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EXPLORING THE ANTI-AGING POTENTIAL OF LAVANDULA PEDUNCULATA EXTRACTS: EFFECT ON CARDIAC CELLULAR SENESCENCE

Dissertação no âmbito do Mestrado em Farmácia – Especialização em Farmacoterapia Aplicada, orientada pela Doutora Mónica da Rocha Zuzarte, pela Doutora Sara Raquel Ramalho Pereira Nunes e pelo Doutor Jorge Miguel Alves Silva e apresentada na Escola Superior de Tecnologia da Saúde do Politécnico de Coimbra para obtenção do grau de Mestre.

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Resumo

O envelhecimento é um declínio progressivo e irreversível das funções fisiológicas do organismo, levando ao comprometimento da saúde e ao aumento do risco de desenvolvimento de doenças ao longo da vida. O envelhecimento pode ser identificado através de diversos marcadores moleculares, celulares e sistémicos. Neste trabalho destaca-se a senescência celular devido ao seu papel central no envelhecimento, tornando-a um fator de risco fundamental para as doenças cardiovasculares.

A senescência celular é um mecanismo fisiológico no qual uma célula interrompe o seu ciclo celular e entra num estado de repouso permanente, permanecendo metabolicamente ativa. Durante a senescência celular ocorre um aumento de múltiplos marcadores, nomeadamente os níveis proteicos de p53/21, a atividade de SA-β-gal e a secreção de SASP. Ao longo dos anos, tem-se verificado um interesse crescente em terapias que modulam estas vias de senescência, em particular alternativas naturais capazes de retardar doenças relacionadas com a idade.

As plantas aromáticas e medicinais apresentam um elevado potencial anti-envelhecimento, pois são ricas em compostos bioativos que apresentam efeitos antioxidantes, anti-inflamatórios e cardioprotetores, podendo ajudar na prevenção e tratamento de doenças cardiovasculares. Os efeitos benéficos relatados para estas plantas podem ser atribuídos aos seus extratos, como os óleos essenciais e os extratos não voláteis ricos em compostos fenólicos. O presente estudo centra-se na *Lavandula pedunculata* (Mill.) Cav. e o potencial anti-senescente dos seus extratos. Estudos anteriores reportaram propriedades antioxidantes, anti-inflamatórias e antifúngicas significativas para esta espécie, o que justifica investimentos adicionais, particularmente na exploração do seu potencial em estratégias antienvelhecimento.

O objetivo deste trabalho foi avaliar o efeito do óleo essencial e do extrato fenólico de *Lavandula pedunculata* na atividade da SA-β-gal, danos na dupla hélice de ADN, área e deformações nucleares e inibidores do ciclo celular. O potencial anti-senescente foi avaliado em cardiomioblastos H9c2 com senescênciainduzida com doxorrubicina. No geral, o óleo essencial de *L. pedunculata* foi capaz de diminuir significativamente a atividade de SA-β-gal e yH2AX, bem como a área e

as deformações nucleares e os níveis das proteínas p53/p21, em concentrações não tóxicas. Estas descobertas destacam o potencial promissor desta espécie na mitigação da senescência cardíaca. No entanto, embora os resultados sejam bastante promissores, são necessários estudos adicionais para elucidar completamente o potencial bioativo destes extratos. No geral, o presente estudo aumenta a valorização de *L. pedunculata*, reforçando a sua relevância industrial e potencial como agente no antienvelhecimento e promotor da saúde cardiovascular.

Palavras-chave: rosmaninho, óleo essencial, cardiovascular, senolítico.

Abstract

Aging is an irreversible and progressive decline of the body's physiological functions, leading to health impairment and increased risk of disease development throughout life. Aging can be identified through several molecular, cellular and systemic markers. Here we highlight cellular senescence due to its central role in aging, making it a key risk factor for cardiovascular diseases.

Cellular senescence is a physiological mechanism in which a cell interrupts its cell cycle and enters a state of permanent rest, but remains metabolically active. During cellular senescence, there is an increase in multiple markers, namely p53/p21 protein levels, SA-β-gal activity and SASP secretion. Over the years, there has been a growing interest in therapies that modulate these senescence pathways, in particular natural alternatives capable of delaying age-related diseases.

Aromatic and medicinal plants have great anti-aging potential, as they are rich in bioactive compounds that have shown antioxidant, anti-inflammatory and cardioprotective effects, which may help prevent and treat conditions such as cardiovascular diseases. The beneficial effects reported for these plants can be attributed to their extracts, such as essential oils and non-volatile extracts rich in phenolic compounds. The present study focuses on *Lavandula pedunculata* (Mill.) Cav. and the anti-senescence potential of its extracts. Previous studies have reported significant antioxidant, anti-inflammatory, and antifungal properties for this species, which justify further investment, particularly in exploring its potential for anti-aging strategies.

The aim of this work was to evaluate the effect of the essential oil and phenolic extract of *Lavandula pedunculata* on SA-β-gal activity, double strand DNA damage, nuclear area and deformations, and cell cycle inhibitors. The anti-senescence potential was assessed using H9c2 cardiomyoblasts and senescence induced resorting to doxorubicin. Overall, *L. pedunculata* essential oil was able to significantly decrease SA-β-gal activity, yH2AX, nuclear area and deformations as well as p53/p21 protein levels, at non-toxic concentrations. These findings highlight its promising potential in mitigating cardiac senescence. Nevertheless, while the results are quite promising, further research is needed to fully elucidate the bioactive potential of these extracts. Overall, the present study enhances the valorisation of

L. pedunculata, reinforcing its industrial relevance and potential as an anti-aging agent and a promoter of cardiovascular health.

Keywords: lavender, essential oil, cardiovascular, senolytic.

List of Abbreviations

ATCC - American Type Cell Collection

CT – Control

DMEM - Dulbecco Modified Eagle Medium

EO – Essential Oil

Ext – Phenolic Extract

FBS - Fetal Bovine Serum

GA - Gallic Acid

GC - Gas Chromatography

GC/MS - Gas Chromatography Coupled to Mass Spectrometry

PBS - Phosphate Buffered Saline

PVDF - Polyvinylidene Difluoride

RI - Retention Indices

SA-β-gal - Senescence-Associated β-Galactosidase

SASP - Senescence-Associated Secretory Phenotype

SDS - Sodium Dodecyl Sulfate

TBS-T - Tris-Buffered Saline with 0.1% Tween

TPC - Total Phenolic Content

yH2AX - phosphorylated H2AX

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1. Introduction

Aging is an irreversible event that unfolds over time (Dziechciaż & Filip, 2014). It is related with a progressive decline of the body's physiological functions, leading to health impairment and increased risk of disease development throughout life (Foley et al., 2012). It involves both epigenetic and genetic factors (Beltrami et al., 2011).

The aging process is considered a risk factor for several diseases including chronic conditions, neurodegenerative diseases and cancer, with its impact remaining highly pronounced in cardiovascular diseases, such as atherosclerosis, hypertension, myocardial infarction and stroke (Foley et al., 2012). It is estimated that at least one in three adults is affected by cardiovascular diseases (Camici et al., 2015) and among the elderly, it remains the main cause of death (Shioi & Inuzuka, 2012). By 2030, nearly a fifth of the world's population will be over 65 years old, which would potentially lead to an exponential increase in the prevalence of cardiovascular diseases (Costantino et al., 2016), representing a significant impact on health systems, both economically and socially.

Cardiovascular aging is associated with structural and functional changes in the cardiovascular system, including left ventricular hypertrophy, decline in diastolic function, atrial fibrillation (Obas & Vasan, 2018), increased arterial stiffness, compromised endothelial function (Foley et al., 2012) and thickening of the arterial wall (Costantino et al., 2016). This occurs as a consequence of phenotypic changes in different types of cells, such as endothelial cells and smooth muscle cells (Costantino et al., 2016).

