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**THE POTENTIAL OF BOTANICALS TO TARGET
INFLAMMATION AND CELLULAR SENESCENCE:
A FOCUS ON NEUROPROTECTION**

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*The end of all our exploring will be to arrive where we started
and know the place for the first time*

T.S. Eliot, *Four Quartets*

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Abstract

Le piante sono considerate la principale risorsa di composti naturali bioattivi. Esse producono un gran numero di metaboliti secondari che hanno il compito principale di difendere la pianta da stress esterni. Queste molecole presentano un'ampia varietà di strutture chimiche e sono caratterizzate da molteplici attività di interesse farmacologico, tra cui attività anti-infiammatoria, antiossidante, antitumorale, antidiabetica, antimicrobica e cardioprotettiva. Per questo motivo, negli ultimi anni, è notevolmente aumentato l'interesse dell'industria farmaceutica per i prodotti naturali come fonte per lo sviluppo di nuovi farmaci e integratori.

Nella prima parte del mio progetto di dottorato, mi sono occupata della caratterizzazione dell'attività biologica di estratti di parti aeree e radici di *Cistus monspeliensis* L. e di olii essenziali del genere *Lavandula*, con potenziale applicazione come agenti neuroprotettivi. Nello studio riguardante il *C. monspeliensis*, è stata effettuata dapprima una caratterizzazione del profilo fitochimico di parti aeree e radici della pianta mediante analisi ^1H NMR. Aspetto fondamentale visto che un diverso contenuto di metaboliti secondari può influenzare l'attività biologica dell'estratto. L'attività anti-infiammatoria e antiossidante sono state successivamente valutate *in vitro* sulle linee cellulari RAW 264.7 e SH-SY5Y, rispettivamente. È stato inoltre analizzato il profilo inorganico degli estratti tramite analisi TXRF, per valutarne efficacia e sicurezza. I risultati hanno mostrato come estratti idroalcolici di parti aeree e radici di *C. monspeliensis* presentino un diverso profilo metabolico, che rispecchia anche una diversa attività biologica. Infatti, solamente il trattamento con le radici ha portato ad una significativa riduzione dell'espressione di citochine infiammatorie, IL-1 β e IL-6, in presenza di LPS. Inoltre, il trattamento della linea cellulare SH-SY5Y con l'estratto di radici ha favorito la sopravvivenza mitocondriale, aumentato la produzione di ATP e l'attività dell'enzima catalasi. Per quanto riguarda la caratterizzazione del profilo inorganico, non sono stata rilevata la presenza di elementi tossici nei due estratti.

La caratterizzazione dell'attività antiossidante di olii essenziali del genere *Lavandula* ha coinvolto *L. angustifolia* subsp. *pyrenaica* (DC.) e due lavandin cultivars (lavandin Emeric ex Loisel cv Grosso e lavandin Emeric ex Loisel cv Super), poco studiati in letteratura, paragonati ad un olio essenziale (OE) di lavanda commerciale. La caratterizzazione fitochimica è stata in questo caso effettuata tramite GS-MS, mentre

l'attività biologica è stata valutata *in vitro* utilizzando la linea cellulare SH-SY5Y. Il contenuto metabolomico è risultato essere pressoché paragonabile tra i quattro olii essenziali, fatta eccezione per alcuni additivi rilevati nell'OE commerciale. Queste molecole vengono spesso aggiunte per esaltare l'aroma del prodotto, ma possono inficiarne le proprietà terapeutiche. Infatti, solamente gli olii essenziali prodotti in laboratorio sono risultati essere protettivi dal danno tossico indotto da H₂O₂ ed in grado di aumentare l'espressione di geni coinvolti nella difesa della cellula dallo stress ossidativo.

La seconda parte del mio progetto di dottorato ha riguardato la valutazione della presenza di uno stato di senescenza nel modello murino di Alzheimer *App*^{NL-G-F}. La senescenza è definita come un blocco irreversibile del ciclo cellulare dovuto a danno al DNA, stress ossidativo e infiammazione cronica. È inoltre strettamente coinvolta nell'invecchiamento e in patologie ad esso associate, come la malattia di Alzheimer.

I topi *App*^{NL-G-F} sono stati valutati a tre diverse età, 4, 12 e 24 mesi e sono stati analizzati i livelli di marker tipicamente usati per studiare la senescenza, quali p16, p21, p53, IL-1β, IL-6 e l'attività della β-galattosidasi. Sono stati inoltre analizzati cambiamenti nella morfologia microgliale. I risultati hanno mostrato come l'andamento della senescenza fosse associato sia all'invecchiamento che alla progressione della patologia. I topi a 12 mesi hanno mostrato le prime differenze tra WT e *App*^{NL-G-F}. Nello specifico hanno mostrato un aumento significativo di microglia ed astrociti senescenti, con associati cambiamenti di morfologia in senso ameboide, oltre che ad un aumento dello stato di infiammazione e di attività della β-galattosidasi.

Nello complesso, questo progetto di dottorato ha posto le basi per il possibile utilizzo del modello murino *App*^{NL-G-F} per lo screening di senoterapeutici con possibile applicazione nella patologia di Alzheimer. Inoltre, molti studi preclinici e clinici stanno valutando il potenziale senoterapeutico di prodotti naturali, come quercetina e fisetina. Ulteriori studi saranno necessari per valutare il potenziale utilizzo dei prodotti naturali oggetto di questa tesi sia come agenti neuroprotettivi che senolitici, per lo sviluppo di integratori volti sia ad un invecchiamento sano, che alla prevenzione e trattamento della malattia di Alzheimer.

Plant kingdom as a source of bioactives

Natural products are compounds obtained from plants, animals, and microorganisms, which were used in traditional medicine for the treatment of various diseases [1]. Plants synthesize an extensive amount of specialized secondary metabolites, including active compounds [2]. In particular, since their structure have been optimized throughout evolution to carry out functions including endogenous defence and interaction with other organisms [3], bioactive compounds comprise a wide pool of different chemical structures that can be used as starting points for the synthesis of new molecules [3]. Natural compounds have always played a central role in drug discovery [2]; taxol, vinblastine, quinine, and artemisinin are some examples of therapeutic agents with natural origin [2]. To date, it has been estimated that more than 25% of all Food and Drug Administration (FDA) and the European Medical Agency (EMA) approved drugs derive from plants [4], [5], [6]. More than 100 natural molecules are currently undergoing clinical trials or preclinical testing [7]. Recently, advances in extraction and identification technologies, together with the development of *in vitro* and *in vivo* testing, have contributed to an increasing interest in the detection of active principles in plants for their direct use as drugs [2]. In addition, natural compounds can also act as lead compounds for the synthesis of novel molecules with higher activity or lower toxicity [8]. Several drugs, e.g. aspirin, codeine, morphine, that can be currently found on the market are synthetic analogues of plant-derived molecules [6], [9]. Interestingly, only 6% of higher plant species have been screened for biologic activity and 15% have been characterized from a phytochemical point of view [7], [8], [10], thus, there is still significant potential for the development of novel drugs based on the large structural diversity of natural molecules. The parts of the plant that can be used to isolate bioactive compounds for drug development include leaf, fruit, flower, pod, seed, bark, and root [11].

Bioactive molecules derived from plants, being an important source of compounds for the pharmaceutical industry [9], have several potential applications for the treatment of diseases [9]. Recently, the biological activity of natural compounds has gained interest for their potential benefit on health [12]. In particular, several studies demonstrated how each family of secondary metabolites is characterized by different biological activities (Figure 1). Various groups of molecules with natural origin have demonstrated anti-oxidant potential. Polyphenols, e.g. epigallocatechin-3-gallate, curcumin, caffeic acid and resveratrol, are

known to inhibit oxidative stress [13] and are involved in the treatment of cancer, cardiovascular, inflammatory, neurodegenerative and age-related diseases [9]. In particular, both phenolic acids and flavonoids can act as radical scavengers; the flavonol quercetin showed, *in vitro* and *in vivo*, to be able to reduce oxidative DNA damage and cell signaling pathways [14]. Terpenes, another group of molecules, target the catalytic activity of enzymes involved in the production of ROS (reactive oxygen species) [9]. Similarly, an anti-inflammatory potential was demonstrated by phenolic acids and terpenes by reducing the production of pro-inflammatory mediators, which are central in asthma and cancer; they are also involved in the treatment of skin diseases, rheumatoid arthritis and inflammatory bowel disease [15], [16], [17], [18]. Saponins showed both antioxidant and anti-inflammatory potential, with Ginsenoside Rb3 known to act on the NK/ NF- κ B signaling pathway [19]. Also, terpenes are known to have antimicrobial potential. Artemisinin, one of the most known molecules belonging to this class, has been used for centuries as an antimalarial agent [20]. Evidence showed that polyphenols, including curcumin, ellagic acid, apigenin, resveratrol, epigallocatechin-3-gallate and quercetin, have also an anti-cancer potential. In particular, they are involved in the prevention of carcinogenesis, by acting on cell proliferation, cell cycle progression, DNA repair, metastasis, angiogenesis, apoptosis, and can be consequently used as chemopreventive agents [9]. Several studies demonstrated that natural compounds, thanks to their bioactivity, can be involved in the treatment of cancer, diabetes, cardiovascular and age-related diseases [9]. Paclitaxel is a well known chemotherapeutic agent belonging to the terpenes class and is widely used for the treatment of breast, ovarian and non-small cell lung cancer [21]. Interestingly, it was demonstrated that terpenoids can be promising agents for the treatment of type-1 and 2 diabetes, insulin resistance and hyperlipidemia as well [22]. Saponins class exhibit a cardioprotective potential by modulating calcium homeostasis, energy metabolism, inflammation and oxidative stress [9]. Secondary metabolites can also have neuroprotective potential. Alkaloids, such as berberine, reduce neuroinflammation, oxidative stress and protect neurons against apoptotic cell death [11], [12].

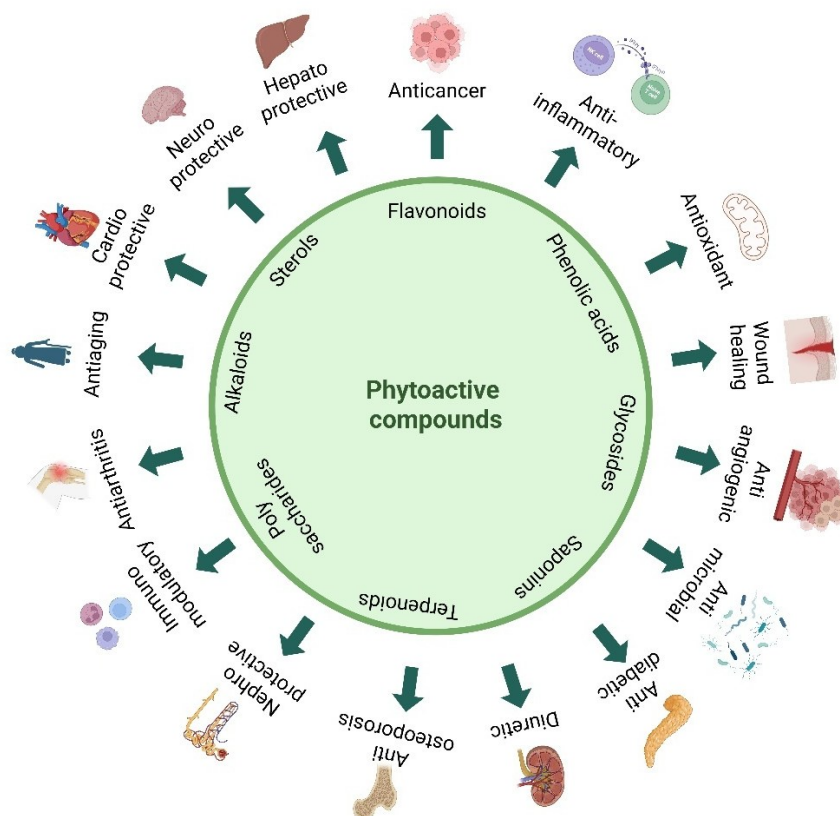


Figure 1. Potential effects of natural compounds on human health. Figure re-designed from Riaz M. et al. (2023).

In this regard, during the last three years I had the opportunity to study the biological activity of several natural extracts. In particular, for the first part of my PhD project (*Chapter 1*), I focused on the characterization of plants included in the Belfrit list, *Cistus monspeliensis* L. and *Lavandula* genus, with the future goal of developing novel neuroprotective agents.

For the second part (*Chapter 2*), I developed a different but complementary research line which focused on the characterization of a senescence state in the murine mouse model of Alzheimer's disease App^{NL-G-F} to establish if it can be used in the future as a model to test senotherapeutic and neuroprotective agents.

Chapter 1

1. Botanicals

The definition of “botanicals” is not internationally shared. According to the Food and Drug Administration (FDA), botanicals are “products that include plant materials, algae, macroscopic fungi, and combinations thereof” and the term is used for plant-derived health products [23]. The European Food Safety Authority (EFSA) specify that botanical materials are “whole, fragmented or cut plants, plant parts, algae, fungi and lichens” and are mainly intended as food supplements [24]. Also, botanical preparations can be obtained “by various processes e.g. extraction, distillation, purification, concentration and fermentation” [24]; specifically, these techniques are used to extract single active compounds or more compounds of a chemical class [25]. Botanical products are available for use as powder, solution, topical formulation, tablet, encapsulated material, tincture or injection and are classified, based on their application, into foods (including dietary supplements), cosmetics, drugs, biologics, and medical devices [26]. If they are applied for the diagnosis, treatment or prevention of human diseases [26], [27], these products are legally classified as drugs and must meet stringent FDA regulatory review [26]. Thus, the broader term herbal products can be used to include both the pharmaceutical and non-pharmaceutical uses of plant-base products.

Medicinal plants are often used as raw materials for the production of botanicals. The WHO defines a medicinal plant as “any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo pharmaceutical semi-synthesis” [28]. On planet Earth there are between 25.000 and 500.000 different plant species and it is estimated that more than 10% are used as therapeutic agents [29]. Plants produce a great amount of secondary metabolites [30], a heterogeneous group of biologically active molecules that can be used to improve human health [31], [32]. Because of their chemical diversity and mechanism of action, natural products play an essential role in drug discovery and development, indeed 80% of all synthetic drugs derive from them [30], [33], [34], [35]; they are also important for food, perfume, agrochemical and cosmetic industries [32].

2. Traditional medicine as a starting point

Since ancient times, medicinal plants have been used as therapeutic agents and were integrated in traditional medicine of many cultures [36]. According to the WHO, traditional medicine includes “diverse health practices, approaches, knowledge and beliefs incorporating plant, animal, and/or mineral based medicines, spiritual therapies, manual techniques and exercises applied singularly or in combination to maintain well-being, as well as to treat, diagnose or prevent illness” (WHO, 2002).

The application of plants for the treatment of human diseases dates back to 60.000 years ago [30]. At the beginning, plants were chosen based on experience and knowledge was transmitted orally [37]. Subsequently, many natural products were reported to have medicinal properties for the healing of pathologies [5] and the information were organized in documents [37]. Traditional medicine included traditional Chinese medicine, Indian Ayurveda medicine, Kampo, traditional Korean medicine and Arabic Unani medicine [38]. One of the most important plant-based therapeutic treatments is *De Materia Medica* written by the Greek physician Pedanius Dioscorides (ca. AD 40–80) [39], [40]. The composition, considered the precursor of all the modern pharmacopeias, comprises 600 medicinal plants and includes information about medical activities, methods of administration, habitat and methods of cultivation, botanical descriptions coupled with plant drawings, contraindications and dosages [41], [42], [43]. In the 18th century, the Swedish naturalist Carolus Linnaeus authored the fundamental composition *Systema Naturae*, which is recognized as the standard classification of botanical species and medicinal plants [4]. Between 19th and 20th century, bioactive compounds from plants started to be isolated and identified leading to the development of essential drugs (e.g. vinblastine, quinine and artemisinin) still central in modern medicine [9].

