LC3. Nuclei were stained with DAPI. Scale bar: 20 µm. (n=1).

9.7.4. *Lavandula viridis* essential oil increases proteins synthesis in pulmonary arterial endothelial cells

As previously shown, *L. viridis* EO clearly increases the autophagic flux in HPAEC. Therefore, it would be expected an increase in the degradation of proteins that are a substrate of autophagy, such as Cx43. Nevertheless, the treatment with *L. viridis* EO induces an accumulation of Cx43 as shown in section 6.7.3., suggesting that this EO might be additionally interfering with Cx43 synthesis. To clarify this question a WB analysis was

carried out in the presence of both, the protein synthesis inhibitor, cycloheximide, and the lysosomal inhibitor, bafilomycin A1 in combination with *L. viridis* EO treatment.

The results in Figure 26 show that protein levels are decreased in the presence of cycloheximide (synthesis inhibited) and are increased in the presence of bafilomycin A1 (degradation inhibited). Importantly, in the presence of cycloheximide, *L. viridis* EO treatment leads to an increase in Cx43 levels, compared to control and cycloheximide alone. Therefore, based in these results it can be speculated that this EO promotes Cx43 synthesis.

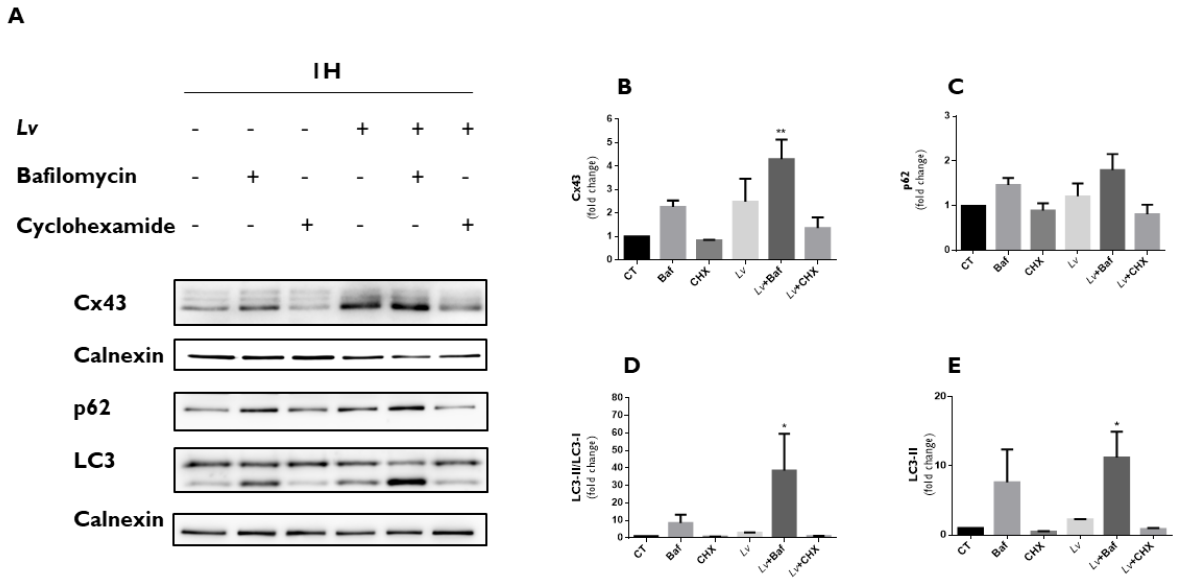


***Figure 26. Lavandula viridis* essential oil increases proteins synthesis in pulmonary arterial endothelial cells**
HPAEC were cultured in EO-depleted medium (CT), selected EO concentration: 25 µg/mL L. viridis EO (Lv), 100 nM of bafilomycin A1 (Baf) and 50 µg/mL cycloheximide (CHX), for 1 hour. Differences in proteins were assessed by WB analysis. (A) Representative WB of protein levels of Cx43, p62, LC3-I and LC3-II. Quantification of Cx43(B), p62 (C), LC3-II/LC3-I (D) and LC3-II (E) protein levels. Quantification of Cx43, p62 and LC3-II was normalized to Calnexin. Results represent mean ± S.E.M. of three independent experiments ($*p < 0.05$, $**p < 0.01$, compared to control).

Discussion

10. Discussion

Pulmonary arterial hypertension is characterized by a persistent elevation of pulmonary artery pressure that leads to right ventricular hypertrophy (RVH). Left untreated, PAH can result in heart failure and death. Although the understanding of the pathogenesis of PAH has expanded significantly in recent years, allowing the development of therapeutic approaches with some efficacy, a cure for this disease is far from being achieved. Therefore, more studies are required to elucidate the molecular mechanisms underlying its pathophysiology, thus opening new avenues for the development of more efficient therapeutic strategies. It has been demonstrated that targeting endothelial dysfunction is one of the most promising strategies for therapeutic development in PAH (Ranchoux *et al.*, 2018).