Aging can be identified through several molecular, cellular and systemic markers, such as: DNA instability, telomere attrition, epigenetic changes, loss of proteostasis, mitochondrial dysfunction, cellular senescence, deregulated nutrient-sensing, compromised macroautophagy, stem cell exhaustion, altered intercellular communication, chronic inflammation and dysbiosis (López-Otín et al., 2023). These mechanisms are considered hallmarks of aging, which have shown to be quite beneficial in pinpointing intervention targets, mainly in diseases associated with aging (Zuzarte et al., 2024). Regardless of the number of hallmarks considered, there is a proven hierarchy among them. Consequently, the hallmarks of aging can be divided into three categories: primary (causes of age-associated damage),

antagonistic (responses to damage) and integrative (consequences of responses to damage; aging phenotype) (McHugh & Gil, 2018). All of these factors are interconnected, with a particular emphasis on cellular senescence, an antagonistic marker, due to its central role in aging, making it a key risk factor for cardiovascular diseases (Evangelou et al., 2023; McHugh & Gil, 2018).

Cellular senescence is a physiological mechanism in which a cell interrupts its cell cycle and enters a state of permanent rest as a response to an extrinsic or intrinsic stimulus (Evangelou et al., 2023; González-Gualda et al., 2021). Senescent cells generally suspend their growth in the G1 phase of mitosis and, despite losing the ability to divide and proliferate, remain metabolically active (Beltrami et al., 2011; Chen et al., 2022; González-Gualda et al., 2021). The senescent response is somewhat complex and can result in both beneficial or harmful effects on the tissue/organism (González-Gualda et al., 2021; Zhang et al., 2022). The main types of senescence known are replicative senescence, oncogene-induced senescence, and stress-induced senescence (Chen et al., 2022), all necessary for normal development, by acting as a response to damage and stress (McHugh & Gil, 2018), contributing to tumour suppression, wound healing and tissue homeostasis in young organisms (Childs et al., 2017; González-Gualda et al., 2021). The negative side of senescence is observed in aged tissues, which exhibit an accumulation of senescent cells (Evangelou et al., 2023), translating into tissue dysfunction, localized chronic inflammation and development of age-associated diseases (González-Gualda et al., 2021). The pathogenic role of cellular senescence can be explained by the senescence-associated secretory phenotype (SASP) (López-Otín et al., 2023), where secretion of bioactive mediators, including cytokines, chemokines, growth factors and enzymes cause local inflammation and tissue damage (Childs et al., 2017; Hernandez-Segura et al., 2018; Matjusaitis et al., 2016). In the cardiac context, cellular senescence acts as a stress response mechanism, which can be triggered by several factors, including oxidative stress, DNA damage, mitochondrial dysfunction, hypoxia, among others (Evangelou et al., 2023; González-Gualda et al., 2021).

There is a set of hallmarks that allow us to distinguish senescent cells from normal cells (***Figure 1***), such as stable cell cycle arrest, increased lysosomal compartment, resistance to apoptosis, among others (González-Gualda et al., 2021). During cellular senescence, an increase in p16, p21 and p53 protein levels

is observed, as well as an increase in senescence-associated β-galactosidase (SA-β-gal) activity and SASP secretion (Chen et al., 2022; Zhang et al., 2022). At a morphological level, senescent cells exhibit a flatter and more elongated appearance, accumulation of lysosomes and mitochondria, and nuclear changes (Hernandez-Segura et al., 2018). As no single characteristic can confirm cellular senescence, several markers are evaluated to validate the senescent phenotype (González-Gualda et al., 2021). At least 3 different markers should be analysed, namely those related to cell cycle arrest (such as p16, p21 or p53), a relevant structural alteration (such as SA-β-gal) and a known feature specific to the senescence subtype (such as morphological alterations or SASP) (González-Gualda et al., 2021).

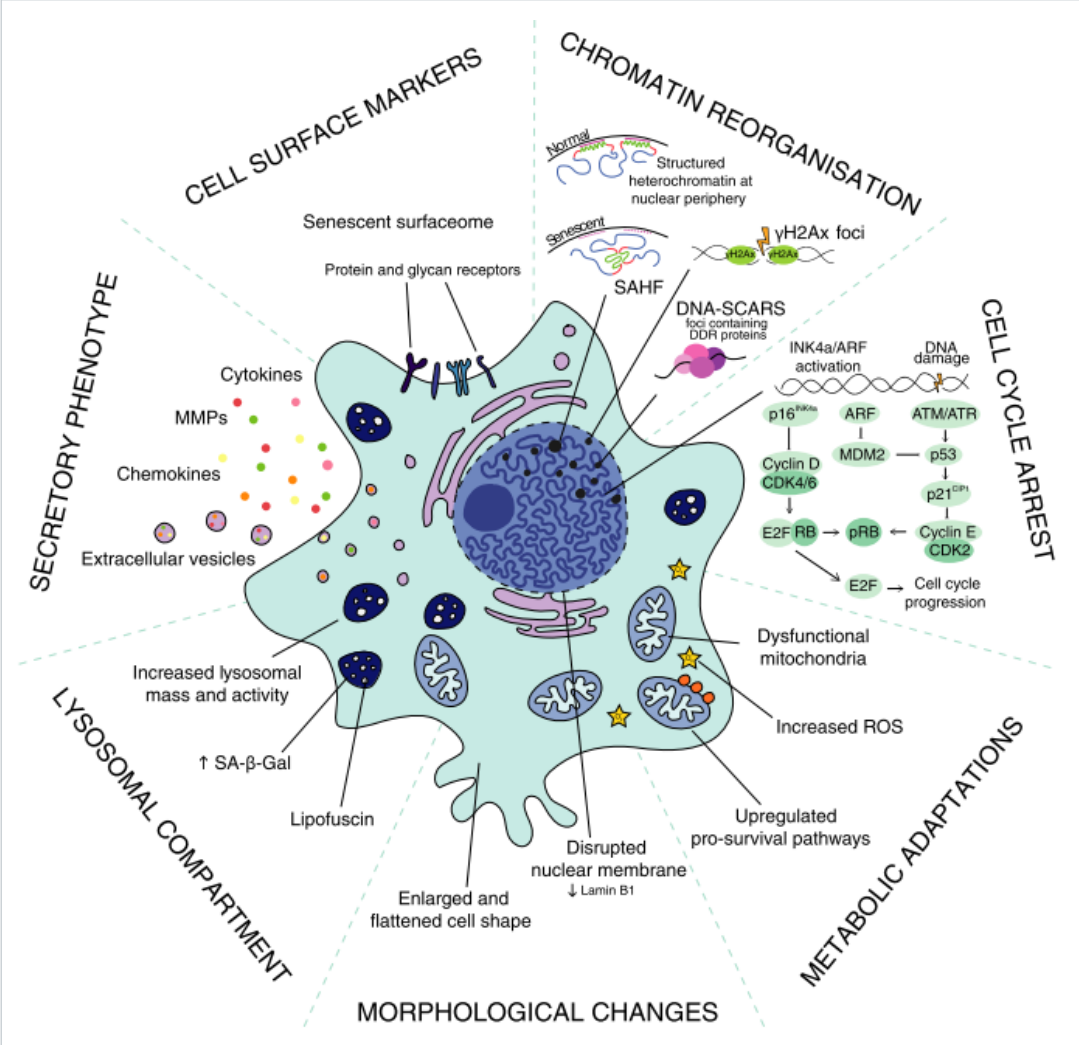

Figure 1. General hallmarks of cellular senescence (González-Gualda et al., 2021).