Nowadays, according to the WHO, almost 65% of the world’s population still includes traditional medicine knowledge in their primary health care [30], [34], [44]. However, despite centuries of use and their great potential in drug discovery, the biological activity and chemical composition of medicinal plants is only partially known [45]. An extensive work still need to be done by the scientific community to determine safety and effectiveness of natural products.

3. Plant bioactive molecules

Plant organic compounds can be sub-divided, based on their function, into primary and secondary metabolites [36], [46]. Primary metabolites (e.g. carbohydrates, lipids and amino acids) are highly conserved due to their central role in plant survival; they are involved in basic life functions such as cell division, cell growth, respiration, storage and reproduction [46], [47], [48]. Plant secondary metabolites are not directly involved in plant survival and were initially considered only by-products of the primary metabolism [36], [49]. Over the years, research has shown that these compounds play an important role in plant growth and development and mediate plant's interaction with the environment [46]. In particular, due to plants' sedentary lifestyle, secondary metabolites are needed to protect them against both biotic and abiotic stressors [49] [50]. Secondary metabolites also contribute to the specific odors, tastes and colours in plants [49]. Plants synthesize a large variety of secondary metabolites, exceeding 200.000, due to their wide genome and specificity for different taxa [36], [49], [51]. Secondary metabolites can be accumulated in different plant's tissues and organs, including roots and stems, leaves, flowers, fruits and seeds [52].

Based on their biosynthetic pathways (Figure 2) and chemical nature, plant secondary compounds can be classified in three major groups: terpenes, alkaloids and phenolics [48].

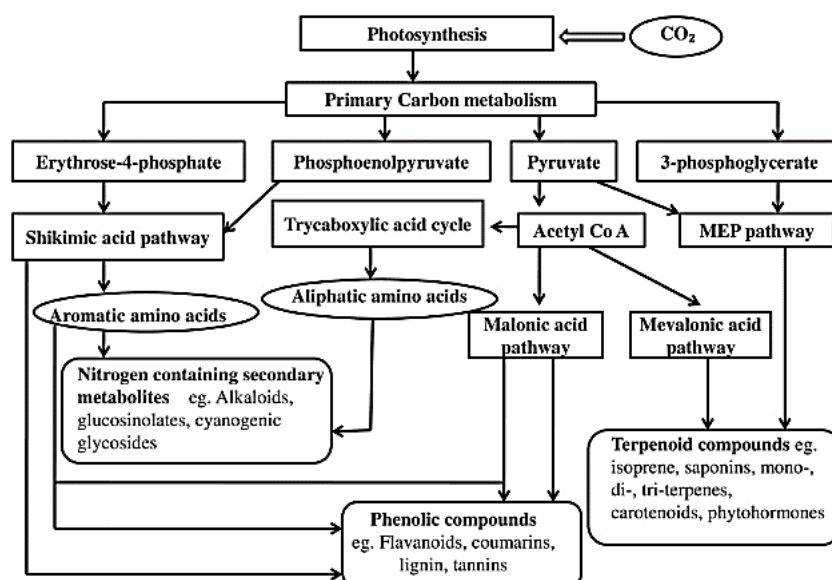


Figure 2. Biosynthetic pathway of plant secondary metabolites (Verma et al., 2015).

3.1 Terpenes

Terpenes are the largest family of secondary metabolites [51], counting approximately 55.000 members [36], [53], and can be found in all taxa, though with differences in quantities. Their general chemical formula is $(C_5H_8)_n$ and can be classified, based on the number of C5 isoprene units (Figure 3), as hemiterpenes (one C5 unit), monoterpenes (two C5 units), sesquiterpenes (three C5 units), diterpenes (four C5 units), sesterpenes (five C5 units), triterpenes (six C5 units), tetraterpenes (eight C5 units), polyterpenes (more than eight C5 units) [36], [53], [54]. Terpenes are described as terpenoids when functional groups as alcohols, aldehydes, or ketones are present in their chemical structure [53]. These compounds are synthesized through two different pathways: the mevalonate (MVA) pathway, that occurs in the cytoplasm, and the methylerythritol 4-phosphate (MEP) pathway, in plastids [51], [55]. Most of these compounds, such as some monoterpenes and sesquiterpenes, can be found in volatile mixtures (essential oils) [36], [51], [55]. Volatile terpenes greatly contribute to the aroma of several fruits [46]. Terpenoids also possess biological and pharmacological properties that are beneficial to humans [54], including antimicrobial, anticarcinogenic, antioxidant and anti-inflammatory activity [46], [53], [55].

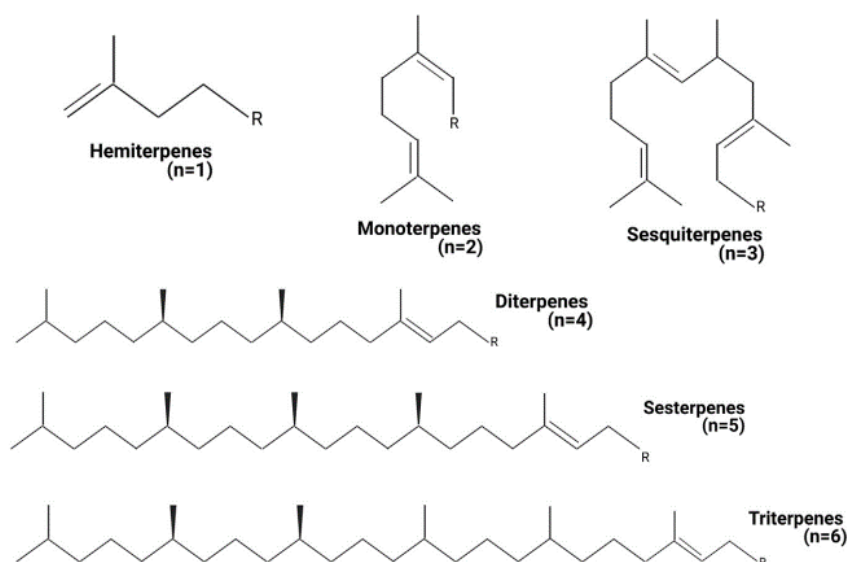


Figure 3. Terpenes classification (del Prado-Audelo et al., 2021).

3.2 Alkaloids

Alkaloids, nitrogen-containing compounds, are the second major group of plant secondary metabolites, consisting of more than 20.000 different elements [36]. They can be classified as: true alkaloids, protoalkaloids and pseudoalkaloids [36]. True alkaloids are the main members of the family and are defined as cyclic organic compounds containing nitrogen [56]. They are biosynthetically derived from amino acids such as ornithine, lysine, tyrosine, tryptophan [51], [57]. Moreover, alkaloids as well play a key role in human medicine and are known to have anaesthetic, cardioprotective, antiplatelet, anticoagulant and anti-inflammatory properties [58], [59].

3.3 Phenolics

Phenolic compounds are the third category of plant secondary metabolites [36] and consists of more than 10.000 soluble and insoluble compounds [51]. Phenolic compounds possess a common chemical structure comprising multiple phenol ring backbones with one or more hydroxyl groups, or other substitutes such as sugar molecules and organic acids [46] [60]. According to the number of phenolic rings and structural elements that link these rings, phenolics can be divided into several groups, that include: phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones (Figure 4) [60], [61]. In particular, phenolic compounds have two basic structures, the C6–C3–C6 ring structure, that is common to flavonoids, partial phenolic acids, and condensed tannins, and the C6–C1 structure, that includes partial phenolic acids and hydrolysed tannins. [60]. Phenolic compounds are produced mainly through the shikimate and phenylpropanoid pathways [46], from L-phenylalanine and L-tyrosine [62]. Phenolics can undergo glycosylation, acylation, or methylation, which changes their chemical properties and biological activity [63]. Phenolic compounds have been found to play an important role in human health. Many phenolics are applied as antioxidants, antimicrobial, anticarcinogenic, anti-inflammatory, antiallergic, antiviral, antithrombotic agents and are used in the prevention of cardiovascular diseases, cancer, diabetes and diseases associated with oxidative stress [60], [62].

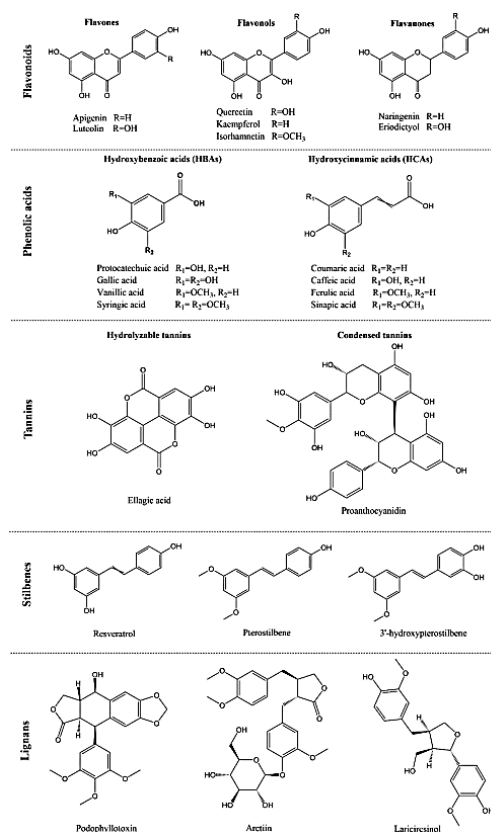


Figure 4. Typical phenolics identified in plants (Zhang et al., 2022).

Because of their vast chemical diversity and biological activity, secondary metabolites are fundamental in drug discovery as well as in the perfume and cosmetic industry; they represent also a unique sources for food additives and flavors [48], [49], [50].

4. Synergistic effect of plant bioactive molecules

Plant extracts differ from synthetic drugs because they consist of hundreds of phytochemicals, at varying abundance, forming the so called phytocomplex [36], [64].

Until recent developments in the field, researchers have always attributed the biological activity of a natural product to the most abundant compound [36]. Recent studies demonstrated that the phytocomplex has a greater and unique bioactivity compared to the single compounds that constitute it, because of the synergistic effect between them; additionally, single molecules were found to exhibit lower activity or increased toxicity [36]. Thus, advantages of using the whole extract, rather than its fractions, are a higher activity, reduced toxicity and the possibility to act on multiple targets for the treatment of diseases [64], [65]. Since several pathologies have multi-factorial causality, the combined action of compounds with different chemical structure and activity might be beneficial [45].

Therefore, together with the evaluation of the pharmacological properties in pre-clinical and clinical studies, is fundamental for drug discovery to chemically characterize natural extracts to identify the compounds responsible for the biological activity and to understand the interactions between them [45], [66].

5. Factors influencing the plant biological activity

Natural extracts composition can vary depending on several factors, including genetics variability, growing conditions and extraction methods [27]. Changes in the phytochemical composition can in turn affect the plant pharmacological properties and beneficial effect on human health. Specifically, the content of plant secondary metabolites varies from species to species and also in plants of the same species [51]. Plant phytochemical composition is also influenced by abiotic and biotic stimuli, since their production is considered an adaptive strategy against environmental stresses [67]. An abiotic stress is caused by environmental factors, such as light intensity, water availability, salinity, temperature, soil type and composition [68]. A biotic factor is defined as the interaction between plants and living organisms, such as herbivores, bacteria, virus, fungi, parasites [68]. Moreover, secondary metabolites' synthesis and accumulation can vary also depending on the growth season, growth year, plant physiological stage and plant developmental stage [50], [69]. In our research group, we compared the phytochemical profile of two *Ficus carica* leaves extracts collected in autumn and spring to determine if seasonality can influence their composition. Our results demonstrated that the two extracts had a different composition in terms of secondary metabolites. In particular, leaves extracted in spring had a higher content of caffeic acid derivatives, glucose, and sucrose, while the autumn collection presented a higher concentration of malic acid and furanocoumarins. Different chemical profiles correlated also with different bioactivity. In particular, leaves collected in autumn outperformed the ones obtained in spring in the modulation of lipid content, adipogenesis pathway and insulin sensitivity [70].

The composition of phytoextracts and, in turn their biological activity, depends also on which part of the plant is used and the method of extraction [27]. Extraction methods can be divided in traditional (e.g. maceration, Soxhlet extraction, and hydrodistillation) and more modern and advanced extraction (e.g. ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction, and enzyme-assisted extraction) [71]. Moreover, extraction efficacy is influenced by solvent type, pH, temperature, extraction duration,

particle size, and mechanical forces applied [71]. Polar solvents (e.g., methanol, ethanol, and water) are a good choice for the extraction of hydrophilic compounds, while non-polar solvents (e.g., hexane and chloroform) are used to obtain hydrophobic molecules [71]. For instance, we analyzed phytoextracts of *Artemisia annua* L. obtained at different percentages of ethanol (25, 50, and 90%) to quantify artemisinin yield. Artemisinin resulted to have a higher concentration in the 90% ethanol extraction. Different concentrations of ethanol influenced also the quantification of other compounds belonging to different chemical classes, as well as the biological activity of the extracts [72].

6. Botanicals and neuroprotection

The term neuroprotection includes all the mechanisms that help to protect the central nervous system (CNS) from neuronal damage related to acute (e.g. stroke or trauma) or chronic (e.g., Alzheimer's and Parkinson's diseases) neurodegenerative diseases [73], [74]. On one hand, neurodegenerative diseases are complex conditions characterized by several hallmarks, including protein aggregation, inflammation, oxidative stress, and loss of neurotransmitters [73]. On the other hand, natural products are considered a good source of bioactive molecules with neuroprotective potential, being able to act on multiple targets [73]. Curcumin, a polyphenol obtained from *Curcuma longa* L. and known to display anti-inflammatory and antioxidant activity, is able to inhibit β secretase and AChE activities, while reducing neuroinflammation, protein oxidation and plaque deposition [75]. Resveratrol, a stilbene polyphenol found in high concentrations in *Polygonum cuspidatum*, was also found to reduce $A\beta$, α -synuclein aggregation and induce tau protein deacetylation through SIRT1 pathway [76], [77], [78]. According to the literature, the alkaloid Berberine acts on APP and the consequently production of $A\beta$ [79]. Ginsenosides are terpenoids isolated from *Panax ginseng* and *Panax notoginseng* characterized by a well-known neuroprotective potential. It was demonstrated *in vitro* that these molecules decrease ROS and cytochrome c production, inhibits caspase-3 activation and the Bax/Bcl-2 ratio. Also, Ginsenoside Rg1 protected cells against H_2O_2 -induced cytotoxicity by acting on ERK1/2 NF- κ B pathway [80]. It was also found to decrease the production of pro-inflammatory markers iNOS, NO and TNF- α *in vivo*, as well as $A\beta$ plaques deposition [81].