Over the years, herbal medicines have gained increasing attention and reputation and are currently successfully used in the treatment of chronic and acute conditions such as CVDs (Liperoti *et al.*, 2017). Bearing in mind the potential of aromatic plants and essential oils or their isolated compounds, the work herein presented aimed to evaluate the effect of selected EOs/terpenes on several biological processes associated with endothelial (dys)function, namely angiogenesis, autophagy and intercellular communication. To address this question, we used two different types of cells, a microvascular neonatal mouse cardiac endothelial cell line MCEC-1 and primary cultures of human pulmonary arterial endothelial cells, HPAEC. The use of a cell line has the advantage of being easy to use, consists of an homogenous population of cells and gives rise to large amount of biological material, however they can functionally differ from primary cultures which better mimic an *in vivo* condition. Therefore, to overcome this limitation, we also used primary cultures of HPAEC. Nevertheless, the amount of material available is scarce, which compromises the data generated. Moreover, it should be kept in mind that MCEC-1 are derived from microvascular cells, while HPAEC are of macrovasculature nature, which may justify some of the differences observed between the two types of cells.

Since herbal medicine´s safety is a crucial issue if a translation approach is envisioned, the cytotoxicity of the EOs and their major compounds was assessed. These assays allowed the selection of safe concentrations to be used in the following experiments. Overall, 1,8-cineole was the safest compound tested and thymol was the most toxic. Moreover, the EOs and thymol showed a more toxic profile in HPAEC, that may be explained by the higher sensitivity of these primary cultures in comparison to cell lines (Pan *et al.*, 2009).

Regarding the bioactivity of the EOs/terpenes, the first set of experiments was designed to assess their impact of different aspects of the angiogenic process, namely cell migration, tube formation, sprouting and proliferation.

In the migration assays only 1,8-cineole showed a significant effect after 10h of incubation in MCEC-1, whereas all EOs/terpenes tested were effective after 4 hours of incubation in HPAEC. A more detailed analysis of these results showed that MCEC-1 migrated slower towards wound closure (> 12 hours) than HPAEC, that completely cover the scratch after 6 hours. It seems that MCEC-1 and HPAEC have distinct migration patterns that may justify the different effects observed. Indeed, the concentration used for 1,8-cineole was higher due to its safety profile and probably higher concentrations of the other EOs/terpenes are needed to exert an effect in MCEC-1. Therefore, further experiments should include dose-response curves to determine the most effective concentrations. As far as it is known, no studies regarding the effect of *L. viridis* and *T. zygis* subps. *sylvestris* EOs on cells migration capacity have been conducted so far. However, *in vivo* studies have demonstrated the wound healing ability of 1,8-cineole in epithelial cells (Rocha Caldas *et al.*, 2015).

Cell migration requires the formation of actin-driven membrane protrusion at the cell front, known as lamellipodia. HPAEC stained with Phalloidin, that binds to F-actin, thus detecting lamellipodia formation, showed a multipolar morphology with several lamellipodia protruding from the cell body in all directions, with an higher number of lamellipodia being formed in the presence of EOs/terpenes. Since lamellipodia formation is a fine tuned process regulated by Rho-family GTPases, namely Rac1 that plays a pivotal role in protrusion formation and forward motion (Kurokawa *et al.*, 2004), it is conceivable that the EOs/terpene have a positive effect in Rac1 activation. These results are in accordance with the previous migration data and strengthen the hypothesis that EOs/terpenes can be a promising therapeutic strategy to induce endothelial cell migration.

Since *in vitro* endothelial cell tube formation on basement membranes (e.g. matrigel) replicates many of the cardinal features involved in angiogenesis, including adhesion, migration, protease activity, alignment and tube formation, it has been widely and consistently used to screen angiogenic compounds (Kleinman *et al.*, 2000; Auerbach, 2003;. Arnaoutova *et al.*, 2009). In this work, no statistical differences were observed between control and treatments in HPAEC. Nevertheless, at 2 hours of treatment *L. viridis* EO seems to enhance tube formation, while 1,8-cineole and thymol, seem to impair this process. Based on these data it can be speculated that these compounds modulate tube formation at an

early stage, namely by modulating cell adhesion. To clarify this, an adhesion assay was performed (data not shown), but no differences were observed. Another important aspect to consider are the signaling pathways implicated in tube formation. For example, angiopoietin (Ang 1) is an important protein involved in the re-organisation of endothelial cells to form tubes. Ang I is produced by surrounding stromal cells and facilitates endothelial cell survival and stabilization of the new capillary tubes (Carmeliet, 2003). It is plausible that the results observed in the present work can be ascribed to an activation of Ang I by *L. viridis* EO whereas the terpenes tested impair its function. However, future studies are needed to clarify this and understand how EOs/terpenes modulate this protein.