Given the fundamental role of cellular senescence in several age-related pathologies, there is a growing interest in the development of senotherapeutics which are capable of delaying its onset (Zhang et al., 2022). Senotherapeutics can be divided into two categories: senolytics that promote the apoptosis of senescent cells and senomorphics that suppress the effects of SASP without causing cell death (Zhang et al., 2023). Among existing therapies, quercetin (polyphenol) and dasatinib stand out as the first senolytics tested for cardiovascular disease treatment. Metformin, a widely studied antidiabetic drug, also demonstrates senomorphic activity, preventing/mitigating the development of cardiovascular diseases (Evangelou et al., 2023). In fact, the interest in anti-aging therapies is increasing and although the existing synthetic senotherapeutics show promising results against cellular senescence, there is growing research into natural alternatives (Imb et al., 2024).

Aromatic and medicinal plants have great anti-aging potential and have been widely used for various purposes since the early days of human history. Herbal extracts remain the primary source of medication for almost 80% of the population in developing countries (Naveed et al., 2020; Rastogi et al., 2016). These plants are rich in bioactive compounds like polyphenols, flavonoids, carotenoids, terpenes, vitamins and others. These bioactive compounds have shown antimicrobial, antioxidant, anti-inflammatory and other beneficial effects that may help prevent and treat conditions such as cardiovascular diseases, cancer, diabetes, infections, arthritis, neurological disorders, and gastric problems (Iweala et al., 2024; Ozkan et al., 2016). The beneficial effects reported for these plants can be attributed to their extracts such as essential oils and non-volatile extracts rich in phenolic compounds (Zuzarte et al., 2023).

Essential oils are volatile extracts obtained from parts of plants such as stems, flowers, seeds, peels, leaves, and roots (Saljoughian et al., 2018). These extracts are obtained, according to the European Pharmacopeia, by distillation using a Clevenger apparatus or by expression in the particular case of *Citrus* spp. fruits (Tongnuanchan & Benjakul, 2014). Chemically, essential oils are mainly composed by low molecular weight terpenes, particularly mono (e.g. limonene) and sesquiterpenes (e.g. caryophyllene), and, in some cases, by phenylpropanoids (e.g. eugenol). These compounds can be hydrocarbons (no oxygen in the structure) or can be oxygenated, giving rise to several subtypes of compounds such as phenols,

alcohols, esters, oxides, ketones, aldehydes, and others (Alves-Silva et al., 2021; Liang et al., 2022). Some studies have shown the potential anti-aging effect of several monoterpenes, as they exhibit various biological characteristics, such as antioxidant, anti-inflammatory, and cardioprotective effects. These compounds can act on oxidative stress, modulate inflammatory responses and promote cell regeneration (Zuzarte et al., 2024). For example, carvacrol (Chenet et al., 2019), limonene (Suh et al., 2017) and 1,8-cineole (Alves-Silva et al., 2022) were shown to inhibit mitochondrial dysfunction, while camphor decreased SA-β-galactosidase activity (Tran et al., 2015) and myrcene modulated SASP (Hwang et al., 2017).

Lavender (*Lavandula* L. spp.) species are widely known for their therapeutic benefits and have been used for centuries. These plants have an extensive history of traditional therapeutic applications, including migraines, anxiety, insomnia, heart disease, gastrointestinal problems, and others (Clara et al., 2017; Domingues et al., 2023; Lopes et al., 2018). Currently, in addition to herbal medicine, lavenders are used in cosmetics, perfumes, aromatherapy and foods. Native to the Mediterranean region, the *Lavandula* genus is now widely cultivated all over the world: Europe, Southwest Asia, the Middle East and North and South America. More than 30 species of *Lavandula* are known and in Portugal the genus is represented by 6 species: *L. latifolia* Medik, *L. multifida L*., *L. stoechas* subsp*. luisieri* (Rozeira) Rozeira, *L. stoechas* subsp. *stoechas* L., *L. pedunculata* (Miller) Cav. and *L. viridis* L'Her (Batilha et al., 2023; Vairinhos & Miguel, 2020). Although *Lavandula* is mostly known for its essential oils, the genus is also rich in polyphenols, coumarins, triterpenes, sterols, and tannins that might contribute to its overall biological effects. Since these bioactive molecules are synthetized as a response to the surrounding environment, factors such as geographic origin and growing conditions can influence the chemical composition of these extracts. (Batilha et al., 2023; Habán et al., 2023; Lopes et al., 2018). The present work focuses on *Lavandula pedunculata* (Mill.) Cav. growing in Castelo Branco region. Previous studies have reported significant antioxidant, anti-inflammatory, and antifungal properties for this species (Lopes et al., 2018; Zuarte et al., 2009; Zuzarte et al., 2022), highlighting its potential for anti-aging strategies and the promotion ofcardiovascular health.

Therefore, the aim of this work is to explore the anti-senescence potential of the essential oil and phenolic extract of *Lavandula pedunculata* (Mill.) Cav by evaluating their effect on SA-β-gal activity, double strand DNA damage (histone

yH2AX), nuclear area and deformations (Lamin A/C), and cell cycle inhibitors (p21 and p53 protein levels).

2. Materials and Methods

2.1. *Chemical characterization of Lavandula pedunculata extracts*

Lavandula pedunculata extracts, namely its essential oil and phenolic extract were previously obtained and characterized. Essential oils were obtained by hydrodistillation according to the European Pharmacopoeia (European Directorate for the Quality of Medicines & HealthCare [EDQM], 2023) and the phenolic extract was obtained by grinding lyophilized plant parts, followed by an extraction with 70% ethanol using ultrasonic and orbital shaking methods. The extracts were centrifuged, filtered, combined, concentrated with a rotary evaporator, and lyophilized for 4 days.

L. pedunculata essential oil was analysed by gas chromatography (GC) and gas chromatography coupled to mass spectrometry (GC/MS). Identification of the volatile compounds was achieved by analysing their retention indices (RIs) on two GC columns [SPB-1 and SupelcoWax-10 (Sigma-Aldrich, St. Louis, MO, USA)] and mass spectra, as previously reported (Zuzarte et al., 2022).

To evaluate the total phenolic content (TPC) of *L. pedunculata*, the Folin-Ciocalteau method was used. Briefly, plant extracts (100 µL; 3 mg/mL) were added to 100 µL of Folin-Ciocalteau reagent, 2 mL of sodium carbonate (Na_2CO_3; 7,5 %, w/v), and the volume was adjusted to 5 mL with distilled water. The mixture was incubated for 1h at room temperature, and the absorbance was read at 760 nm using a microplate reader (BMG LABTECH GmbH, FLUOstar Omega). Gallic acid was used as standard, and TPC was expressed as gallic acid equivalents (mg GA eq/g lyophilized extract).

2.2. Cell culture

The rat cardiomyoblast cell line, H9c2, was purchased from ATCC (American Type Cell Collection, ATCC CRL-1446). Cells were maintained in Dulbecco Modified Eagle Medium (DMEM, Gibco, Thermo Fisher, NY, USA) with high glucose (25mM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

2.3. Assessment of cell viability

The safety profile of both extracts was assessed on H9c2 cells through the resazurin assay. Briefly, cells were plated at a density of 50,000 cells/mL and after an overnight incubation, treated with different concentrations of *L. pedunculata* extracts (800 – 50 µg/mL) for 24h in standard culture conditions. The cells were then incubated with resazurin (final concentration 50 µM) for 3h at 37 ºC, after which the absorbance was recorded at 570 nm with a reference filter at 620 nm using a spectrophotometer (Biotek Synergy HT; Winnoski, VT, USA). A cell-free control was performed to exclude unspecific effects of the extracts on resazurin (data not shown). DMSO used to dilute the essential oil never exceeded 0.1%.