7. Our contribution: *Cistus monspeliensis* L. anti-inflammatory and antioxidant potential

Cistus monspeliensis L. (Montpelier rock-rose) (Figure 5) belongs to the Cistaceae family and is a lowland shrub dominant in evergreen garrigue vegetation. Also, this plant is able to grow in fire-degraded soils [82]. Is characterized by white flowers and is one of the most common *Cistus* species that can be found in the Mediterranean region and, in particular, on Sardinia Island (Italy) [82], [83]. Plants belonging to the *Cistus* species present glandular trichomes on the leaves that produce ladano, a typical resin containing compounds of pharmacological interest [84], [83]. In Sardinian traditional medicine, poultice obtained from the leaves were used for wound healing, to treat skin diseases and for their analgesic properties, while the infusion was known to treat tick bites [85], [86].

To date, only few studies have been conducted on *C. monspeliensis* biologically active compounds. In particular, researchers demonstrated an antibacterial activity of the plant aerial parts against various bacteria and fungi strains and anti-inflammatory activity of aerial parts *in vivo* [87], [88]; antioxidant potential was also determined but just *in vitro* with colorimetric tests [89], [90].



Figure 5. *C. monspeliensis* L. (<https://www.gbif.org/>).

To the best of our knowledge, no previous studies investigated the phytochemical profile and biological activity of *C. monspeliensis* roots. In addition, the few studies

conducted to characterize the bioactivity of the aerial parts did not include analysis on cell models.

Given these premises, our project aimed to broaden the understanding on the anti-inflammatory and antioxidant activity of *C. monspeliensis* aerial parts and roots, in comparison, by testing them on cell models (RAW 264.7 and SH-SY5Y cell lines). The phytochemical content of the two organs was profiled by ^1H NMR. Since the plant metal content can affect the efficacy and safety of natural products [91], the plant inorganic content was also characterized using TXRF analysis.

This research was published in an international scientific journal:

Mac Sweeney E, Chiocchio I, Mandrone M, Sanna C, Bilo F, Maccarinelli G, Popescu VS, Pucci M, Morandini S, Memo M, Uberti DL, Borgese L, Trincia S, Poli F, Mastinu A, Abate G. Exploring the Anti-Inflammatory and Antioxidant Potential, Metabolite Composition and Inorganic Profile of *Cistus monspeliensis* L. Aerial Parts and Roots. *Antioxidants* (Basel). 2024 Jun 21;13(7):753. doi: 10.3390/antiox13070753.

Article

Exploring the Anti-Inflammatory and Antioxidant Potential, Metabolite Composition and Inorganic Profile of *Cistus monspeliensis* L. Aerial Parts and Roots

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Abstract: This work focuses on *Cistus monspeliensis* L. aerial parts (AP) and roots (R) extracts, investigating the anti-inflammatory and antioxidant potential of the two organs in comparison. At dosages between 1.56 and 6.25 µg/mL, both extracts showed a protective effect against LPS inflammatory stimulus on a macrophage cell line (RAW 264.7). Interestingly, only R was able to significantly reduce both IL-1β and IL-6 mRNA gene expression in the presence of LPS. Moreover, the treatment of a neuroblastoma cell line (SH-SY5Y) with AP and R at 6.25 µg/mL increased the cell survival rate by nearly 20% after H₂O₂ insult. However, only R promoted mitochondria survival, exhibited a significantly higher production of ATP and a higher activity of the enzyme catalase than the control. Both AP and R had similar primary metabolites; in particular, they both contained 1-O-methyl-*epi*-inositol. Labdane and methoxylated flavonoids were the most characteristic compounds of AP, while R contained mainly catechins, gallic acid, and pyrogallol derivatives. Considering the importance of elemental composition in plants, the inorganic profile of AP and R was also investigated and compared. No potentially toxic elements, such as Pb, were detected in any sample.

Keywords: Cistaceae; RAW 264.7; antioxidant defense; mitochondria; TXRF; cytokines; ¹H NMR profiling; methoxy flavonols; inorganic profile

1. Introduction

Cistus L. is a genus of dicotyledonous perennial plants with pink or white flowers and viscid stems [1–3]. It belongs to the rockrose family Cistaceae and includes 21 species [4]. The most common *Cistus* species in the Mediterranean region are *C. albidus* L., *C. creticus* L., *C. crispus* L., *C. parviflorus* Lam., *C. monspeliensis* L., *C. populifolius* L., *C. salvifolius* L., *C. ladanifer* L., *C. laurifolius* L., and *C. clusii* Dunal [1].

These plants can be found from the sea level to the mountainous regions, both in acidic and basic soils; they are resistant to summer droughts and able to grow in fire-degraded

soils [1,4–6]. In particular, on Sardinia Island (Italy), *Cistus* is one of the most representative shrub genera, accounting for six taxa [2].

The glandular trichomes present on the leaves of these plants produce a characteristic resin named *ladano*, which is responsible for the aromatic scent and contains compounds of pharmacological interest [1,3,7]. Although each species has a distinct phytochemical profile, terpenoids (sesquiterpenes, diterpenes, monoterpenes), polyphenols (flavonoids, tannins, and phenolic acids), coumarins, and alkaloids are commonly found in the aerial parts of *Cistus* species [8–11]. Moreover, their essential oils contain mainly monoterpenes, sesquiterpenes, and diterpenes [8].

This work is focused on *Cistus monspeliensis* L. (Montpelier rockrose), a lowland shrub dominant in evergreen garrigue vegetation [1,4–6] belonging to the white-flowered *Cistus* lineage [1]. It is one of the most common *Cistus* species in the Mediterranean basin [7], where it is used as a traditional remedy to treat wounds [6]. In Sardinian folk medicine, the poultice obtained from fresh leaves is topically used for wound healing, skin diseases, and as an analgesic; additionally, the infusion is employed to treat tick bites [12–14].

Aerial parts of *C. monspeliensis* showed anti-inflammatory efficacy *in vivo* against paw edema [5], as well as antimicrobial activity against several bacteria and fungi strains [15–17]. Moreover, its antioxidant activity, determined through simple *in vitro* colorimetric tests (DPPH and BCB), was also explored in comparison with other *Cistus* species [18,19], and *C. monspeliensis* resulted the most active one. Attaguile et al. [19] found that in addition to showing antioxidant and superoxide *in vitro* scavenging activity, *C. monspeliensis* aqueous extract was also able to inhibit lipoperoxidation in rat liver microsomes. Regarding its essential oil, this resulted in antioxidant and enzyme inhibitory activity [3]. Despite the ethnobotanical relevance and these promising results obtained for *C. monspeliensis* aerial parts, to the best of our knowledge, there are no studies focused on its antioxidant and anti-inflammatory activity in cell models, deepening the mechanisms of action. Moreover, there are no previous studies on the roots of this plant, which are not mentioned in any ethnobotanical reports and still remain unknown in terms of both phytochemical profile and biological activity.

Hence, the present study aims to extend the knowledge of *C. monspeliensis*, explore its antioxidant and anti-inflammatory potential in cell models, and test both aerial parts and root extracts in comparison. An overview of the phytochemical profile of the two organs was acquired by ¹H NMR profiling, followed by further phytochemical analysis. Moreover, to have a complete overview of this plant composition, the inorganic profile was also investigated, considering the importance of elementals for human health and safety and the fact, widely reported in the literature, that plant metal content can also influence its biological activity [20].

1. Materials and Methods

1.1. Reagents

All reagents used for cell cultures were purchased from Sigma Aldrich (Merck KGAA, Darmstadt, Germany), including Roswell Park Memorial Institute medium (RPMI 1640), Dulbecco's Modified Eagle Medium (DMEM), Ham's F12 medium, fetal bovine serum, L-glutamine, penicillin and streptomycin. For the cell treatments and antioxidant assays, dimethyl sulfoxide (DMSO), Phosphate Buffered Saline (PBS), Lipopolysaccharide (LPS), H₂O₂, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), Ammonium molybdate and purified Catalase enzyme (20100U) were obtained from Sigma Aldrich (Merck KGAA, Darmstadt, Germany). MitoTracker™ Green FM, for the mitochondrial content, was from Thermo Fisher Scientific (Waltham, MA, USA). To evaluate the ATP production, Seahorse XFe24 culture plates and Agilent Seahorse XF base medium DMEM were purchased from Agilent Technologies (Agilent, Santa Clara, CA, USA); poly-L-lysine, sodium pyruvate, Glucose, glutamine, HEPES, oligomycin, rotenone, and antimycin A were from Sigma Aldrich (Merck KGAA, Darmstadt, Germany). The Qubit protein assay organic dye was obtained from Invitrogen (Buenos Aires, Argentina). Regarding qPCR,

TRIzol was from Sigma Aldrich (Merck KGAA, Darmstadt, Germany), while oligo (dT) primers, M-MLV reverse transcriptase, dNTPs, RNase inhibitor, and 1X RT buffer were obtained from Promega (Madison, WI, USA); SYBR Green Master Mix was from Bio Rad Laboratories (Richmond, CA, USA).

1.1. Plant Collection and Extraction

Aerial parts (AP) and roots (R) from five plants of *C. monspeliensis* (Figure 1) were collected during the spring of 2016 from the Paringianu area, Sardinia, Italy (39°10′19.0″ N 8°27′31.9″ E). The plant is not threatened or endangered and it is not protected by national or international regulations. The whole plant was manually removed from the soil, and the aerial parts and roots were separated and carefully stored in zip-locked polyethylene bags with silica gel for transport. Subsequently, samples were dried at 40 °C in a ventilated oven and kept in sealed recipients in the dark. Subsequently, hydroalcoholic extraction and all the biological and chemical analyses were performed between 2017 and 2018. A voucher specimen (CAG 135v) was deposited in the Herbarium of the University of Cagliari (Italy).



Figure 1. *Cistus monspeliensis* L.

1.2. Preparation of Plant Extracts for Bioactivity Tests and for NMR Profiling

For biological assays, plant material was extracted following the method proposed by Cappadone et al. [13] with slight modifications. Briefly, 120 mg of dried and powdered plant material were extracted by sonication for 20 min using 6 mL of MeOH/H₂O (1:1). Subsequently, samples were centrifuged (1700 × g) for 20 min, and the supernatant was separated from the pellet, aliquoted in tests tubes, and dried in vacuum concentrator (SpeedVac SPD 101b 230, Savant, Italy) yielding in total 38 mg of AP and 6 mg of R extracts. This procedure is optimized to obtain a wide range of metabolites with a minimal consumption of both solvents and plant material.

To obtain the fingerprinting of the phytochemical composition of the extract, firstly, ¹H NMR was employed. An extraction procedure similar to that used for the bioassay was carried out, using 30 mg of sample and as solvents 0.5 mL of MeOH-*d*₄ and 0.5 mL phosphate buffer (90 mM, pH 6.0) in H₂O-*d*₂ containing standard 0.01% trimethylsilylpropionic-2,2,3,3-

*d*₄ acid sodium salt (TMSP). This time the supernatant was directly transferred to NMR tubes and subjected to ¹H NMR profiling and HSQC NMR experiments.

1.1. Fractionation for Compounds Identification

With the aim of obtaining an adequate yield to carry out the structure elucidation of the main secondary metabolites, in this stage, a different extraction procedure was employed.

One hundred g of dried and grounded AP were extracted through a maceration for 72 h using 700 mL of CH₃OH/H₂O (70:30). The extraction procedure was repeated three times on the same plant material, and the extract was filtered and dried in rotary evaporator yielding 34.8 g of dried extract. This latter was then suspended in 350 mL of water to undergo liquid–liquid partition (four times with each solvent). After anhydri-fication, the fractions were dried in a rotary evaporator, obtaining hexane (APHex = 1 g), chloroform (APCl = 5 g), ethyl acetate (APEt = 2.1 g), and water (APW = 20 g) fractions. 8-Hydroxylabdan-15-oic acid was partially purified from APCl, while flavonols were obtained from both APCl and APEt; both were further subjected to Medium-Pressure Liquid Chromatography (MPLC).

APCl was dissolved in 7 mL of chloroform and mixed with 5 g of silica gel (60 g Kieselgel[®], Merck KGaA, Darmstadt, Germany), then the solvent was evaporated in rotavapor, and the fraction was injected by dry loading in MPLC system (Reveleris[®], Büchi, Switzerland) connected to a silica column (80 g, 40 µm). The eluent consisted of an isocratic phase of CHCl₃:EtOH (97:3), the flow rate was 20 mL/min, and the run length was 60 min. The fractions were collected by volume (15 mL in each tube) and were successively reduced to 11 fractions, unifying on the basis of Thin Layer Chromatography (TLC) profiles.

The TLC was carried out using silica gel slides with 254 nm fluorescence indicator and as mobile phase CHCl₃:EtOH (97:3). Compound 8-hydroxylabdan-15-oic acid was directly crystallized from subfraction 8 in the same subfraction also eluted quercetin 3,4'-dimethylether and myricetin 3,4',5'-trimethyl ether.

Analogously, APEt was dry-loaded in the MPLC instrument connected to the same column. The eluent consisted of an isocratic phase of CHCl₃:MeOH (95:5), the flow rate was 35 mL/min, and the run length was 80 min. The fractions were collected by volume (25 mL in each tube) and successively reduced to sixteen fractions, unifying on the basis of the TLC profiles as previously described. Compound myricetin 3,7,4',5'-tetramethyl ether was obtained from subfraction 1 (and also found in APCl subfraction 4). Gallic acid was obtained from subfraction 14, and catechin and 1-(2-hydroxy-6-methylphenyl) ethanone 2-*O*-β-hexoside were obtained from subfraction 15.

Two hundred grams of dried and grounded R were extracted through maceration of 72 h using 1400 mL of CH₃OH/H₂O (70:30). The extraction procedure was repeated four times on the same plant material, and the extract was filtered and freeze-dried (yield 12 g). The extract was suspended in 500 mL of water to undergo liquid–liquid partition. After anhydri-fication, the fractions were dried in a rotary evaporator, obtaining chloroform (RCl = 110.9 mg), ethyl acetate (Ret = 865 mg), and water (RW = 8.18 g) fractions. 1-*O*-methyl-*epi*-inositol was detected in RW and prepurified through a Solid Phase Extraction (SPE) followed by size exclusion chromatography. Briefly, 1.2 g of RW was solubilized in the minimum volume of water and loaded into the Supelco Discovery[®] DPA-6S SPE Tube (Merck KGaA, Darmstadt, Germany), eluting first with methanol and then with water. The water eluate was dried in a rotary evaporator. Subsequently, 300 mg of it was then solubilized in the minimum volume of methanol and loaded into a chromatography column (900 mm × 16 mm) filled with 60 g of Sephadex (LH-20). This latter chromatography was carried out using methanol as an eluent with a flow rate of 0.6 mL/min. A total of 80 tubes were collected, and 1-*O*-methyl-*epi*-inositol was eluted in tubes 19–21 and was chemically characterized from tube 20.

1.1. NMR Measurement and Analysis

^1H NMR spectra and inverse detected ^1H - ^{13}C correlation experiments (HMBC, HSQC) were recorded at 25 °C on a Bruker Avance Neo Ascend 600 instrument equipped with the cryoprobe Prodigy. For ^1H NMR profiling, the instrument was operating at a ^1H NMR frequency of 600.13 MHz, and $\text{MeOH-}d_4$ was used as an internal lock. Each ^1H NMR spectrum consisted of 16 scans (corresponding to 5 min) with a relaxation delay (RD) of 2 s and spectral width of 9595.8 Hz (corresponding to δ 16.0). A presaturation sequence (PRESAT) was used to suppress the residual water signal at δ 4.83. The spectra were manually phased and baseline corrected and calibrated to the internal standard trimethyl silyl propionic acid sodium salt (TMSP) at δ 0.0 using Mestrenova software (version 15.0, Mestrelab Research, Spain). The standard was also used to perform a semiquantitative analysis of 1-*O*-methyl-*epi*-inositol and gallic acid. The analysis of the extracts ^1H NMR profiles was performed based on an in-house library and compared with the literature [21,22]. All the spectra were uploaded onto the Zenodo repository (<https://doi.org/10.5281/zenodo.11281288>).