Cell based angiogenic assays should be carefully interpreted since different cell lines or primary cultures may present distinct endothelial phenotypes (Unger *et al.*, 2002). Therefore, the results were confirmed in a more reliable system such as the *ex vivo* aortic ring assay, considered to better mirror the changes occurring during *in vivo* angiogenesis. Moreover, the vascular outgrowths in the aortic rings show a morphology similar to newly formed blood microvessels, since they also recruit perivascular smooth muscle cells and pericytes (Nicosia and Villaschi, 1995). Our results show that *T. zygis* subps. *sylvestris* EO, 1,8-cineole and thymol induce sprout formation, being the latter more effective, suggesting that EOs/terpenes can be effective in promoting sprouting angiogenesis. It is well known that sprout initiation involves one endothelial cell responding to angiogenic stimuli by extending filopodia, and then migrating outward from the parent vessel while still connected to its neighbors. These neighbor cells are largely unresponsive in terms of morphogenesis but respond to growth factors, such as vascular endothelial growth factor (VEGF) by dividing (Chappell, Wiley and Bautch, 2011). It is conceivable that EOs/terpenes may stimulate pro-angiogenic factors, which in turn trigger signaling pathways in ECs that leads to the formation of EC sprouts. Indeed, there are studies that demonstrate that EOs from *Citrus* sp. and *Chamaecyparis* sp. enhances VEGF. However, these results were performed in other cells and different assays (Lee *et al.*, 2010; Lee, 2017).

Regarding cell proliferation, all treatments showed a negative impact. These results are quite unexpected since cell proliferation is required for new vessel formation. Nevertheless, it is important to refer that proliferation was assessed after 6 hours to compare the results with migration assays, and further experiments should be carried out at latter time points. Moreover, different proliferation assays, such as BrdU staining, should be performed to corroborate these results.

Taken together, these experiments (migration, tube formation, sprouting and proliferation assays) suggest a pro-angiogenic effect for the EOs/terpenes tested. At a first sight, these results seem to conflict with several studies that describe EOs and their compounds as anti-carcinogenic, due to their anti-angiogenic effects (Chen *et al.*, 2011; Gautam, Mantha and Mittal, 2014). Nevertheless, anti-carcinogenic assays are performed in tumor cell lines whereas angiogenesis assays are carried out in endothelial cells, which may justify the opposite results (Gautam, Mantha and Mittal, 2014).

Further experiments are being performed to corroborate these results in different endothelial primary cultures and *in vivo* experiments will be considered to validate the effects of the most promising EO/terpene.

The second set of experiments of this work was performed to assess the effect of EOs/terpenes on the expression of proteins associated with autophagy and intercellular communication, using WB and immunofluorescence assays.

Autophagy is a highly conserved cellular process where organelles and cytosolic proteins are encapsulated within double-membraned autophagosomes that ultimately fuse with lysosomes for degradation. Concomitantly, a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II). This conversion of LC3-I into LC3-II and the recruitment of LC3-II to the autophagosome constitute a reliable marker of autophagic activity (Wu *et al.*, 2006; Liu *et al.*, 2010). Our results indicate that the EOs and 1,8-cineole lead to an accumulation of LC3-II in MCEC-1, that can result from an increased conversion of LC3-I in LC3-II which would suggest an enhanced autophagosome formation. This was confirmed by immunofluorescence, where MCEC-1 subjected to EOs/terpenes treatments led to more puncta formation that positively stained for LC3, reinforcing the hypothesis of an enhancement of autophagic vesicles' formation. Moreover, it is described in the literature that under basal conditions LC3 is found evenly distributed in both, the nucleus and the cytoplasm, and that under nutrient-deprived conditions, LC3 suffers a redistribution from the nucleus to the cytoplasm (Huang and Liu, 2015). Similarly, in our experiments, in the control condition we observed LC3 uniformly distributed, while in cells subjected to EOs/terpenes it was notorious an increase in the accumulation of LC3 in the cytoplasm. Treatments with EOs/terpenes are not exerting any effect on the levels of p62, a canonical substrate of autophagy, while the levels of Cx43, also shown to be degraded by macroautophagy, increase in cells incubated with *L. viridis* EO and 1,8-cineole, suggesting that this protein is being incorporated into autophagic vesicles. This result was confirmed by immunofluorescence where a co-

localization between Cx43 and LC3 was observed. Contrarily, with *T. zygis* subps. *sylvestris* EO and thymol stimuli, the levels of Cx43 tend to decrease. Since it is well known that autophagy is an important connexin-degradation pathway (Ribeiro-Rodrigues *et al.*, 2014; (Martins-Marques *et al.*, 2015); Martins-Marques *et al.*, 2015; Iyyathurai *et al.*, 2016) it is plausible that the later treatments promote Cx43 degradation. An important aspect to consider is that the hypoxic stimuli showed opposite results in two sets of experiments which can be explained by the volatility of the EOs/terpenes, tested at the same time in each experimental setup, which may interfere with the results. Therefore, in further experiments a separate multiwell for each treatment should be considered.