2.4. Anti-senescence potential of the extracts

2.4.1. Senescence-associated β-galactosidase activity

H9c2 were plated at 7,500 cells/mL and 30,000 cells/mL for untreated and doxorubicin-treated cells, respectively, and left to stabilize for 48h. Then, doxorubicin, a standard senescent inducer, (0.3 µM) was added for 24h. Following this period, culture medium was removed and fresh medium was added with or without the essential (200 and 100 µg/mL) or the phenolic extract (200 µg/mL) and cells were left to recover for 7 days. β-galactosidase activity was assessed using a commercially available kit, according to the manufacturer's instructions (#9860, Cell Signaling Technology Inc., Danvers, MA, USA). After colour development, with the distinct blue-green colour indicating β-galactosidase activity, the cells were photographed for subsequent image analysis. ImageJ software was used for quantitative analysis, by assessing the percentage of senescent cells. A minimum of 5 images were analysed for each condition, with a minimum of 100 cells counted in each independent assay.

2.4.2. Nuclear morphological alterations

For the nuclear staining of histone γH2AX and lamin A/C, H9c2 cardiomyoblasts were seeded at 7,000 cells/mL for untreated cells and 20,000 cells/mL for doxorubicin-treated cells in glass coverslips and treated, as reported in Section 2.4.1. At the end of the treatment, cells were fixed with 4% paraformaldehyde for 15min, followed by three washes with sterile PBS (137 mM

NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Then, cells were permeabilized with 0.1% Triton X-100 for 15min and washed with PBS (three times). Cells were incubated with blocking solution (3% bovine serum albumin, 10% goat serum in PBS) for 1h. Then, primary antibodies against yH2AX (1:500, #9718 Cell Signalling, Danvers, MA, USA) and Lamin A/C (1:500, sc-376248, Santa Cruz Biotechnology, Dallas, TX, USA), prepared in blocking solution, were added and the cells were incubated overnight at 4°C. Afterwards, coverslips were washed with PBS (three times), and incubated for 1h at room temperature with the corresponding secondary antibody (1:500, goat anti-rabbit Alexa Fluor 564 or 1:500, goat anti-mouse Alexa Fluor 633) and 4′,6-diamidino-2-phenylindole (DAPI;1:1000) prepared in blocking solution. After washes with PBS, coverslips were mounted in glass slides with Mowiol mounting medium. Images were acquired using a confocal point-scanning microscope (Zeiss LSM710; Carl Zeiss, Oberkochen, Germany) with a 63× objective.

2.4.3. Expression levels of p21 and p53 proteins

H9c2 cardiomyoblasts plated at 17,500 cells/mL for untreated cells and 60,000 cells/mL for doxorubicin-treated cells in 6-well plates were treated, as reported in Section 2.4.1. At the end of the experimental procedure, cells were collected in Laemmli buffer 2x (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH≈6.8). Whole cell lysates were separated in 12% acrylamide gels, at 120V for 1.5h and electrophoretically transferred onto PVDF membranes using a wet transfer system, at 150 mA for 2h. 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 4ºC with the relevant p21 (1:1000, Abcam ab188224) and p53 (1:1000, Proteintech 10442-1-AP) antibodies. Then, membranes were washed with TBS-T followed by 1h incubation at room temperature with horseradish peroxidase-conjugated secondary antibodies (1:20,000). Afterwards, proteins were detected with a chemiluminescence scanner (Image Quant LAS 500, GE Life Sciences, Marlborough, MA, USA). Calnexin was used as loading control. Densiometric analysis of the bands was carried out using ImageLab version 6.1.0 (Bio-Rad Laboratories, Inc.), and the density of the proteins of interest were normalized to the density of the loading control.

2.5. Statistical analysis

All statistical analysis were performed using GraphPad Prism version 10.1.2 (GraphPad Software, San Diego, CA, USA) and the results are presented as mean values ± SEM (standard error of the mean) from at least three independent experiments performed in duplicate. Sample distribution was assessed by Kolmogorov-Smirnov or Shapiro-Wilk normality tests. When samples had a normal distribution, one-way ANOVA followed by Tukey's or Dunnet's multiple comparisons test was performed. When normality tests failed, statistical significance was determined using Kruskal-Wallis test followed by Dunn's multiple comparisons test. Results with a p value <0.05 were considered statistically significant.

3. Results

3.1. Chemical composition of Lavandula pedunculata extracts

The essential oil of *L. pedunculata* was predominantly characterized by fenchone (63.8%) and camphor (20%) (***Erro! A origem da referência não foi encontrada.***). The total content of phenolic compounds of *L. pedunculata* phenolic extract was 155.64 mg GAL eq/g extract.

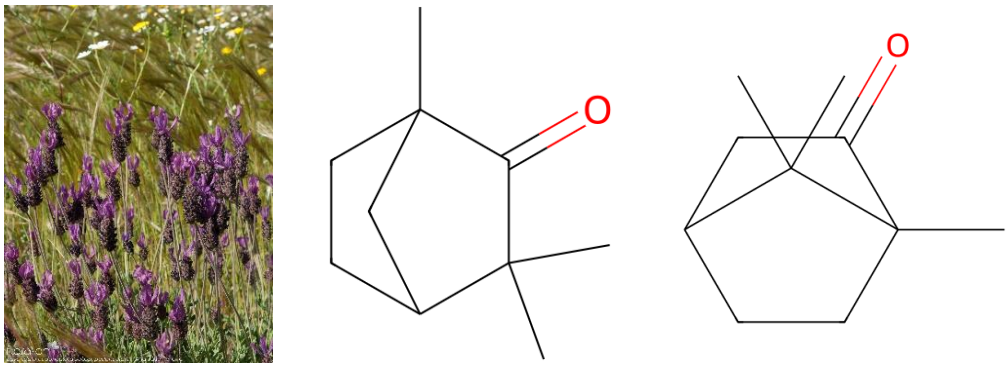

Figure 2. *L. pedunculata* and chemical structures of its main compounds. The chemical structures were downloaded from Chemical Structure Search–ChemSpider (**https://www.chemspider.com/StructureSearch.aspx**, accessed on 21 March 2025).

3.2. Effect of Lavandula pedunculata on cell viability

Envisioning a potential application of *L. pedunculata* extracts in the health sector, we first evaluated their impact on H9c2 cardiomyoblasts viability. As shown in ***Figure 3***, the essential oil was devoid of toxicity in all tested concentrations, whereas the phenolic extracts presented toxicity at the higher concentrations tested, being devoid of toxic effects at concentrations below 200 µg/mL. For the following assays, concentrations that did not reduce cell viability below 75% were selected, as recommended by Gautam et al., 2022.

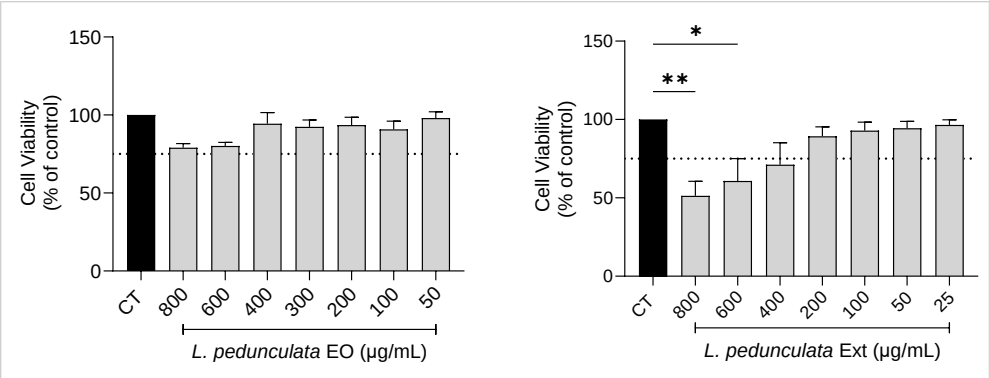

Figure 3. Effect of *L. pedunculata* essential oil and phenolic extract on H9c2 cell viability. Cell viability was assessed using the resazurin assay. * $p < 0.05$ and ** $p < 0.01$, compared to control (black bars); CT – control, EO – essential oil, Ext – phenolic extract.