1.2. Analytical Chromatography

UHPLC-MS analysis was run on a Waters ACQUITY ARC UHPLC/MS system consisting of a QDa mass spectrometer equipped with an electrospray ionization interface and a 2489 UV/Vis detector. The detected wavelengths (λ) were 225 nm and 260 nm. The analyses were performed on an XBridge BEH C18 column (10 mm $\times$ 2.1 mm i.d., particle size 2.5 μm) with an XBridge BEH C18 VanGuard Cartridge precolumn (5 mm $\times$ 2.1 mm i.d., particle size 1.8 μm). The mobile phases were H_2O (0.1% formic acid) (A) and MeCN (0.1% formic acid) (B). Electrospray ionization in positive (QDa 1) and negative (QDa 2) modes was applied in the mass scan range of 50–1200 Da. REt was diluted to 1 mg/mL, and a volume of 4 μL was injected. The extract was eluted with an explorative gradient starting with 5% B and reaching 95% B in 15 min. The flow rate was 0.8 mL/min. This analysis allowed gallic acid, catechin, and gallocatechin to be detected (Figure S1).

Analogously, APeT subfractions 1 and 15 and APcI subfraction 8 were chromatographed following the same procedure. This led to the identification of myricetin 3,7,4',5'-tetramethyl ether, catechin, 1-(2-hydroxy-6-methylphenyl) ethanone 2-*O*- β -hexoside, myricetin 3,4',5'-trimethyl ether, and quercetin 3,4'-dimethyl ether.

Finally, to confirm the presence of the characterized compounds in AP and R extracts, two aliquots prepared for the biological assays (one AP and one R) were solubilized at 10 mg/mL and subjected to the same UHPLC-MS analysis (values reported in the next paragraph).

1.3. Compounds Identification

Below, the ^1H and ^{13}C NMR resonances of the characterized compounds are reported, together with the m/z values and retention times (RT) found in AP and R.

1.3.1. 8-Hydroxyabdan-15-oic Acid (1)

^1H NMR spectral data (600 MHz, DMSO) δ : 3.82 (1 H, s, OH), 2.22 (1 H, dd, J = 14.88, 5.87 Hz, H14a), 1.96 (1 H, dd, J = 14.88, 8.3 Hz, H14b), 1.78 (1 H, m, H13), 1.67 (1 H, dt, H7a), 1.55 (1 H, H1b), 1.52 (1 H, H6b), 1.44 (1 H, H12b), 1.35 (1 H, H11b), 1.34 (1 H, dt, H7b), 1.32 (1 H, H3b), 1.20 (1 H, H6a), 1.14 (1 H, H11a), 1.13 (1 H, H12a), 1.10 (1 H, H3a), 0.98 (3 H, s, H17), 0.93 (1 H, t, H9), 0.88 (3 H, d, J = 6.66 Hz, H16), 0.87 (1 H, H1a), 0.84 (1 H, d, J = 2.4 Hz, H5), 0.83 (3 H, s, H18), 0.75 (3 H, s, H19), 0.73 (3 H, s, H20). ^{13}C NMR, δ : 175.11 (C15), 72.67 (C8), 62.07 (C9), 56.37 (C5), 44.43 (C7), 42.11 (C3), 41.87 (C14), 40.72 (C12), 39.95 (C1), 39.57 (C10), 33.76 (C18), 33.54 (C4), 31.33 (C13), 24.18 (C17), 22.67 (C11), 21.75 (C19), 20.60 (C6), 20.06 (C16), 18.23 (C2), 15.66 (C20). m/z value in QDa 2 = 323 $[\text{M-H}]^-$ found in AP at RT 9.48 min.

1.1.1. Gallic Acid (2)

^1H NMR spectral data (600 MHz, D_2O), δ : 7.03 (2H, s, H_1' , H_6') ^{13}C NMR, δ : 172.03 (C7), 144.64 (C4), 137.18 (C3, C5), 123.30 (C1), 110.0 (C2, C6). m/z value in QDa 2 = 169 $[\text{M}-\text{H}]^-$ found in R at RT 0.53 min and in AP at RT 0.58 min.

1.1.2. Myricetin 3,7,4',5'-Tetramethyl Ether (3)

^1H NMR spectral data (600 MHz, DMSO) δ : 9.63 (1H, s, $3'\text{OH}$), 7.38 (1H, d, $J = 2.11$ Hz, H_2'), 7.22 (1H, d, $J = 2.11$ Hz, H_6'), 6.76 (1H, d, $J = 2.09$ Hz, H8), 6.40 (1H, d, $J = 2.09$ Hz, H6), 3.88 (3H, s, 7 OCH_3), 3.87 (3H, s, $5'\text{OCH}_3$), 3.83 (3H, s, 3 OCH_3), 3.78 (3H, s, $4'\text{OCH}_3$). ^{13}C NMR, δ : 178.55 (C4), 165.85 (C7), 164.50 (C5), 156.84 (C9), 155.83 (C2), 153.49 (C5'), 151.03 (C3'), 139.42 (C4'), 139.22 (C3), 110.50 (C2'), 105.84 (C10), 104.3 (C6') 98.4 (C6), 92.9 (C8), 60.64 ($4'\text{OCH}_3$), 60.38 (3 OCH_3), 56.63 (7 OCH_3), 56.40 ($5'\text{OCH}_3$). m/z value in QDa 1 = 375 $[\text{M}+\text{H}]^+$ found in AP at RT 7.49 min.

1.1.3. Quercetin 3,4'-Dimethyl Ether (4)

^1H NMR spectral data (600 MHz, DMSO) δ : 12.68 (1H, s, 5OH), 10.88 (1H, s, 7OH), 9.63 (1H, s, $3'\text{OH}$), 7.65 (1H, d, $J = 2.03$ Hz, H_2'), 7.58 (1H, dd, $J = 8.43$, $J = 2.03$ Hz, H_6'), 6.97 (1H, d, $J = 8.43$ Hz, H_5'), 6.48 (1H, d, $J = 2.10$ Hz, H8), 6.22 (1H, d, $J = 2.10$ Hz, H6), 3.86 (3H, s, $4'\text{OCH}_3$), 3.81 (3H, s, 3 OCH_3). ^{13}C NMR, δ : 178.41 (C4), 164.44 (C7), 161.66 (C5), 155.79 (C2), 149.90 (C3'), 148.58 (C4'), 138.79 (C3), 122.62 (C2'), 121.24 (C1'), 116.30 (C5'), 112.60 (C6'), 104.64 (C10), 98.93 (C6), 94.25 (C8), 60.09 (3 OCH_3), 56.24 ($4'\text{OCH}_3$). m/z value in QDa 2 = 329 $[\text{M}-\text{H}]^-$ found in AP at RT 5.82 min.

1.1.4. Myricetin 3, 4',5'-Trimethyl Ether (5)

^1H NMR spectral data (600 MHz, DMSO) δ : 12.60 (1H, s, 5OH), 10.88 (1H, s, 7OH), 9.92 (1H, s, $3'\text{OH}$), 7.24 (1H, d, $J = 2.10$ Hz, H_2'), 7.19 (1H, d, $J = 2.10$ Hz, H_6'), 6.46 (1H, d, $J = 2.10$ Hz, H8), 6.21 (1H, d, $J = 2.10$ Hz, H6), 3.85 (3H, s, $5'\text{OCH}_3$), 3.80 (3H, s, 3 OCH_3), 3.77 (3H, s, $4'\text{OCH}_3$). ^{13}C NMR, δ : 178.78 (C4), 164.44 (C7), 161.66 (C5), 156.42 (C9), 155.54 (C2), 152.39 (C5'), 150.77 (C3'), 139.30 (C4'), 138.2 (C3), 125.27 (C1'), 110.36 (C2'), 104.84 (C10), 104.31 (C6'), 98.93 (C6), 94.25 (C8), 60.34 ($4'\text{OCH}_3$), 60.09 (3 OCH_3), 56.24 ($5'\text{OCH}_3$). m/z value in QDa 2 = 359 $[\text{M}-\text{H}]^-$ found in AP at RT 6.02 min.

1.1.5. 1-(2-Hydroxy-6-methylphenyl) Ethanone 2-O- β -Hexoside (6)

^1H NMR spectral data (600 MHz, D_2O), δ : 7.26 (1H, t, H4), 6.98 (1H, d, $J = 8.37$ Hz, H3), 6.94 (1H, d, $J = 8.37$ Hz, H5), 5.01 (1H, d, $J = 7.71$ Hz, H_1'), 3.45 (1H, H_2'), 3.50 (1H, H_3'), 3.40 (1H, H_4'), 3.39 (1H, H_5'), 3.65 (1H, $\text{H}_6'\text{a}$), 3.83 (1H, $\text{H}_6'\text{b}$), 2.51 (3H, s, H9), 2.18 (3H, s, H7). ^{13}C NMR, δ : 218.51 (C8), 152.56 (C2), 135.51 (C6), 131.43 (C1), 130.10 (C4), 125.29 (C5), 112.72 (C3), 100.35 (C1'), 76.10 (C3'), 75.75 (C5'), 72.78 (C2'), 69.32 (C4'), 60.82 (C6') 31.96 (C9), 17.91 (C7). m/z value in QDa 1 = 335.12 $[\text{M}+\text{Na}]^+$ found in AP at RT 3.40 min.

1.1.6. Catechin (7)

^1H NMR spectral data (600 MHz, D_2O), δ : 6.83 (1H, d, $J = 1.89$ Hz, H_6'), 6.82 (1H, d, $J = 8.28$ Hz, H_3'), 6.72 (1H, dd, $J = 1.89$, $J = 8.28$ Hz, H_2'), 5.93 (1H, H6), 5.83 (1H, H8), 4.54 (1H, d, $J = 7.75$ Hz, H2), 4.01 (1H, m, H3), 2.76 (1H, dd, $J = 5.43$, 15.85 Hz, H_4a), 2.37 (1H, dd, $J = 8.36$, 15.85 Hz, H_4b). ^{13}C NMR, δ : 155.21 (C5), 154.75 (C9), 156.42 (C9), 144.29 (C5'), 143.93 (C4'), 130.17 (C1'), 119.96 (C2'), 116.19 (C3'), 114.94 (C6'), 100.48 (C10), 95.92 (C6), 94 (C8), 80.88 (C2), 66.44 (C3), 26.53 (C4). m/z value in QDa 1 = 290 $[\text{M}^+]$ found in R at RT 1.81 min and m/z value in QDa 1 = 291 $[\text{M}+\text{H}]^+$ found in AP at RT 1.86 min.

1.1.7. 1-O-methyl-*epi*-inositol (8)

^1H NMR spectral data (600 MHz, D_2O), δ : 4.00 (1H, *m*, H2), 3.96 (1H, *m*, H5), 3.92 (1H, *m*, H3), 3.77 (1H, *m*, H4), 3.62 (1H, *m*, H6), 3.43 (1H, *m*, H1), 3.37 (3H, s, H7). ^{13}C NMR, δ : 84.77 (C1), 75.22 (C4), 72.72 (C3, C5), 71.29 (C2), 66.39 (C6), 60.41 (C7).

1.1. TXRF Analysis

Samples were processed in the form of suspensions. Approximately 10 mg of powder samples were suspended in nearly 1000 mg of ultrapure de-ionized water obtained from a Milli-Q purifier system (Millipore DirectQ-5 TM, Millipore S.A.S., 67120, Molsheim, France). The suspensions underwent homogenization using a vortex shaker for 1 min at 2500 rpm. A 10 µL drop of an internal standard solution containing gallium (Ga) was placed at the center of a siliconized quartz sample carrier. This internal standard served as a reference element for quantitative analysis. To create a hydrophobic surface, a silicon solution in isopropanol (Serva Electrophoresis, Heidelberg, Germany) was applied to all quartz glass sample carriers. The sample carriers were then dried on a hot plate at 50 °C under the laminar hood. Subsequently, 10 µL of the prepared sample suspension was deposited onto the previously dried residue on the quartz glass sample carrier and re-dried. Two sample carriers were prepared for each sample, and each set was irradiated for 600 s live time.

TXRF analysis of prepared samples was performed using a commercial benchtop TXRF spectrometer equipped with a Mo low-power X-ray tube (S2 PICOFOX, Bruker AXS Microanalysis GmbH, Berlin, Germany). It is equipped with air-cooled low-power X-ray tubes operating at 750 µA and 50 kV and a Peltier-cooled silicon drift detector (SDD), and thus, no cooling media and gas consumption are required. Spectra evaluation and calculation of the analyte net peak area were performed using the provided software (Spectra Plus 5.3, Bruker AXS Microanalysis GmbH, Berlin, Germany) linked to the system. For peak integration, the Spectra Plus 5.3 software applies a deconvolution routine that uses measured mono-element profiles for the evaluation of peak areas.

1.2. Cell Culture and Treatments

Murine macrophage cells (RAW 264.7) (CLS cell lines service, Germany) were cultured in RPMI 1640, supplemented with 10% fetal bovine serum, 1% L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin, in an incubator at 37 °C and 5% CO₂.

Human neuroblastoma cells (SH-SY5Y) were cultured in DMEM and Ham's F12 medium 1:1, supplemented with 10% fetal bovine serum, 0.5% L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin, in an incubator at 37 °C and 5% CO₂.

For the treatments, aerial parts (AP) and roots (R) extracts were solubilized in DMSO and diluted in PBS to obtain an initial concentration of 1 mg/mL. The concentrations used for the treatments were then prepared by diluting the extracts in cell culture medium.

To test the biocompatibility of the extracts, RAW 264.7 cells were seeded at a density of 2.4×10^3 cells/mL in 96-well plates. Cells were treated for 48h with AP and R extracts at concentrations of 100 µg/mL, 50 µg/mL, 25 µg/mL, 6.25 µg/mL, 3.12 µg/mL, and

1.56 µg/mL. Untreated cells were used as control. Regarding the SH-SY5Y cell line, the seeding density used was 2×10^4 cells/well in 96-well plates. SH-SY5Y cells were treated for 48h with AP and R extracts using the following dosages: 100 µg/mL, 50 µg/mL, 25 µg/mL, 6.25 µg/mL, 3.12 µg/mL, and 1.56 µg/mL. Untreated cells were used as control.

The extract's anti-inflammatory activity was tested by treating RAW 264.7 cells with a pro-inflammatory stimulus elicited by LPS for 48 h. Briefly, LPS 500 ng/mL was administered in the presence or absence of AP and R extracts at concentrations between 1.56 µg/mL and 25 µg/mL.

To determine AP and R antioxidant activity, SH-SY5Y cells were pre-treated for 24 h with the extracts at different dosages between 1.56 µg/mL and 6.25 µg/mL. The day after, cells were challenged with 0.2 mM H₂O₂ for 24 h.

1.3. Cell Viability

After cellular treatments, cell viability was assessed by incubating cells with 500 mg/mL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) for 90 min at 37 °C. After removing the supernatant, cells were lysed with DMSO. The absorbance was measured at 570 nm using an EnSight Multi-mode Plate Reader (PerkinElmer, Waltham, MA, USA).

Data were expressed as a percentage of cell viability over the control group. The results were expressed as mean $\pm$ SEM.