In HPAEC *L. viridis* EO significantly increased the levels of LC3 and Cx43. To further characterize the effect of *L. viridis* EO in autophagic flux, we evaluated the levels of LC3-I and LC3-II either in the presence or absence of the lysosome inhibitor (Bafilomycin A1). Increases in LC3-II simply indicates the accumulation of autophagosomes but does not guarantee autophagic degradation. If, however, the amount of LC3-II further accumulates in the presence of bafilomycin A1, this indicates an enhancement of the autophagic flux. The results obtained in the present study showed that *L. viridis* EO promoted an increase in this activity. A proper autophagic flux relies on an equilibrium between autophagosome formation and its clearance by the lysosomes. Therefore, it would be expected that compounds that activate autophagy promote degradation of proteins that are a substrate for autophagy, such as Cx43. However, surprisingly, the levels of this protein were increased, which could be ascribed to an increased protein synthesis. To test the hypothesis that *L. viridis* EO enhance Cx43 synthesis, the cells were incubated with the EO in the presence of an inhibitor of protein synthesis (cycloheximide). Since in this circumstance the levels of Cx43 increased, it is suggested that *L. viridis* EO induces protein synthesis. Future studies, such as RT-PCR, are being considered to quantify the transcript levels and confirm these results. Nevertheless, other explanations for these results should also be taken into account, such as the possible effect of the EO on endocytosis. During endocytosis the pH is a crucial factor and the V-ATPase is largely responsible for the accumulation of H^+ (Huynh and Grinstein, 2007; Scott and Gruenberg, 2011). Based on previous studies demonstrating that the inhibition of vacuolar H^+-ATPases causes an accumulation of Cx43 in early endosomes (Leithe, Brech and Rivedal, 2006), it is likely that the effect of *L. viridis* EO on Cx43 is mediated by the inhibition of this vacuolar H^+-ATPases.

Up to date, studies on the autophagic effect of EOs/terpenes on ECs are lacking. Also, in PAH, studies on autophagy remain inconclusive but it appears that this process has

an important function, through regulation of hypoxic cell proliferation (Fraidenburg and Yuan, 2013). Furthermore, a study demonstrated that knockdown of LC3 enhanced the proliferation of pulmonary arterial endothelial cells (PAEC) and pulmonary arterial muscle cells (PASMC) in hypoxic conditions, and overexpression of LC3 inhibited the proliferation in both (Lee *et al.*, 2011). From this perspective, our results suggest that *L. viridis* EO can be a promising therapeutic agent for PAH, since cell proliferation is an hallmark of this disease and the increase of autophagy could be a solution. Interestingly, we can suggest that the EOs and 1,8-cineole act as a calorie restriction mimetic, since caloric restriction (CR) is a potent inducer of autophagy. Importantly, it is well established that CR delays the progression of age-related diseases, including CVDs. Indeed, clinical and experimental evidences in numerous species have demonstrated that CR can exert protective effects on the cardiovascular system (Thomas *et al.*, 1993; Weindruch, 1996; Han and Ren, 2010), which reinforces the promising potential of the EOs/terpenes herein tested.

Overall, the results presented in this work bring new insights to the field and the EOs/terpenes used showed promising cardiovascular effects. Nevertheless, future studies are required to better elucidate the mechanisms involved. Thus, to enlighten relevant questions that arised during this study, further experiments are being conducted to determine dose-response curves. Additionally, other studies are planned to evaluate angiogenesis, namely the endothelial sprouting assay with EC coated beads and the *ex vivo* chick chorioallantoic membrane assay (CAM). Also, to validate if the EOs/terpenes are enhancing autophagic flux, ECs will be transfected with a fluorescent-tagged LC3 (mCherry-GFP-LC3) that is a suitable assay for monitoring autophagic flux based on different pH stabilities of GFP and mCherry fluorescent proteins. Moreover, intercellular communication will be evaluated by Scrape Loading/Dye Transfer assay.

Taken together, we believe that some EOs/terpenes could represent a promising new therapeutic strategy for PAH, by mediating pro-angiogenic and autophagic responses. Moreover, bearing in mind that EOs are complex mixtures of several compounds, namely terpenes, synergistic effects of the compounds could lead to more effective outcomes, thus impacting, in the long run, on patient's quality of life.

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