3.3. Determination of optimal cells´ recovery period following doxorubicin treatment

Considering the diverse bioactivities described for *L. pedunculata* that may be beneficial in anti-senescence therapeutics, we assessed whether its essential oil and phenolic extract could directly impact cellular senescence. To accomplish this, we evaluated the effect of these extracts on several senescence markers, including senescence-associated β-galactosidase activity, nuclear accumulation of yH2AX histone, nuclear size and deformations, and the expression levels of p21 and p53. First, the optimal timeline for cell recovery was determined by comparing 24-hour and 7-day recovery periods, with the latter showing an increased expression of senescence markers. Indeed, regarding the activity of β-galactosidase, we observed that treating cells with doxorubicin for 24h, followed by a recovery period of either 24h or 7 days led to an increase in the activity of this enzyme, as observed by the distinct blue colour in ***Figure 4***. However, after 7 days of recovery, the percentage of X-galactose was significantly increased when compared to 24h. In addition, cells presented an increased size after 7 days of recovery when compared to those that recovered only for 24h.

To complement these findings, we then observed how the two different time-points of recovery influenced the nuclear accumulation of yH2AX histone. As shown in ***Figure 5***, after 7 days of recovery, there´s a slightly higher yH2AX puncta/nuclei, when compared to 24h. Considering these results, the following experiments were conducted using the 7 days recovery window.

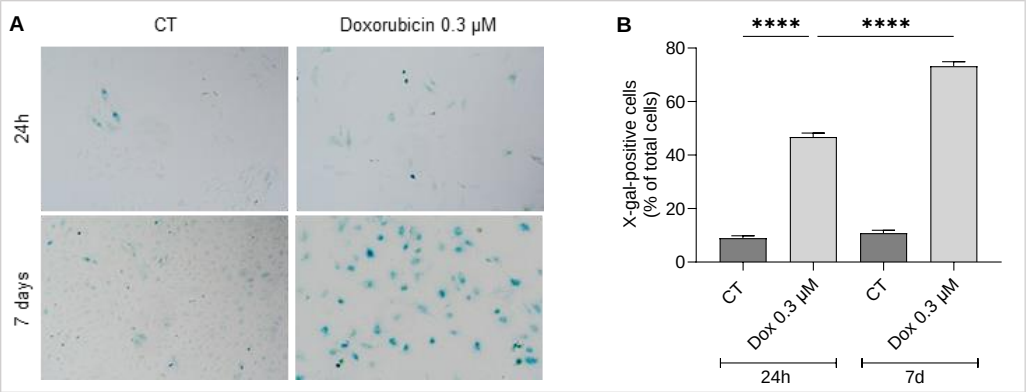

Figure 4. (**A**) Representative bright-field images of H9c2 cardiomyoblasts treated for 24h with 0.3 µM doxorubicin, followed by 24h and 7 days in doxorubicin-free medium. (**B**) Percentage of X-gal positive H9c2 cardiomyoblasts treated for 24h with 0.3 µM doxorubicin, followed by 24h and 7 days in doxorubicin-free medium. **** $p < 0.0001$, compared to control; CT – control, Dox – doxorubicin.

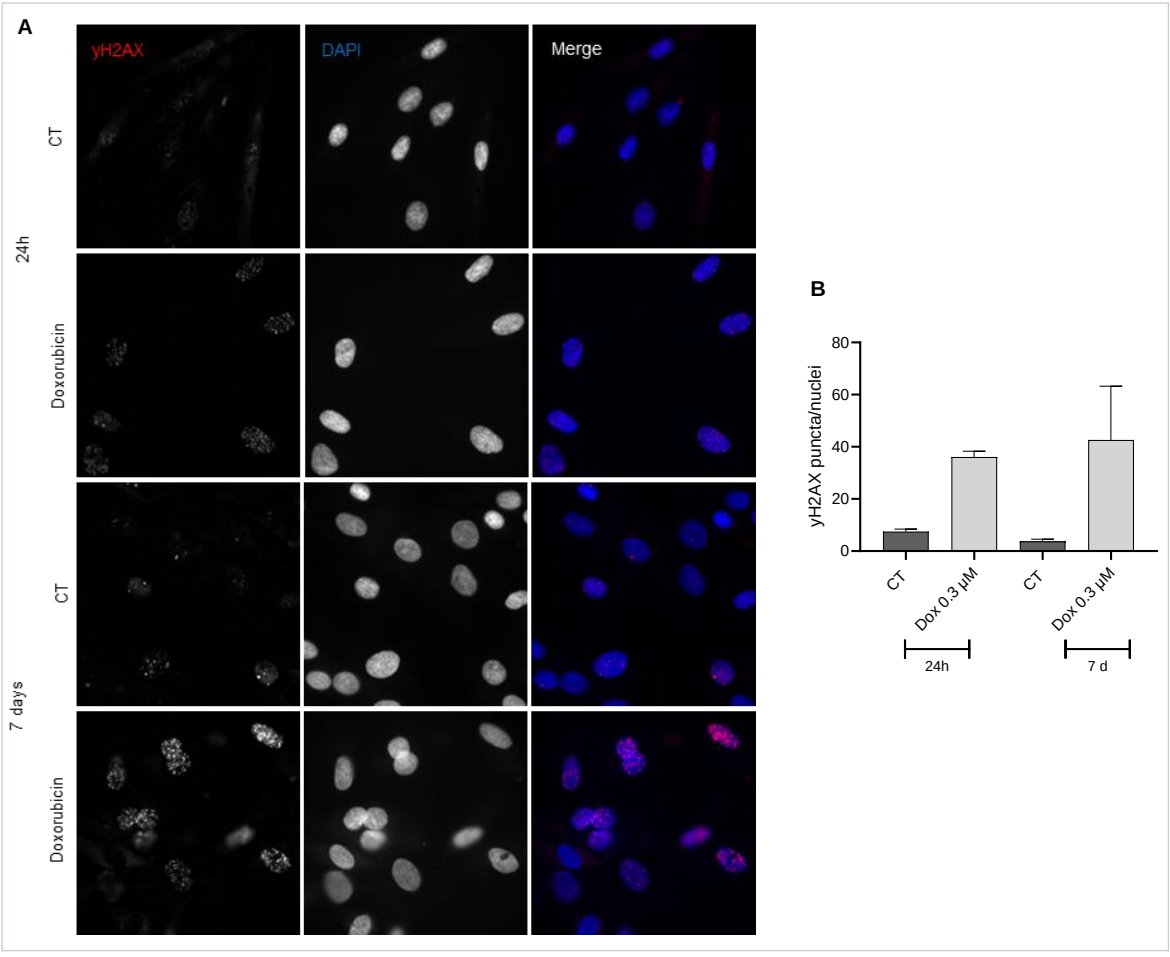

Figure 5. (**A**) Representative confocal images of H9c2 cardiomyoblasts treated for 24h with 0.3 µM doxorubicin, followed by 24h and 7 days in doxorubicin-free medium. γH2AX was stained with Alexa Fluor 568 and nuclei were counterstained with DAPI. (**B**) γH2AX puncta/nuclei in H9c2 cardiomyoblasts treated for 24h with 0.3 µM doxorubicin, followed by 24h and 7 days in doxorubicin-free medium; CT – control, Dox – doxorubicin.