1.1. Catalase Activity

To test catalase activity, SH-SY5Y cells were plated at a density of 2×10^5 cells/well in 24-well plates and treated with AP and R extracts at the most protective dosage. After 48h of incubation, cells were collected and lysate to obtain a total protein extract. Briefly, cells were harvested in 100 μ L of lysis buffer containing 1M Tris-HCl pH 7.6, 5N sodium chloride (NaCl), NP-40, and protease and phosphatase inhibitors. Samples were sonicated, and the total protein content was determined using the Bradford assay.

Catalase activity was measured by monitoring the decomposition of H_2O_2 , according to Shangari and O'Brien [23]. Specifically, 5 μ L of total protein extracts were incubated with the substrate (65 μ M hydrogen peroxide in 6.0 mM PBS buffer pH 7.4) at 37 °C for 60 s. The enzymatic reaction was stopped by adding 32.4 mM ammonium molybdate, and spectrophotometrically measured at 405 nm. The results were extrapolated by a standard curve (ranging from 12 U/mL to 0.5 U/mL) performed with purified Catalase enzyme (20100 U). Data were finally normalized on the total protein content, and the results were expressed as U/mg.

1.2. Mitochondrial Content

SH-SY5Y cells, seeded at a density of 2×10^5 cells/well, were pre-treated for 24 h with AP and R extracts at the most protective dosage and then challenged with 0.2 mM H_2O_2 for an additional 24 h. The day after, cells were washed twice with PBS and incubated with MitoTracker™ Green FM 100 nM for 45 min at 37 °C 5% CO_2 . Subsequently, cells were washed again with PBS and then fixed with 3.7% formaldehyde in PBS for 15 min at RT. The observation was performed with the LSM880 Zeiss confocal laser microscope (Carl Zeiss S.p.A., Milan, Italy). Analysis was performed with an excitation wavelength of 490 nm and an emission wavelength of 516 nm. Fluorescence quantification was conducted using Fiji software, an ImageJ2-based program (<https://imagej.net/software/fiji/> (accessed on 28 May 2024)).

1.3. ATP Production

ATP production in SH-SY5Y cells was analyzed using a Seahorse XFe24 Extracellular Flux Analyzer (Agilent, Santa Clara, CA, USA). SH-SY5Y cells were seeded at a density of 5×10^4 cells/well in Seahorse XFe24 culture plates, previously coated with poly-L-lysine 2 μ g/mL, and treated with AP and R 6.25 μ g/mL for 48h. Agilent Seahorse XF base medium DMEM (pH 7.4), added with 1 mM sodium pyruvate, 5 mM Glucose, 2 mM glutamine, 5 mM HEPES, was used. At the end of the treatment, cells were incubated at 37 °C in a non- CO_2 incubator for 1 h before running the assay. To determine mitochondrial ATP production, the ATP synthase inhibitor oligomycin 1.25 μ M and a mixture of the respiratory complex I inhibitor rotenone and the complex III inhibitor antimycin A were sequentially injected. Basal respiration was measured in the absence of inhibitors. At the end of each experiment, cells were lysed, and their total protein content was used for the normalization. Protein quantification was assessed, as reported by Tonello et al. [24], by incubating samples in the dark for 15 min with a fluorescent labeling reagent (Qubit protein assay organic dye), and then the fluorescence signal derived from the stained protein was acquired with the Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

1.4. Quantitative Real-Time PCR

The most promising dosage of the extracts able to protect cells against the LPS stimulus was employed to treat the cells and assess the expression of pro-inflammatory genes such as interleukin-1 β (IL-1 β) and interleukin-6 (IL-6). Specifically, RAW 264.7 cells were treated with AP and R extracts in the presence or absence of LPS for 6 h. Then, total RNA was extracted from 4×10^4 cells/mL following the TRIzol reagent protocol. Two micrograms

of total mRNA were then reverse-transcribed. The reaction mix was composed of oligo (dT) primers 5 mM, 10 U/mL M-MLV reverse transcriptase, 1 mM dNTPs, 1 U/mL RNase inhibitor, and 1X RT buffer. The reaction was conducted at 70 °C for 10 min, followed by a 2 min step at 4 °C and a 60 min step at 37 °C. Real-time PCR was performed using the ViiA7 Real-Time PCR Detection System (Applied Biosystems, Foster City, CA, USA). The reaction mix contained 6 µL of SYBR Green Master Mix, 6 pmol of each forward and reverse primer, and 2 µL of diluted cDNA. It was incubated at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. For each gene, samples were plated in triplicate. Primers sequences were IL-1 β (F: CTTCAGGCAGGCAGTATC, R: TAATGGGAACGTCACACACC), IL-6 (F: CCTACCCCAATTTCATGCT, R: TATTTTCTGACCACAGTGAGGAAT), β -actin (F: AGCCATGTACGTAGCCATCC, R: TCTCAGCTGTGGTGGTGAA). β -actin was used as a calibrator. Relative quantification was performed using the comparative Ct method. Data were presented as the fold change in target gene expression and expressed as mean $\pm$ SEM.

1.1. Statistical Analysis

The results are presented as mean $\pm$ standard error mean (SEM) of three independent replicates. Data were analyzed using a one-way ANOVA test, followed by Dunnett's test for multiple comparisons. A p -value < 0.05 was considered significant. Statistical analyses were performed using GraphPad Prism 9.0.0 (GraphPad Prism Software, San Diego, CA, USA).

2. Results

2.1. Anti-Inflammatory Potential of *C. monspeliensis* Extracts

Firstly, the cytocompatibility of aerial parts (AP) and roots (R) extracts was investigated on RAW 264.7 by performing an MTT assay. Figure 2 depicts the percentage of viable cells after a 48 h treatment. Specifically, cells were treated with *C. monspeliensis* AP and R extracts at concentrations ranging from 100 µg/mL to 1.56 µg/mL for 48h. Treatments that reduced cell viability by more than 70% were considered cytotoxic. The results showed that the AP extract significantly reduced cell viability at concentrations above 50 µg/mL (EC_{50} 59.4 µg/mL) (Figure 2a), while the R extract showed toxicity from 25 µg/mL (EC_{50} 24.88 µg/mL) (Figure 2b).

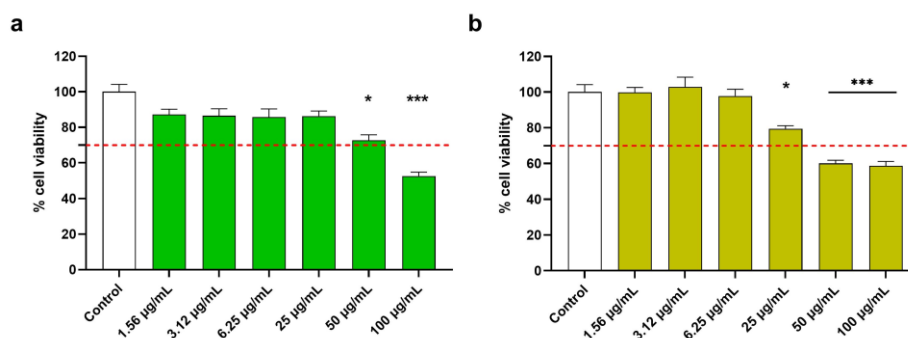


Figure 2. Effect of *C. monspeliensis* aerial parts (a) and roots (b) extracts on RAW 264.7 cell line viability. Cells were treated with the extracts at concentrations ranging from 100 µg/mL to 1.56 µg/mL for 48 h. Cell viability was assessed with an MTT assay. Data are presented as a percentage of cell viability compared to untreated cells, which are used as a control. The red dotted line indicates the percentage of cell viability under which the treatments were considered cytotoxic. Data are shown as mean $\pm$ SEM of three replicates: *** $p < 0.001$, * $p < 0.05$ vs. control group.

In order to test the anti-inflammatory action of *C. monspeliensis*, cells were co-treated with the AP and R extracts at concentrations that did not reduce cell viability by more than 70% (1.56–25 µg/mL). LPS (500 ng/mL) [25,26] was used as an inflammatory stimulus for 48h. According to the results shown in Figure 3, the LPS treatment reduced cell viability by 40%. The treatment with the AP extract in the presence of LPS significantly reduced

cytotoxicity at concentrations between 1.56 and 6.25 $\mu\text{g/mL}$. However, the highest dose used (25 $\mu\text{g/mL}$) failed to protect against the LPS stimulus, possibly due to the dose-dependent cytotoxicity of the extract previously found (Figure 3a). Likewise, also in the co-treatment with R extract and LPS, the extract showed a protective effect against the LPS inflammatory stimulus at dosages between 1.56 and 6.25 $\mu\text{g/mL}$, while cell mortality increased at 25 $\mu\text{g/mL}$ (Figure 3b).

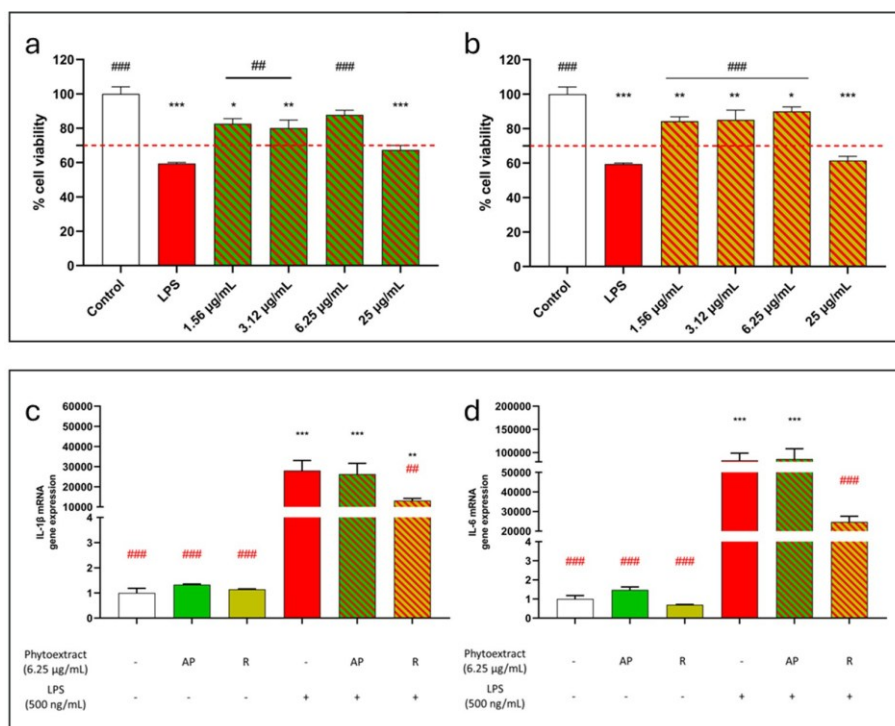


Figure 3. (a,b) Protective effect of *C. monspeliensis* aerial parts (a) and roots (b) extracts on RAW 264.7 cells subjected to LPS-induced inflammation. Cells were co-treated with the extracts at concentrations ranging from 25 $\mu\text{g/mL}$ to 1.56 $\mu\text{g/mL}$ and LPS (500 ng/mL) for 48 h. Cell viability was assessed with an MTT assay. Data are presented as a percentage of cell viability compared to untreated cells, which are used as a control. The red dotted line indicates the percentage of cell viability under which the treatments were considered cytotoxic. Data are shown as mean $\pm$ SEM of three replicates: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ vs. control group; ### $p < 0.001$, ## $p < 0.01$ vs. LPS. (c,d) *C. monspeliensis* roots extract reduces the expression of the inflammatory cytokines IL-1 β (c) and IL-6 (d) in the presence of LPS. RAW 264.7 cells were co-treated with *C. monspeliensis* aerial parts (AP) and roots (R) extracts at a concentration of 6.25 $\mu\text{g/mL}$ and LPS (500 ng/mL) for 6 h. Cells were then processed to measure IL-1 β and IL-6 mRNA levels by real-time PCR. β -actin was used to normalize the results. Data are shown as mean $\pm$ SEM; *** $p < 0.001$, ** $p < 0.01$ vs. control group; ### $p < 0.001$, ## $p < 0.01$ vs. LPS.

To further investigate the anti-inflammatory action of *C. monspeliensis* extracts, cells were treated with the AP and R extracts and LPS for 6 h. Based on the results concerning the protection against the LPS stimulus, the extracts were used at a concentration of 6.25 $\mu\text{g/mL}$, which was the highest dose that resulted to protect against LPS. Subsequently, the mRNA gene expression of two pro-inflammatory cytokines, IL-1 β and IL-6, was determined. Interestingly, only the R extract was able to significantly reduce both IL-1 β (Figure 3c) and IL-6 (Figure 3d) mRNA gene expression in the presence of LPS. Contrarily, the treatment with the AP extract did not counteract the LPS action for any of the two cytokines (Figure 3c,d).

1.1. Anti-Oxidative Activity of *C. monspeliensis* Extracts

To assess the anti-oxidative potential of *C. monspeliensis* extracts, SH-SY5Y cells have been selected due to their heightened susceptibility to oxidative damage. This characteristic renders them particularly useful in exploring the efficacy of antioxidant compounds in mitigating oxidative stress-induced cellular damage.

Initially, the cytocompatibility of *C. monspeliensis* AP and R extracts with SH-SY5Y cells was assessed before examining their antioxidant properties. Also, in this case, cells were treated with *C. monspeliensis* AP and R extracts at concentrations ranging from 100 $\mu\text{g/mL}$ to 1.56 $\mu\text{g/mL}$ for 48 h. Comparable results were observed to those obtained with RAW 264.7 cells; thus, dosages from 6.25 $\mu\text{g/mL}$ to 1.56 $\mu\text{g/mL}$ were further tested (Figure 4).

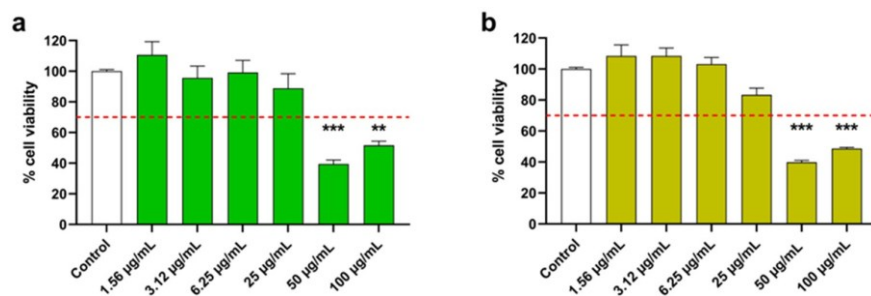


Figure 4. Effect of *C. monspeliensis* aerial parts (a) and roots (b) extracts on SH-SY5Y cell line viability. Cells were treated with the extracts at concentrations ranging from 100 $\mu\text{g/mL}$ to 1.56 $\mu\text{g/mL}$ for 48 h. Cell viability was assessed with MTT assay. Data are presented as percentage of cell viability compared to untreated cells used as a control. The red dotted line indicates the percentage of cell viability under which the treatments were considered cytotoxic. Data are shown as mean $\pm$ SEM of three replicates: *** $p < 0.001$, ** $p < 0.01$ vs. control group.

To induce cellular damage, H_2O_2 0.2 mM was utilized as an oxidative stimulus. As depicted in Figure 5a,b, H_2O_2 24 h treatment was able to reduce cell viability by nearly 40%. Cellular pre-treatment with both *C. monspeliensis* AP and R at 6.25 $\mu\text{g/mL}$ was found able to significantly enhance by nearly 20% the cell survival rate after H_2O_2 insult, with R ($p < 0.001$) extract outperforming AP ($p < 0.01$).