3.4. Effect of Lavandula pedunculata extracts on senescence markers

3.4.1. Senescence-associated β-galactosidase activity

Having selected the best recovery time after damage induced by doxorubicin, we then proceed to assess whether the essential oil and the phenolic extract could mitigate senescence. As observed in ***Figure 6A*** *and* ***6B***, a clear and significant decrease of X-galactose-positive cells was detected in cells where 200 µg/mL of the essential oil was added during the recovery period. For the phenolic extract, no effect was observed at non-toxic concentrations. Having this in mind, we continued to explore the anti-senescent potential of *L. pedunculata* using the essential oil.

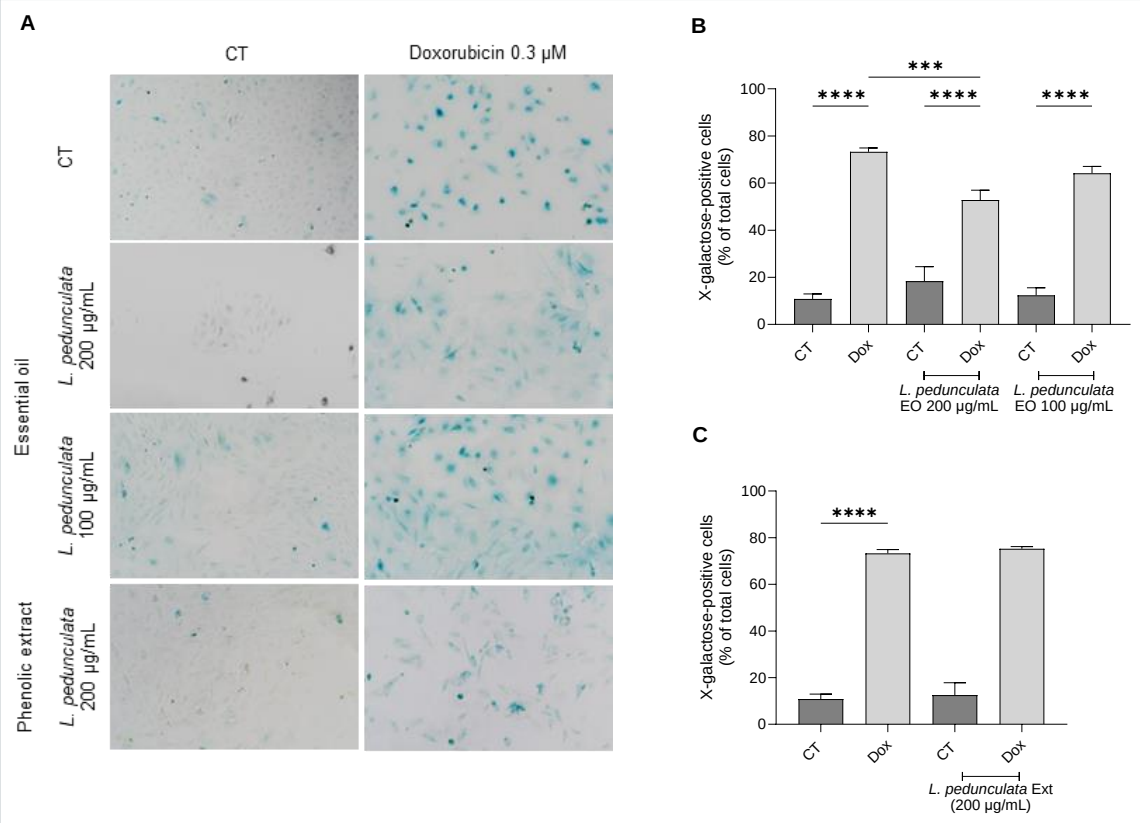

Figure 6. *L. pedunculata* essential oil decreases SA-β-gal activity. (**A**) Representative bright-field images of H9c2 cardiomyoblasts treated for 24h with 0.3 µM doxorubicin, followed by 7 days in doxorubicin-free medium in the absence or presence of 200 and 100 µg/mL of *L. pedunculata* essential oil or 200 µg/mL of *L. pedunculata* phenolic extract. (**B**) Percentage of X-gal positive H9c2 cardiomyoblasts treated for 24h with 0.3 µM doxorubicin, followed by 7 days in doxorubicin-free medium in the absence or presence of 200 and 100 µg/mL of *L. pedunculata* essential oil. (**C**) Percentage of X-gal positive H9c2 cardiomyoblasts treated for 24h with 0.3 µM doxorubicin, followed by 7 days in doxorubicin-free medium in the absence or presence of 200 µg/mL of *L. pedunculata* phenolic extract. *** $p < 0.001$ and **** $p < 0.0001$, compared to control cells or cells treated with doxorubicin; CT – control, Dox – doxorubicin, EO – essential oil, Ext – phenolic extract.

3.4.2. Nuclear alterations

Similarly to other senescence inducers, doxorubicin induces damage in the DNA double strand, that leads to the recruitment of H2AX histone aiming at repairing the damage, however when the damage is permanent, a subset of this histone becomes phosphorylated (γH2AX) and serves as a marker of persistent DNA damage, contributing to the activation of senescence pathways (Mah & Karagiannis, 2010). Therefore, we next assessed the nuclear accumulation of this histone in our experimental conditions. As expected, a marked and significant increase in this marker in doxorubicin-treated cells was observed when compared to untreated cells (**Erro! Autorreferência de marcador inválida.**). Nevertheless, when the essential oil was added during the recovery period, a significant decrease of γH2AX was detected, thus validating the anti-senescent potential of *L. pedunculata* essential oil (**Erro! Autorreferência de marcador inválida.*A*** and ***7B***).

Besides the accumulation of γH2AX, an increase in nuclear area, as well as in nuclear deformations are often seen in senescent cells (Pathak et al., 2021). With this in mind, we investigated whether the essential oil could prevent these features. Indeed, we observed that in doxorubicin-treated cells, the percentage of deformed nuclei (invaginations, blebbing or crumpled) was increased when compared with untreated cells. Interestingly, in the presence of the essential oil, the percentage of deformed nuclei was reduced when compared to doxorubicin alone (**Erro! Autorreferência de marcador inválida.*A*** and ***7D***). Regarding nuclear area, it was observed that doxorubicin-treated cells presented increased nuclear area, whereas the presence of the essential oil during the recovery phase led to modest, yet significant, decrease in nuclei area (**Erro! Autorreferência de marcador inválida.*A*** and ***7C***).

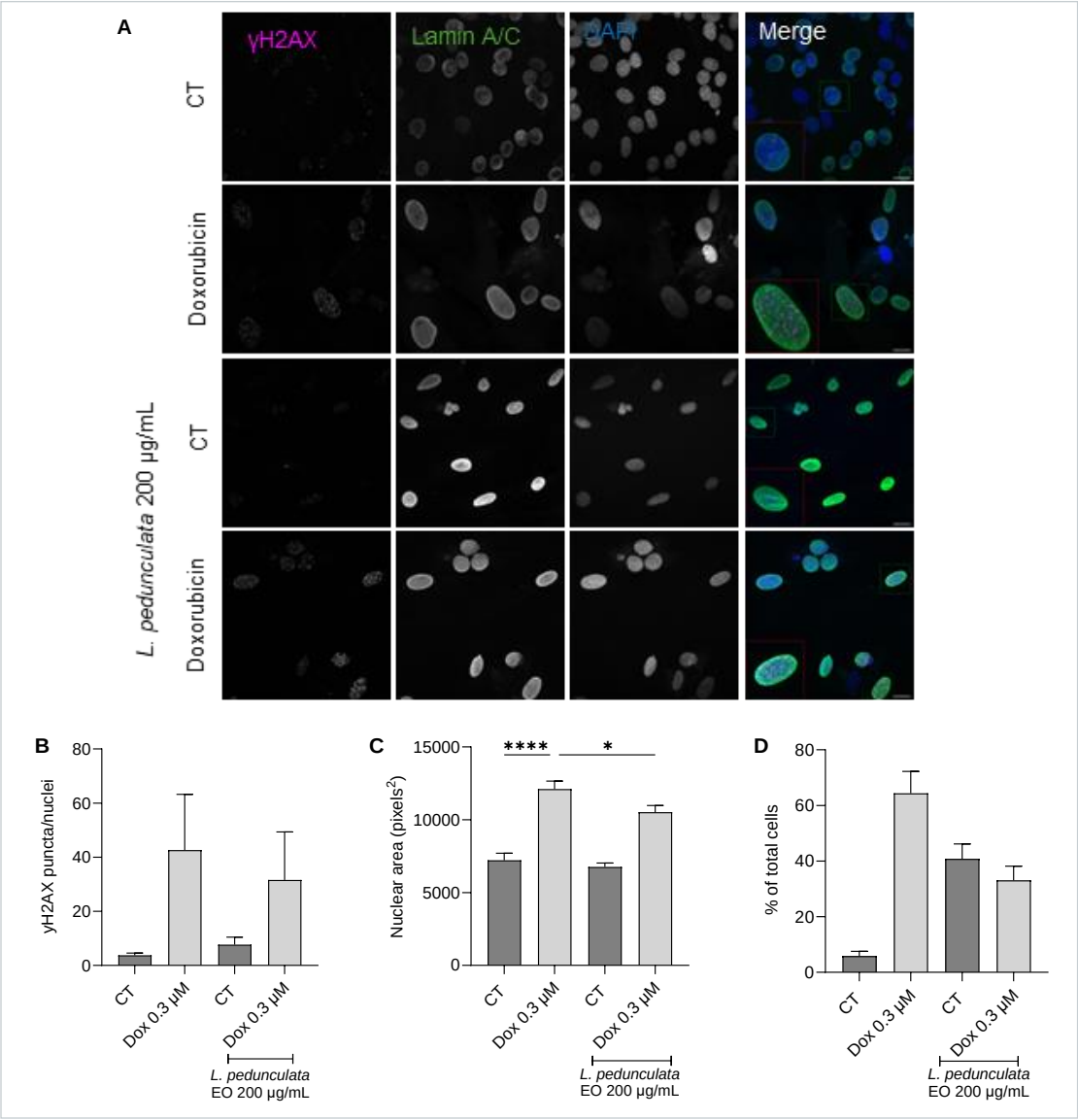

Figure 7. *L. pedunculata* essential oil improves various markers of senescence. (**A**) Representative confocal images of H9c2 cardiomyoblasts treated for 24h with 0.3 µM doxorubicin followed by 7 days in doxorubicin-free medium in the absence or presence of 200 µg/mL of *L. pedunculata* essential oil. γH2AX was stained with Alexa Fluor 568, Lamin A/C was stained with Alexa Fluor 633 and nuclei were counterstained with DAPI. (**B**) γH2AX puncta/nuclei in H9c2 cardiomyoblasts treated for 24h with 0.3 µM doxorubicin, followed by 7 days in doxorubicin-free medium in the absence or presence of 200 µg/mL of *L. pedunculata* essential oil. (**C**) Nuclear area of H9c2 cardiomyoblasts treated for 24h with 0.3 µM doxorubicin followed by 7 days in doxorubicin-freed medium in the absence or presence of 200 µg/mL of *L. pedunculata* essential oil. (**D**) Percentage of deformed nuclei in H9c2 cardiomyoblasts treated for 24h with 0.3 µM doxorubicin followed by 7 days in doxorubicin-freed medium in the absence or presence of 200 µg/mL of *L. pedunculata* essential oil. * $p < 0.05$ and **** $p < 0.0001$, compared to doxorubicin alone. CT – control, Dox – doxorubicin, EO – essential oil.

3.4.3. Expression of p21 and p53 protein levels

It is known that the p53/p21 pathway plays a crucial role in cellular senescence, having a higher expression in senescent cells (González-Gualda et al., 2021). Having this in consideration, we next tested if adding *L. pedunculata* essential oil during the cells´ recovery period would decrease the expression of p21 and p53 proteins when compared to untreated cells. As expected, in cells treated with doxorubicin and left to recover in essential oil-free medium an increase in both p53 and p21 protein levels was observed (**Figure *8***). In agreement with the previous results regarding other senescence markers, a decrease in both proteins was detected in cells where 200 µg/mL of the essential oil was added, although no statistical significance was attained.

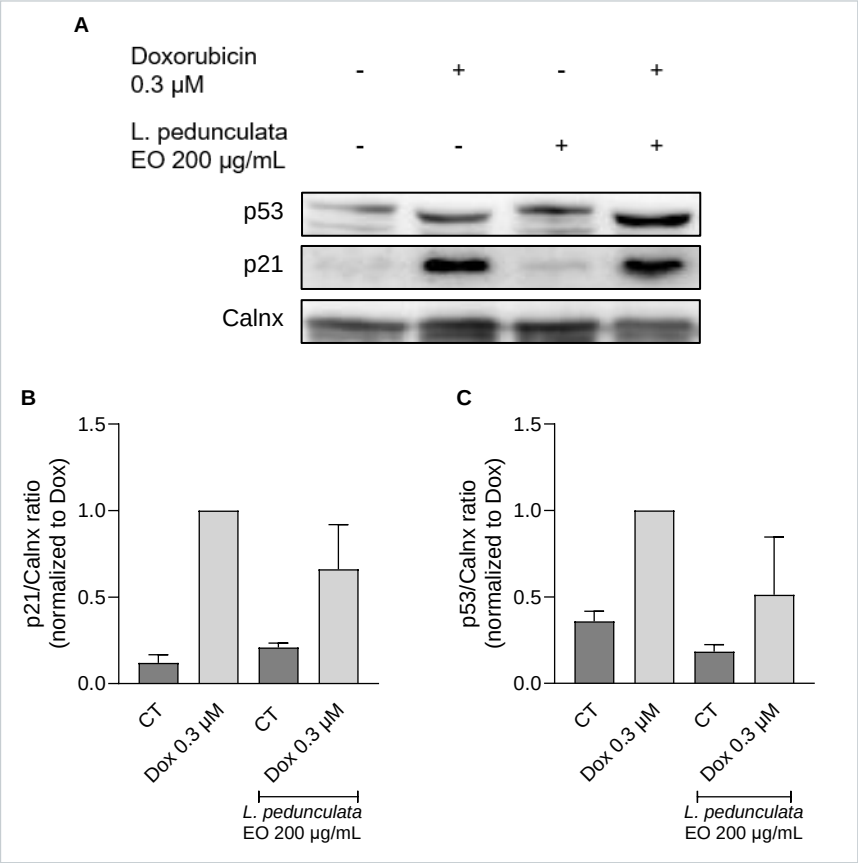

Figure 8. (**A**) Representative Western blots for p21 and p53 proteins. (**B**) p21 protein levels in H9c2 cardiomyoblasts treated for 24h with 0.3 µM doxorubicin, followed by 7 days in doxorubicin-free medium in the absence or presence of 200 µg/mL of *L. pedunculata* essential oil. (**C**) p53 protein levels in H9c2 cardiomyoblasts treated for 24h with 0.3 µM doxorubicin, followed by 7 days in doxorubicin-free medium in the absence or presence of 200 µg/mL of *L. pedunculata* essential oil; CT – control, Dox – doxorubicin. EO – essential oil.