To explore the potential impact of *C. monspeliensis* extracts on mitochondrial functionality, cells were treated with AP and R at 6.25 $\mu\text{g/mL}$ and subjected to H_2O_2 exposure. Mitochondria were then assessed using the MitoTracker fluorescent dye, which selectively stains mitochondria in live cells. Interestingly, we found that pre-treatment with *C. monspeliensis* R extract showed significant increases in MitoTracker Green fluorescent emission when compared to the H_2O_2 signal. These data suggest that R extract can promote mitochondria survival when cells are exposed to oxidative stress. On the contrary, no difference in terms of fluorescent signal was observed for the AP extract (Figure 5c,d).

Moreover, it was observed that cells treated with R extract (6.25 $\mu\text{g/mL}$) exhibited a significantly higher production of ATP compared to untreated cells, suggesting that R extract can exert a positive impact on the cell energy metabolism, improving mitochondria functionality (Figure 5e,f).

To further explore the anti-oxidative properties of AP and R, we investigated the enzymatic activity of Catalase, a crucial antioxidant enzyme that catalyzes the decomposition of H_2O_2 into water and molecular oxygen. Data reported in Figure 5g showed that cells treated with R extract (6.25 $\mu\text{g/mL}$) exhibited a higher catalase enzyme activity when compared to untreated cells. Conversely, cells treated with AP extract (6.25 $\mu\text{g/mL}$) exhibited higher catalase levels, although the difference was not statistically significant (Figure 5g).

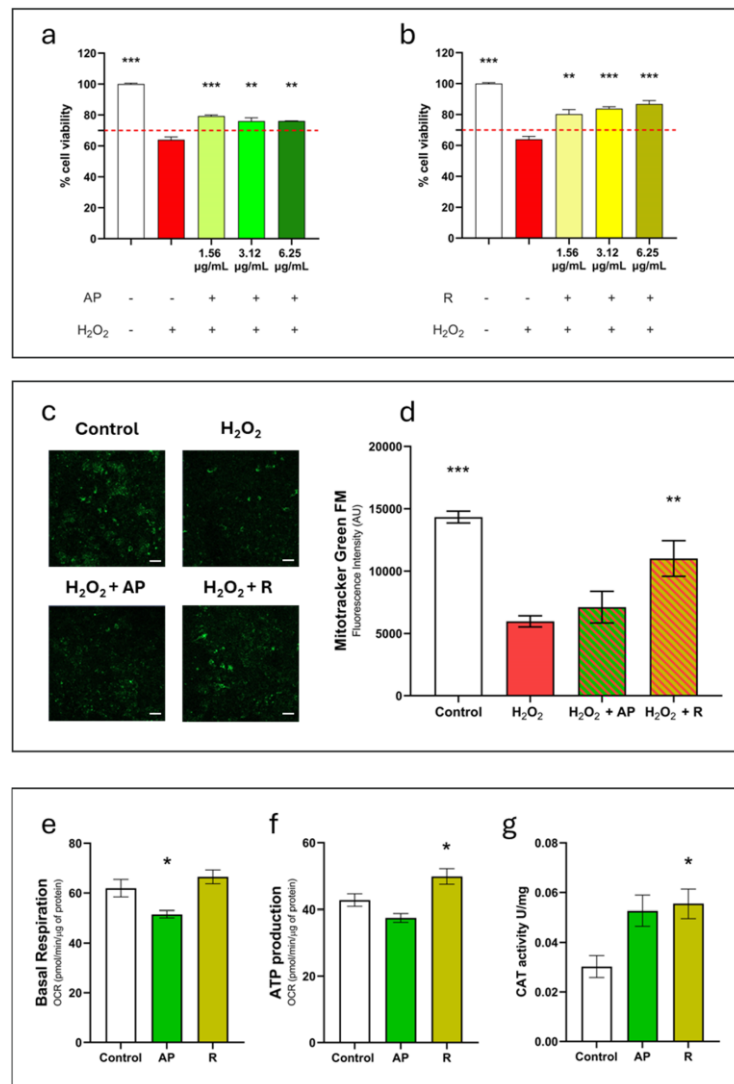


Figure 5. (a,b) Protective effect of *C. monspeliensis* aerial parts (a) and root (b) extracts on SH-SY5Y cells subjected to the H₂O₂ pro-oxidant stimulus; the red dotted line indicates the percentage of cell viability under which the treatments were considered cytotoxic. Data are shown as mean $\pm$ SEM of three replicates: *** $p < 0.001$, ** $p < 0.01$ vs. H₂O₂ group. (c,d) Effect of *C. monspeliensis* aerial parts and roots extracts on mitochondrial functionality. Cells were pre-treated with the extracts at 6.25 μ g/mL; H₂O₂ was added after 24 h. Mitotracker Green representative figures are shown in (c), while in (d), the normalized fluorescence intensity graph is shown. Images have been acquired with 10 $\times$ magnification, white scale bar 50 μ m. Data are shown as mean $\pm$ SEM of three replicates: *** $p < 0.001$, ** $p < 0.01$ vs. H₂O₂ group. (e,f) Graphs relative to basal respiration and ATP production. Cells were treated with *C. monspeliensis* aerial parts and roots extracts at 6.25 μ g/mL; cells were evaluated by using the Seahorse XF96e Extracellular Flux Analyzer; (g) cellular Catalase enzymatic activity in the presence or absence of AP and R extracts (6.25 μ g/mL). Results obtained are normalized on total protein content and expressed as unit/mg of protein. Data are shown as mean $\pm$ SEM of three replicates; p -value is * $p < 0.05$ vs. control group.

1.1. Phytochemical Analysis

A first overview of the phytochemical composition of AP and R extracts was acquired by ^1H NMR profiling (Figure 6), supported by further pre-purification procedures, 2D NMR, and UHPLC-MS experiments on the obtained fractions (Figures S1–S8).

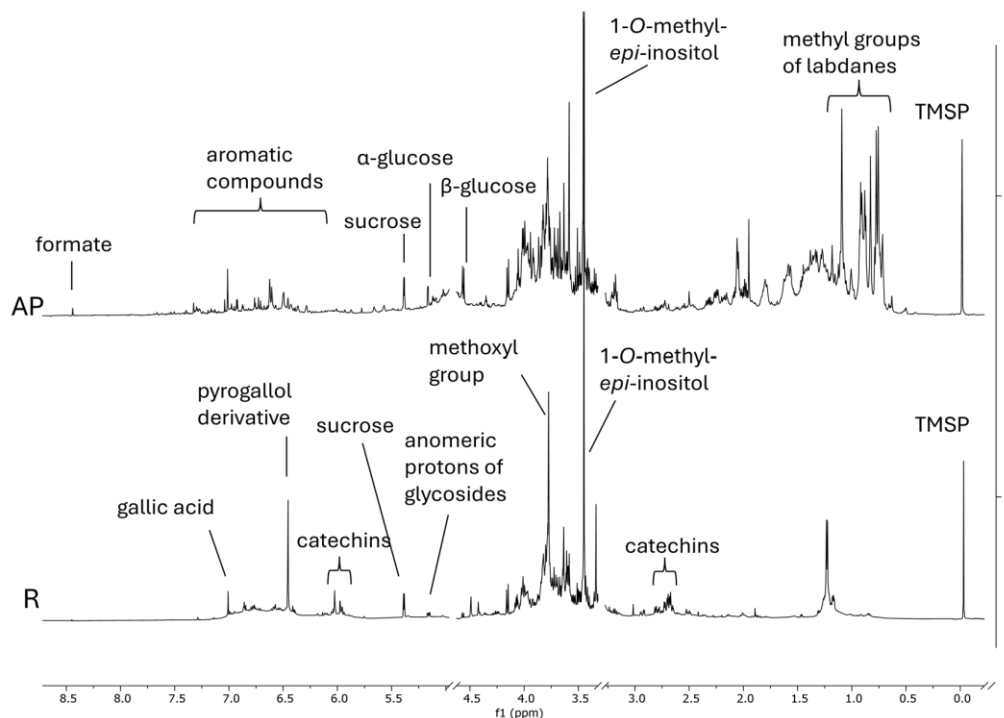


Figure 6. ^1H NMR profiles of *C. monspeliensis* extracts; aerial parts (AP) on the top and roots (R) on the bottom.

A singlet at δ 3.46 was prominent in both AP and R ^1H NMR profiles, and it was attributed to the methoxy group of *O*-methyl-cyclitol; indeed, by HSQC, it correlated with a carbon at δ 58.40. This compound was further purified, and its structure was fully elucidated (Figure S8) as 1-*O*-methyl-*epi*-inositol, a stereoisomer of pinitol. According to the semiquantitative NMR analysis, the concentration of this metabolite was 34.5 $\mu\text{g}/\text{mg}$ of AP dry weight (DW) and 22.3 $\mu\text{g}/\text{mL}$ of R DW.

Among the primary metabolites, sucrose was also present in both extracts, while α -glucose, β -glucose, and formate were detected only in AP.

Considering the secondary metabolites, the AP and R ^1H NMR profiles were strongly different. In particular, the AP profile was characterized by several signals in the aliphatic region from δ 0.8 to 2.2. According to the literature, diterpenes are characteristic metabolites of *C. monspeliensis* aerial parts [9], together with methoxylated flavonoids [27,28]. In fact, the AP ^1H NMR profile showed several singlets diagnostic of labdane terpenes and aromatic signals, potentially ascribable to flavonoids (Figure 6). However, their structure could not be unambiguously characterized only by NMR profiling.

Through the pre-purification procedure, 8-hydroxylabdan-15-oic acid (**1**) [9] (Figure S2), gallic acid, catechin, methoxylated flavonols, and an acetophenone glycoside (**6**) (Figure 7) were found in AP.

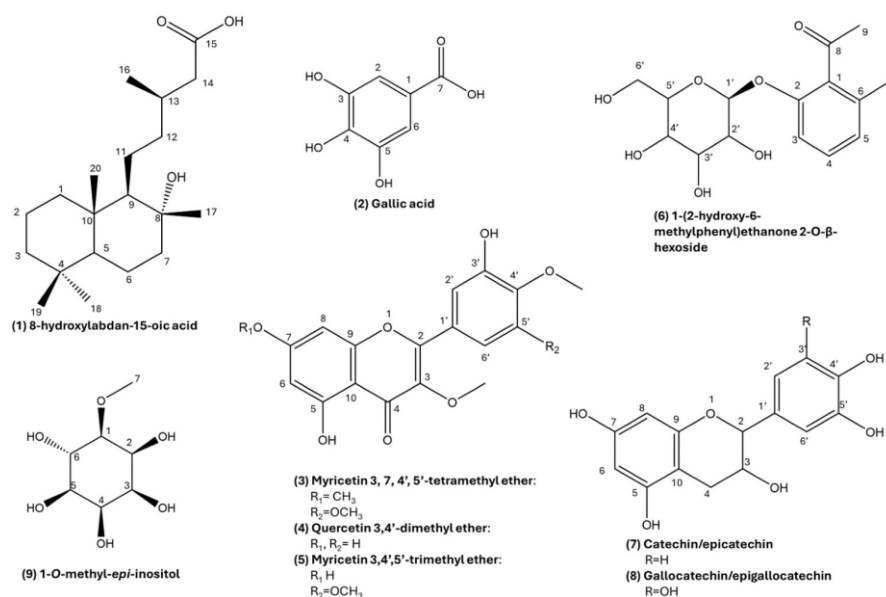


Figure 7. Structures of the most prominent compounds identified.

Among the flavonoids found in AP, myricetin 3,7,4',5'-tetramethyl ether (3) has already been reported in *C. monspeliensis* [9,18]. It was easily identified based on the HMBC correlations given by its four methoxyl groups (Figure S3). In particular, the protons of the methoxyl at δ 3.88 correlated with carbon at δ 168, typical of flavonoids A ring. This was, in turn, equally correlated with protons 6 and 8, suggesting that the methoxyl substitution was in position 7. The protons of the other two methoxyl groups (δ 3.83 and 3.78) correlated in HMBC with the carbons at δ 139.2 and 139.4, respectively; these values are diagnostic of carbon 3 and carbon 4'. Indeed, the carbon at δ 139.4 was also correlated with the proton in 6' (δ 7.22) and the hydroxyl proton in 3' (δ 9.63) (visible because the sample was prepared in DMSO- d_6), confirming that also position 4' was methoxy substituted. Considering the splitting pattern of the protons of the B ring (two doublets with $J = 2.11$ Hz), this aromatic ring was asymmetric; hence, the other methoxyl group (protons at δ 3.87 and carbon at δ 56.40) was positioned on carbon 5' (δ 153.5). The presence of compound 3 in the total extract was further confirmed by UHPLC-MS analysis, which showed in QDa1 mode a m/z value 375.24 interpreted as $[M+H]^+$.

Compounds 4 and 5 (Figure 7) eluted together in APCL subfraction 8 (Figure S4). The UHPLC-MS analysis of this subfraction (Figure S5) suggested the presence of a quercetin dimethyl ether (m/z 331.22) and a myricetin trimethyl ether (m/z 361.23), interpreted both as $[M+H]^+$. Actually, the ^1H NMR spectrum showed signals of protons belonging to two distinct flavonoid A rings (two doublets around δ 6.2 and 6.4, with $J = 2.10$ Hz), as well as five singlets around δ 3.8, ascribable to the protons of five methoxyl groups. Moreover, the spectrum also showed three signals diagnostic of a catechol moiety and two doublets with both $J = 2.10$ Hz. The protons of two methoxyl groups correlated with carbons at δ 138.2 and 138.8 (Figure S4), diagnostic of position 3. This information was crucial to assess that both compounds were methoxylated in position 3. One more methoxyl signal correlated with a carbon resonating at δ 139.3, attributed to that in position 4' of compound 5, which was identified as myricetin 3,4',5'-trimethyl ether. Finally, the doublet at δ 6.97 ($J = 8.43$ Hz) ascribable to proton 5' of compound 4 (Figure 7) gave HMBC correlation with carbon at δ 148.58, which in turn correlated with the methoxyl protons at δ 3.86 (Figure S4). This allowed us to identify compound 4 as quercetin 3,4'-dimethyl ether.

Compound **6** was found in APeT subfraction 15 (Figure S7). The ^1H NMR spectrum of this molecule showed three aromatic protons, several signals ascribable to a sugar moiety, and two characteristic singlets resonating at higher fields (δ 2.51 and 2.18).

The HMBC correlations of the proton resonating at δ 2.51 with the carbon at δ 218 and the carbon at δ 131.4 (C1) were diagnostic of an acetophenone structure. Moreover, the carbon at δ 131.43 also correlated in HMBC with the singlet at δ 2.18 (whose carbon established by HSQC correlation resonated at δ 17.91) and was interpreted as a methyl group in position 6. These methyl protons gave, in turn, HMBC correlation also with carbon 6 (δ 135.51) and carbon 5 (δ 125.29) of the aromatic ring.

Moreover, carbon 2 (resonating at δ 152.6) gave HMBC correlation not only with the aromatic protons in 3 (δ 6.98) and in 4 (δ 7.26), but also with a doublet at δ 5.01, which carbon resonates at δ 100.4 (by HSQC). This latter is a typical anomeric proton; hence, the HSQC and HMBC spectra revealed the presence of other signals ascribable to a hexose. By the coupling constant of the anomeric proton ($J = 7.7$ Hz), it was possible to define that the glycosidic bond was in the β orientation. Further purifications are ongoing to determine the stereochemistry of the sugar. Hence, compound **6** was characterized as 1-(2-hydroxy-6-methylphenyl) ethanone 2- O - β -hexoside. When searching this structure on the online database Reaxys (independently from the sugar stereochemistry), only juniperoside III was found, where the sugar moiety is glucose. This compound was first identified in *Juniperus occidentalis* [29]. Venditti et al. [27] found a compound with a structure similar to **6** in *C. monspeliensis*, with one more hydroxyl substituent.