3. Discussion and Conclusions

Cellular senescence is a complex biological process, characterized by irreversible cell cycle arrest, that is vital in the aging process and the onset of age-associated diseases, including cardiovascular diseases (Chen et al., 2022). The accumulation of senescent cells in the heart is linked to cardiac dysfunction, fibrosis, and a diminished ability for tissue regeneration, contributing to a handful of diseases, such as heart failure, arrhythmias, atherosclerosis, and others (Chen et al., 2022; Shimizu & Minamino, 2019). Consequently, comprehending the mechanisms that regulate cardiac cellular senescence and identifying strategies to modulate this process is highly relevant for public heath, particularly regarding the aging population and its economic and social impact. In fact, there is a growing interest in finding natural anti-aging therapies that meet the safety and effectiveness of long-term use. Aromatic and medicinal plants have been widely used for their bioactive potential since the beginning of human civilization (Naveed et al., 2020; Rastogi et al., 2016). In fact, medicinal plants emerge as alternative and/or complementary preventive/therapeutic strategies with lower adverse effects when compared to synthetic drugs (Chen et al., 2024). Additionally, the extracts obtained from these plants are rich in bioactive compounds that often exhibit pleiotropic pharmacological activities due to their multimodal actions, as they target oxidative stress, inflammation, mitochondrial dysfunction, among other senescence inducers (Chen et al., 2024; Liang et al., 2022). Moreover, these extracts are able to target multiple senescence-related pathways and generally present synergistic effects among their bioactive compounds, potentially enhancing their overall anti-aging efficacy (Chen et al., 2024).

In this context, *Lavandula* L. spp. extracts appear to be a promising approach due to their antioxidant, anti-inflammatory and potential anti-aging effects (Lopes et al., 2018; Zuzarte et al., 2022; Zuzarte et al., 2024). Moreover, previous studies suggest that lavenders´ bioactive compounds, namely monoterpenes can modulate signalling pathways to reverse cellular senescence (Zuzarte et al., 2024). In addition, *Lavandula* spp. extracts, namely essential oils and phenolic compounds may offer a therapeutic advantage compared to isolated compounds, due to the synergistic effects between their components, thus contributing to improved effectiveness (El Omari et al., 2023; Wagner & Ulrich-Merzenich, 2009). Therefore,

discovering the impact of such extracts on cardiac cellular senescence may pave the way for the development of new natural therapies to prevent cardiac aging and its complications.

The present study focuses on *Lavandula pedunculata* (Mill.) Cav. from Castelo Branco region with an essential oil characterized by high amounts of fenchone and camphor (Vairinhos & Miguel, 2020) and a phenolic extract rich in caffeic acid, luteolin-7-O-glucuronide and rosmarinic acid (Lopes et al., 2018). Importantly, previous studies have shown the antioxidant and anti-inflammatory potential of these compounds (El Omari et al., 2023; Tran et al., 2015), highlighting their potential in cellular aging.

To evaluate the anti-senescence potential of the essential oil and phenolic extracts of *L. pedunculata* we assessed SA-β-gal activity, double strand DNA damage (histone γH2AX), nuclear area and deformations (Lamin A/C), and cell cycle inhibitors (p21 and p53 protein levels). SA-β-gal is one of the most commonly used markers of cellular senescence, as its activity increases in senescent cells (González-Gualda et al., 2021). In accordance, we observed a significant increase in SA-β-gal activity in H9c2 cardiomyoblasts treated with a senescence-inducing agent (doxorubicin), which was significantly reduced in the presence of *L. pedunculata* essential oil. We reported a dose-dependent effect for the essential oil, with 200 µg/mL showing the best results. These results are in line with previous studies that assessed the effect of other species of *Lavandula* on cellular senescence. For example, *L. angustifolia* showed anti-aging potential related to the presence of *β*-cyclocitral, a non-volatile carotenoid compound, responsible for modifying telomeres, oxidative stress, and autophagy (Shan et al., 2024). On the other hand, fenchone and camphor, the main compounds of *L. pedunculata*, have already been shown to exhibit beneficial anti-senescent effects (Tran et al., 2015; Zuzarte et al., 2022), and, therefore, the observed effects in *L. pedunculata* essential oil may be attributed to the high concentrations of these compounds.

Another relevant senescence marker is the histone γH2AX, a double strand DNA damage marker. Since cellular senescence can be triggered by persistent or irreparable DNA damage, γH2AX nuclear accumulation can be considered as a specific marker for senescence associated with genotoxic damage (Mah & Karagiannis, 2010). Herein, we report for the first time that lavender extracts are able to modulate this histone. Indeed, our results show an increased formation of

γH2AX foci in cells treated with doxorubicin, which was attenuated when*L. pedunculata* essential oil was added. Similar to these results, *Ferulago lutea* essential oil was able to decrease the phosphorylation of H2AX, among other senescence markers such as SA-β-gal activity and p21/p53 protein levels (Alves-Silva et al., 2023). Moreover, other compounds have shown influence on γH2AX, for example, quercetin, a natural bioactive flavonoid, known for its anti-inflammatory and antioxidant properties, has shown the ability to alleviate senescence by decreasing γH2AX in bone marrow mesenchymal stem cells with senescence induced by H_2O_2(Sun et al., 2025).

Lamin A/C, a nuclear protein that plays an important role in maintaining nuclear integrity (Pathak et al., 2021), was also used to assess nuclear deformations. The results show that the percentage of deformed nuclei was higher in doxorubicin-treated cells when compared to control cells. Nevertheless, following treatment with *L. pedunculata* essential oil, the percentage of deformed nuclei decreased significantly. Increased nuclear area is also an important hallmark of senescent cells. Herein we report for the first time that *Lavandula* L. spp. extracts are able to modulate this lamin. Indeed, we report that cells treated with doxorubicin presented larger nuclei in doxorubicin-treated cells when compared to control. Interestingly, when treated with *L. pedunculata* essential oil, the area decreased significantly, suggesting once again that this extract has anti-senescence properties.

Activation of p53 and, consequently, of p21 occurs as a response to DNA damage in order to induce cell cycle arrest, thus allowing for the repair of the genotoxic damage (Chen et al., 2022). If DNA damage is efficiently repaired, p53 and p21 proteins are degraded and the cell is able to re-enter the cell cycle. However, if the damage is irreparable, p21 and p53 levels stay high, leading to a permanent cell cycle arrest, a characteristic of senescence (Gorgoulis et al., 2019). Our results show a significant decrease in p21 and p53 expression when *L. pedunculata* was added, reinforcing the anti-senescent potential of its essential oil. Similar to these results, *Mentha piperita* L. and *Zataria multiflora* Boiss. extracts, also members of the *Lamiaceae* family, have demonstrated to decrease p53 protein levels on senescent mesenchymal stem cells (Sarikhani et al., 2021).

Based on the reported findings, it seems that *L. pedunculata* essential oil may have a beneficial effect on cardiac senescence. This promising potential is due

to the observed decrease in SA-β-gal activity, yH2AX, nuclear area and deformations and p53/p21 protein levels, highlighting the possible therapeutic application of the essential oil as an anti-aging agent and promoter of cardiovascular health, thus improving overall health and well-being. Although there is a lack of specific studies regarding the anti-senescence potential of *L. pedunculata*, there is a study that shows that camphor, one of its main compounds, reduces SA-β-gal activity (Tran et al., 2015), which may also justify the positive effects herein reported for the essential oil.

Overall, these findings contribute to the valorisation of *L. pedunculata,* increasing its industrial relevance and importance in the health sector. This is in alignment with the rising demand for natural compounds that reflects a global trend towards seeking safer, more effective therapies with fewer side effects compared to synthetic drugs.

Despite the promising results, we acknowledge that more studies are needed to fully elucidate the bioactive potential of these extracts. Moreover, other senescence markers should be considered, namely SASP inhibition (levels of IL-6, IL-8 and IL-10), and the effect of these extracts on other relevant signalling pathways such as autophagy and apoptosis, should be disclosed.

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