Other aromatic compounds were found in APeT, particularly gallic acid and catechin (Figure 7 and Figure S6). This latter eluted together with compound **6**. To establish that this compound was catechin and not its isomer epicatechin, the ^1H NMR signal of proton 2 was diagnostic. In fact, this doublet resonating at δ 4.54 had $J = 7.75$ Hz, indicating an opposite orientation of the coupling protons in positions 2 and 3.

Gallic acid was also found in R (Figure 6), and it was responsible for the singlet at δ 7.03; its concentration by semiquantitative analysis was 1.7 $\mu\text{g/mL}$ (DW). In addition, the R ^1H NMR profile showed a particularly abundant derivative of pyrogallol, responsible for the singlet at δ 6.48, correlating with a methoxyl group; however, to fully characterize this compound, other pre-purification procedures are ongoing.

R profile also showed signals ascribable to catechins. The UHPLC-MS analysis revealed the presence of both catechin or its isomer epicatechin (exact mass 290.26 Da), giving 289.11 $[\text{M}-\text{H}]^-$ in QDa 2, and galocatechin or its isomer epigallocatechin (exact mass 306.267 Da), which gave a m/z value of 305.11 $[\text{M}-\text{H}]^-$ in QDa 2 (Figure S1).

1.1. Total Reflection X-ray Fluorescence (TXRF) Analysis

TXRF spectra of *C. monspeliensis* AP and R extracts are shown in Figure 8.

The inorganic content of *C. monspeliensis* AP and R extracts was determined by means of TXRF analysis. Table 1 shows a clear difference between the two extracts in terms of both qualitative and quantitative determination. The identified elements included macronutrients (K and Ca) and micronutrients (Ti, Mn, Fe, Co, Ni, Cu, Zn, Br, Rb, Sr). The majority of the elements were present in both extracts, except for Ti, which was not detected in the R extract, and Co, not found in the AP extract. From a quantitative point of view, concentrations of K, Ca, and Br were higher in the AP, while Ni, Cu, and Sr were more abundant in the R extract. Minor variations were detected for Mn, Fe, Zn, and Rb. Potentially toxic elements, such as Pb, were not detected in any sample. The differences underscore the selective accumulation of elements within distinct plant tissues [30].

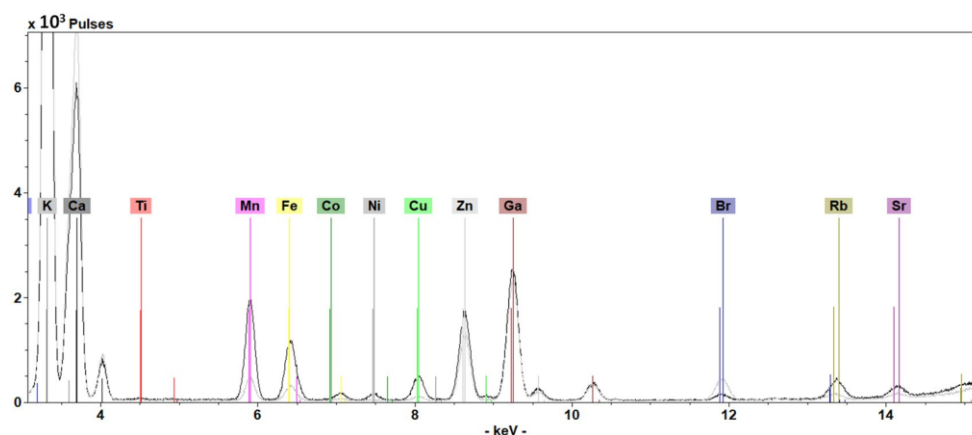


Figure 8. TXRF spectra of *C. monspeliensis* AP (grey line) and R (black line) acquired for 600 s live time.

Table 1. Elemental concentration (mg/kg) of AP and R extracts of *C. monspeliensis*.

Element	AP (mg/kg)	R (mg/kg)
K	9000 ± 2600	1800 ± 550
Ca	1400 ± 400	400 ± 120
Ti	2.1 ± 1.7	-
Mn	38 ± 11	40 ± 13
Fe	34 ± 11	21 ± 7
Co	-	1.5 ± 0.4
Ni	2.3 ± 1.8	5.7 ± 1.6
Cu	5.0 ± 1.5	18 ± 6
Zn	21 ± 6	27 ± 8
Br	8 ± 2	0.9 ± 0.3
Rb	2.7 ± 0.8	2.3 ± 0.8
Sr	0.9 ± 0.3	1.3 ± 0.4
Pb	-	-

1. Discussion

Natural product-derived compounds have always been considered of great importance for drug discovery [31], and ethnobotanical knowledge has often contributed to laying the basis for finding natural molecules and extracts endowed with specific bioactivity [32–34].

Due to its large use in Mediterranean traditional medicines [6], the *Cistus* genus has been widely studied, revealing the presence of specialized metabolites endowed with antioxidant, antimicrobial, anti-inflammatory, and neuroprotective properties [3–6,10,11].

In recent years, interesting data have been reported on *Cistus incanus* L., one of the most well-known *Cistus* species, which grows in the innermost areas of Europe. This plant has shown antioxidant and anti-inflammatory properties [19,35,36].

This work was focused on *Cistus monspeliensis*, from which the aerial parts are used in Sardinian traditional medicine for wound healing [13], a condition with an inflammatory basis [37]. The antioxidant properties of *C. monspeliensis* aerial parts are supported by the literature; however, limited studies have been conducted [5,18,19], and none of them are on cell models.

Hence, this work was addressed to deepen the bioactivity of the ethnobotanically relevant organ (aerial parts: AP), as well as investigate its roots (R), which still remain unexplored both in terms of bioactivity and phytochemistry.

Prior to investigating their anti-inflammatory potential, the biocompatibility of the extracts was assessed for the RAW 264.7 cell line. AP and R did not cause cytotoxicity at concentrations lower than 50 µg/mL and 25 µg/mL, respectively.

When tested for their ability to protect against the LPS inflammatory stimulus, the extracts (AP and R) were active in a concentration range between 1.56 and 6.25 $\mu\text{g/mL}$.

During inflammation, the pro-inflammatory response is mediated by cytokines, including IL-6, IL-1 β , and TNF- α . Their production can be triggered by the recognition of bacterial products, such as LPS and toxins, through the toll-like receptor (TLR) family present in monocytes, macrophages, and endothelial cells [26,38].

Hence, to assess the ability of the extracts to modulate the gene expression of pro-inflammatory cytokines involved in the inflammatory response, the gene expression of IL-6 and IL-1 β was investigated. Interestingly, only the treatment with R (at 6.25 $\mu\text{g/mL}$) was able to reduce the expression of these two pro-inflammatory cytokines in the presence of LPS. In future studies, it will be valuable to assess the anti-inflammatory action of R on specific targets like hyaluronidase and tyrosinase, similar to the approach taken by Graczyk et al. [39].

The data obtained in this study suggest that R has notable antioxidant capabilities, effectively protecting cells from oxidative stress-induced death. These findings suggest that the antioxidant action of R may be mediated by its effects on mitochondria: indeed, the treatment enhanced energy capacity, as evidenced by higher ATP levels in the cells. Additionally, R acted on multiple levels, enhancing the cell's ability to detoxify an excess H_2O_2 through increased catalase enzyme activity. Interestingly, although AP extract protects against oxidative stress-induced cell death, it did not confirm mitochondrial-mediated action. The overall findings let us hypothesize that AP could exert its action through other mechanisms that have not been investigated here.

The metabolite composition of the extracts was first explored by ^1H NMR profiling, a technique often used to analyze complex mixtures and commonly applied in metabolomics [40–42]. Because of the NMR robustness and repeatability, due to the easy sample preparation procedure, the ^1H NMR fingerprinting is particularly appealing considering data recycling and reuse; for instance, spectral profiles produced in different works can be collected in order to create a database. For this reason, we deposited all the spectra in a data repository (<https://doi.org/10.5281/zenodo.11281288>).

By ^1H NMR profiling, it was found that the AP and R have only some primary metabolites in common, such as sucrose and cyclitol, which resulted in 1-*O*-methyl-*epi*-inositol. This latter was one of the most prominent compounds in both AP and R, with a concentration of 34.5 $\mu\text{g/mg}$ (DW) and 22.3 $\mu\text{g/mL}$ (DW), respectively. 1-*O*-methyl-*epi*-inositol is a stereoisomer of the most common pinitol, which was already reported in *C. monspeliensis* [27].

Cyclitols generally act as osmoprotectants in plants, allowing them to survive under water stress due to drought and high salinity [43,44]. They are also endowed with several bioactivities; for instance, pinitol was proven active as antidiabetic, antioxidant, anti-inflammatory, anti-cancer, and chemopreventive [45].

The ^1H NMR profiling also showed a high amount of labdane terpenes in AP; in fact, *C. monspeliensis* is renowned for its aromatic resin that coats the leaves, constituted mainly of labdanes and clerodanes [27].

Deepening the phytochemical investigation, several methoxy flavonols were found exclusively in AP, while catechins were found in both AP and R. 8-hydroxyabdanoic acid and myricetin 3,7,4',5'-tetramethyl ether were already detected in Sardinian *C. monspeliensis* ecotypes by Salomè-Abarca et al. [9], while quercetin 3,4'-dimethyl ether and myricetin 3,4',5'-trimethyl ether are here reported for the first time in a species of *Cistus* genus.

The phytochemical investigation conducted in this work on AP also yielded another compound, which is apparently rare in plants and new to *Cistus*, namely 1-(2-hydroxy-6-methylphenyl) ethenone 2-*O*- β -hexoside. No studies on the biological activity of this compound are available.

The high degree of methylation appears to be a characteristic phytochemical signature of the *C. monspeliensis* metabolome; in fact, from 1-*O*-methyl-*epi*-inositol to many aromatic compounds found in this plant (both AP and R), they all bear methyl groups on their structure, either C-bonded or O-bonded (methoxy).

Although further studies will be necessary to assess which compounds are responsible for the bioactivities measured, it is reasonable to hypothesize that flavonoids, found both in AP and R, might make an important contribution since they are renowned natural antioxidants. In particular, among flavonoids, catechins, which appear more abundant in R than AP by ^1H NMR profile, might have a prominent role in determining the bioactivities observed [46,47]. Moreover, the pyrogallol moiety was found both on metabolites contained in AP and R. This moiety is renowned for enhancing antioxidant activity [48], and recently, pyrogallol derivatives have also been associated with in vitro antiproliferative activity [49].

Even if the bioactivity of a plant extract is generally associated with its metabolites, the bioactivity and the content of plant secondary metabolites can also be influenced by the presence of essential heavy metals [20].

Plant elements can be subdivided in macronutrients, e.g., Nitrogen (N), Phosphorus (P), Potassium (K), Calcium (Ca), Sulfur (S), and Magnesium (Mg) and micronutrients, e.g., Iron (Fe), Zinc (Zn), Copper (Cu), Boron (B), Manganese (Mn); the former are needed in large amounts, while the latter in traces and are beneficial for plant growth and development [50]. Since plants take essential and non-essential elements from the soil, including potentially toxic heavy metals, their content depends on both the environment and human activities, such as mines, foundries, and agriculture [51,52]. Also, it is established that roots function as a barrier, limiting the transfer of heavy metals to the upper sections of the plant. The accumulation of heavy metals is more pronounced in the roots, gradually decreasing as it moves towards the upper parts (roots > stems > leaves > crops) [52].

It is worth mentioning that the antioxidant activity of some flavonoids, e.g., quercetin, rutin, galangin, complexed with metal ions, was found to be higher than that of free flavonoids; moreover, metal ions coordinated with rutin can potentiate the anti-inflammatory activity [20]. Several studies have analyzed the influence of heavy metals on the metabolomic profile and biological action of plants. Lala showed that the application of Cu nanoparticles at sub-toxic doses increased the whole secondary metabolites content in *Bacopa monnieri* [53]; Slaven Juric' and colleagues applied microparticles containing Ca and Cu ions to augment the secondary metabolites production in *Lactuca sativa* L. [54].

According to Mucha and colleagues [20], the antioxidant activity of metal ions, specifically Al(III), Zn(II), Cu(II), and Fe(II) complexed with flavonoids was higher than that of the single compounds. Complexes of Cu(II), Fe(II), Fe(III), Ni(II), and Zn(II) with rutin also showed better anti-inflammatory activity [20].

Hence, considering the importance of the inorganic composition of a plant, the extract's elemental content was also determined. Also in this case, AP and R differed from both a qualitative and quantitative point of view. The TXRF analysis revealed the presence of K and Ca (as macronutrients) and Ti, Mn, Fe, Co, Ni, Cu, Zn, Br, Rb, and Sr (as micronutrients). Certain elements were exclusively identified in specific extracts; for instance, Ti was found in the AP, while Co was solely present in the root extract. In addition, K, Ca, and Br were more concentrated in the AP, while Ni, Cu, and Sr had a higher concentration in the R.

In conclusion, it is here shown that the roots of this plant are even more promising as anti-inflammatory than aerial parts, which are traditionally used for this purpose.

None of the extracts were found to accumulate harmful metals. Further studies are ongoing to test single metabolites in bioassays, with the final aim of establishing their contribution to the bioactivity of the raw extracts and to further support the potential use of this plant and its metabolites as active ingredients for anti-inflammatory and antioxidant phytotherapeutic formulations.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/antiox13070753/s1>, Figure S1: UHPLC-MS analysis of R. Mass spectra in negative ion mode -QDa 2. (A) Mass spectrum at retention time 0.535 min interpreted as gallic acid (exact mass 170.12 Da) which gave m/z value 169.08 $[M-H]^-$. (B) Mass spectrum at retention time 0.760 min interpreted as galocatechin or epigallocatechin (exact mass 306.267 Da) which gave m/z value 306.01 $[M^+]$. (C) Mass spectrum at retention time 1.818 min interpreted as catechin or epicatechin (exact mass 290.26 Da) which gave m/z value 290.12 $[M^+]$; Figure S2: NMR-based structural elucidation of 8-hydroxylabdan-15-oic acid (A) Superimposed HSQC and HMBC spectra of APCI subfraction 8 from which the compound was characterized (green dots = HMBC correlations; blue and red = HSQC correlations). (B) Structure of the compound (C) Spectral range between δ 0.60–1.03 highlighting the signals of the methyl groups; Figure S3: Structural elucidation of myricetin 3,7,4',5'-tetramethyl ether (A) Superimposed HSQC (green dots) and HMBC (red dots) spectra in deuterated DMSO of APet subfraction 1 from which the compound was characterized. (B) Mass spectra in QDa 1 of the same subfraction showing m/z 375.24 interpreted as $[M+H]^+$ (exact mass of myricetin 3,7,4',5'-tetramethyl ether 374.10 Da); Figure S4: Structural elucidation of quercetin 3,4'-dimethyl ether (A) and myricetin 3,4',5'-trimethyl ether (B) Superimposed HSQC and HMBC spectra of APCI subfraction 8 from which the two compounds were characterized (A and B bottom). The blue arrows indicate the HMBC correlations which were crucial to determine the position of methoxyl groups on the molecules; Figure S5: UHPLC-MS analysis of APCI subfraction 8 (A) Chromatogram at 260 nm (B) Mass spectra of peak at RT 5.84 min showing m/z 331.22 interpreted as $[M+H]^+$ (exact mass of quercetin 3,4'-dimethyl ether = 330.0740 Da) (C) Mass spectra of peak at RT 6.045 min showing m/z 361.23 interpreted as $[M+H]^+$ (exact mass of myricetin 3,4',5'-trimethyl ether = 360.0845 Da); Figure S6: NMR-based structural elucidation of gallic acid and catechin (A) Structures of the two molecules of interest, (B) HMBC spectrum of subfraction 14 of APet showing the heteronuclear correlations in gallic acid molecule. (C,D) superimposed HSQC and HMBC spectra of APet subfraction 15 from which catechin was characterized; Figure S7: NMR-based structural elucidation of 1-(2-hydroxy-6-methylphenyl) ethanone 2-O- β -hexoside (A) Molecular structure (B) Superimposed HSQC and HMBC spectra of APet subfraction 15 from which the compound was characterized. The extended regions (C,D) show the correlations between aromatic protons and glycosidic protons, respectively; Figure S8: NMR-based structural elucidation of 1-O-methyl-*epi*-inositol in D₂O (A) Molecular structure, (B) HMBC spectrum of RW subfraction 20 from which the compound was characterized, (C) 2D-NOESY, (D) COSY (the diagonal was suppressed).

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8. Our contribution: variables impacting *Lavandula* genus essential oils biological activity

Lavandula genus (lavender) plants are small perennial shrubs, typically growing in rocky and sunny habitats, and belong to the Lamiaceae family [92]. These plants are native to the Mediterranean basin, in particular they can be found in Northern Africa, Turkey, France, Spain, and Italy; they are also present in the Canary Islands, Southern Africa, England, India, Australia, and the USA [51], [92], [93]. Lavender plants are mainly cultivated for their essential oils (EOs) [94], which are complex volatile organic mixtures used in the cosmetic, perfume and pharmaceutical industry [95], [96]. Lavender natural products were used in traditional medicine for the treatment of respiratory disorders [97]. Moreover, studies have showed that EOs obtained from these plants exhibit anti-inflammatory, antioxidant, antimicrobial, antifungal, and antiviral activity [97], [98], [99], [100].

Since only few studies characterized *L. intermedia* (lavandin) cultivars, the subjects of our study were *L. angustifolia* subsp. *pyrenaica* (DC.) (Figure 6) and two cultivars of lavandin (lavandin Emeric ex Loisel cv Grosso and lavandin Emeric ex Loisel cv Super). Specifically, the goal of our study was to analyze their EOs from a phytochemical and bioactivity point of view and to compare them to a commercially available lavender EO. Indeed, it is known that, during the production, incorrect identification and adulteration (e.g. addition of unauthorized substances for cost-saving, improvement of fragrance and conservation) can occur, leading to a modification in EOs biological properties [27], [101]. The phytochemical profile was determined by gas chromatography-mass spectrometry (GS-MS) analysis, while antioxidant activity was tested using the SH-SY5Y cell line.



Figure 6. *L. angustifolia* (<https://www.gbif.org/>)

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Phytochemical Characterization and Potential Anti-Oxidative Activity of *Lavandula angustifolia* subsp. *pyrenaica* (DC.), *Lavandula x intermedia* Emeric ex Loisel cv Grosso, and cv Super Essential Oils Compared to a Commercial Lavender Essential Oil

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ABSTRACT

This study investigates the phytochemical profiles and antioxidant properties of three lavender essential oils (LEOs) from *Lavandula angustifolia* subsp. *pyrenaica* (DC.), *Lavandula x intermedia* Emeric ex Loisel cv Grosso, and *Lavandula x intermedia* Emeric ex Loisel cv Super, and compares them to a commercial one. LEOs were extracted by hydrodistillation, analyzed using gas chromatography-mass spectrometry, and tested for antioxidant effects on human SH-SY5Y neuroblastoma cells. The major components identified in all the four LEOs were linalool and linalyl acetate; however, in the commercial LEO differences in minor compounds and the presence of additives were found. Antioxidant activity assays revealed significant protection against H₂O₂-induced oxidative stress for hydrodistilled EOs, while the commercial EO showed no protective effect. Gene expression analysis indicated upregulation of antioxidant-related genes in cells treated with “Grosso” and “Super” LEOs. This research highlights the potential therapeutic applications of LEOs, with a particular emphasis on the differences between naturally derived oils and commercial variants.

1 | Introduction

Lavandula L. genus, commonly known as lavender, belongs to the Lamiaceae family [1] and includes 39 species as well as hundreds of cultivars and hybrids [2–4]. Plants belonging to this genus consist mostly of small perennial shrubs; *Lavandula* species can differ in height, color, and shape of their leaves, flowering period,

natural habitat, geographical distribution, and essential oils (EOs) composition [4–7]. Lavender plants commonly reach a height of 30–80 cm [2] and grow in rocky and sunny habitats. They are characterized by linear and semievergreen leaves, presenting a grey-green color in summer, and becoming silvery during the winter [7]. Lavender plants also exhibit purple flowers, distributed in spike shapes at the end of long stalks [2]. *Lavandula* genus plants

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are native to the Mediterranean area, including Northern Africa, Turkey, France, Spain, and Italy; in addition, they can also be found in the Canary Islands, Southern Africa, England, India, Australia, and the USA [5–7]. The most researched and known *Lavandula* species are *Lavandula angustifolia* Miller, also known as *Lavanda vera* or *Lavanda officinalis*, *Lavandula stoechas* L. (French lavender) [6], and *Lavandula latifolia*, commonly known as spike lavender [4–7]. *L. x intermedia* (lavandin), is a hybrid cross between two natural-growing lavenders, *L. latifolia* and *L. angustifolia* [4–7]. *L. angustifolia* subsp. *pyrenaica* (DC.) and two different cultivars of lavandin, namely lavandin Emeric ex Loisel cv Grosso and lavandin Emeric ex Loisel cv Super, are the focus of this research. *L. angustifolia* is a hardy perennial plant, that can reach a height of 50 cm; this plant is characterized by narrow and linear grey leaves that become greener as they mature, with blue, white, and violet flowers. It can be found mainly in mountainous regions in Italy, France, and Spain at 600–1200 m above sea level, in sunny and drained calcareous soils [5, 7]. *L. latifolia* height can vary from 50 to 100 cm; the leaves are grey and silvery in color and linear to spatulate in shape, with blue to pale purple-colored flowers; *L. latifolia* has a similar distribution to that of *L. angustifolia*, differing only for the altitude (300 m above sea level) [5, 7]. *L. “Grosso”* and *L. “Super”*, as hybrid plants derived from *L. angustifolia* and *L. latifolia*, grow in the territories the parents naturally habit; these shrubs can reach heights of 60–150 cm and are characterized by purple to white flowers and grey leaves with shape from linear to spatulate [5]. A noteworthy feature of *L. intermedia*, and hybrid plants in general, is hybrid vigor (also known as heterosis), which means that they exhibit some added emerging benefits compared to their parent plants. In the case of the lavandin subspecies, they are sturdier than true lavender and they contain a higher amount of EOs thanks to their secretory structures being more present than the ones of true lavender [7–9]. All these characteristics render lavandin cultivars easier and cheaper to cultivate and more profitable as they give more EOs per hectare compared to *L. angustifolia* and other natural lavenders, making them ideal for commercial uses. However, since *L. intermedia* is a cross-hybrid, is considered sterile [4–7]. Lavender plants share similar ethnobotanical properties and the main EOs compounds are conserved throughout the whole genus [4–7]. Since the Middle Ages, lavender and its extracts have been used in the perfume industry, for aromatherapy, skincare, sleep aid, and washing clothes [10, 11]. Other traditional uses include the treatment of respiratory disorders, such as cough, cold, asthma, and sinus congestion [10, 11]. Lavender EOs have been proven to have many pharmacological applications, including anti-inflammatory and antioxidant properties, along with antimicrobial, antifungal, and antiviral activity [11–15].

To the best of our knowledge, only a few studies have compared different *Lavandula* species with each other, especially considering *L. intermedia* cultivars. The main objective of this research is to analyze the phytochemical profile of three different lavender EOs (LEOs) (*L. angustifolia*, *L. “Grosso”*, and *L. “Super”*) and to characterize them from a biological point of view while comparing them to a commercially available LEO.

The three LEOs were obtained by hydrodistillation, and their phytochemical content was analyzed by means of gas chromatography-mass spectrometry (GS-MS) analysis. The

antioxidant activity was evaluated using a human neuroblastoma cell line (SH-SY5Y).

1 | Results

1.1 | LEOs’ Phytochemical Profile

The LEOs’ chemical composition was analyzed by means of GC-MS. Specifically, for *L. angustifolia*, the main compounds found were linalool (21.8%) and linalyl acetate (20.1%) (Table 1), belonging to oxygenated monoterpenes, which interestingly represented the most abundant class of compounds found in this EO (71%), followed by oxygenated sesquiterpenes (13.3%), sesquiterpene hydrocarbons (7.9%), monoterpene hydrocarbons (5.7%), and other non-terpene derivatives (1%) (Table 1).

Linalool (27%) and linalyl acetate (19.1%) were, also, the major components of *L. “Grosso”* EO (Table 1), and even in this case, the major detected class of compounds was oxygenated monoterpenes, representing 78.2%, besides oxygenated sesquiterpenes (13.3%), sesquiterpene hydrocarbons (7.9%), monoterpene hydrocarbons (5.7%), and other non-terpene derivatives (1%) (Table 1).

Interestingly, the chemical profile of *L. “Super”* EO showed that the main components were the oxygenated monoterpenes linalool (19.6%) and linalyl acetate (10.2%), and the monoterpene hydrocarbon terpinolene (12.7%) (Table 1). The main class of components was, also in this case, oxygenated monoterpenes, accounting for 68.7%; unlike the previously described EOs, monoterpene hydrocarbons were the second class, representing 16.9%, followed by oxygenated sesquiterpenes (11.9%), and sesquiterpene hydrocarbons (2.4%) (Table 1).

Finally, the chemical composition of the commercially available LEO was analyzed. The main compounds were linalyl acetate (36.1%) and linalool (28.6%) (Table 1). Interestingly, compounds like 2-*p*-menthen-1-ol, *cis-p*-menth-2-en-1-ol, and 1-octen-3-ol, not present in the previous three LEOs, were also identified. Once again, oxygenated monoterpenes represented the great majority of the chemical composition (87.1%), followed by sesquiterpene hydrocarbons (8.4%), monoterpene hydrocarbons (2%), other non-terpene derivatives (1.1%), oxygenated sesquiterpenes (0.7%), and phenylpropanoids (0.5%) (Table 1).

1.2 | LEOs Cytocompatibility

The cytocompatibility of *L. angustifolia*, *L. “Grosso”*, *L. “Super”* EOs, and the commercially available LEO was established on the SH-SY5Y cell line by performing a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cells were treated with the four LEOs at concentrations ranging from 0.0001% to 0.2% for 48 h. Treatments were considered cytotoxic if they reduced cell viability by more than 80%. As shown in Figures 1A,B, the treatment with *L. angustifolia* and *L. “Grosso”* EOs resulted to be cytotoxic at concentrations above 0.1%; considering *L. EO*, only the treatment with the 0.2% concentration significantly reduced cell viability below 80% (Figure 1C). No

TABLE 1 | Chemical composition of *L. angustifolia*, *L. "Grosso"*, *L. "Super"*, and commercially available lavender essential oils (LEOs).

Compounds	Class	Linear Retention index	Relative abundance (%) $\pm$ Standard deviation			Commercially available LEO
			<i>L. angustifolia</i>	<i>L. "Grosso"</i>	<i>L. "Super"</i>	
α -pinene	mh	933	0.2 $\pm$ 0.00	0.3 $\pm$ 0.01	0.5 $\pm$ 0.16	—
Camphene	mh	948	0.2 $\pm$ 0.00	0.2 $\pm$ 0.01	0.3 $\pm$ 0.11	—
1-octen-3-ol	nt	976	—	—	—	0.2 $\pm$ 0.01
β -pinene	mh	977	0.2 $\pm$ 0.01	0.3 $\pm$ 0.01	0.4 $\pm$ 0.17	—
3-octanone	nt	985	0.2 $\pm$ 0.04	—	—	0.2 $\pm$ 0.00
Myrcene	mh	991	0.5 $\pm$ 0.00	0.4 $\pm$ 0.01	0.5 $\pm$ 0.15	0.2 $\pm$ 0.00
δ -3-carene	mh	1011	0.1 $\pm$ 0.01	—	—	0.2 $\pm$ 0.00
1-hexyl acetate	nt	1012	0.3 $\pm$ 0.02	0.2 $\pm$ 0.02	—	—
p-cymene	mh	1024	0.1 $\pm$ 0.01	—	0.2 $\pm$ 0.23	—
Limonene	mh	1029	0.5 $\pm$ 0.01	0.5 $\pm$ 0.04	0.7 $\pm$ 0.09	0.4 $\pm$ 0.03
1,8-cineole	om	1031	1.6 $\pm$ 0.09	3.9 $\pm$ 0.05	6.2 $\pm$ 1.46	4.3 $\pm$ 0.11
(Z)- β -ocimene	mh	1036	2.0 $\pm$ 0.22	0.5 $\pm$ 0.01	0.8 $\pm$ 0.36	1 $\pm$ 0.04
(E)- β -ocimene	mh	1047	2.1 $\pm$ 0.09	0.5 $\pm$ 0.04	0.3 $\pm$ 0.10	0.3 $\pm$ 0.00
γ -terpinene	mh	1058	—	—	0.4 $\pm$ 0.11	—
cis-linalool oxide (furanoid)	om	1072	0.2 $\pm$ 0.03	0.1 $\pm$ 0.00	—	—
Terpinolene	mh	1089	0.3 $\pm$ 0.03	0.3 $\pm$ 0.00	12.7 $\pm$ 12.41	—
Linalool	om	1101	21.8 $\pm$ 1.15	27.0 $\pm$ 0.42	19.6 $\pm$ 9.45	28.6 $\pm$ 0.58
1-octen-3-yl acetate	nt	1113	0.5 $\pm$ 0.05	0.1 $\pm$ 0.01	—	0.3 $\pm$ 0.01
cis-p-menth-2-en-1-ol	om	1122	—	—	—	0.2 $\pm$ 0.01
2-p-Menthen-1-ol	om	1126	—	—	—	0.3 $\pm$ 0.01
Camphor	om	1145	0.5 $\pm$ 0.04	6.4 $\pm$ 0.05	5.9 $\pm$ 1.89	4.4 $\pm$ 0.02
Hexyl isobutyrate	nt	1149	—	0.2 $\pm$ 0.03	—	—
Isoborneol	om	1157	—	—	—	1.9 $\pm$ 0.03
Borneol	om	1165	2.1 $\pm$ 0.22	4.7 $\pm$ 0.11	3.3 $\pm$ 0.97	4.7 $\pm$ 0.04
Lavandulol	om	1166	0.8 $\pm$ 0.1	—	—	—
4-terpineol	om	1177	4.4 $\pm$ 0.36	4.3 $\pm$ 0.12	3.6 $\pm$ 0.44	0.2 $\pm$ 0.00
Cryptone	om	1186	0.5 $\pm$ 0.02	—	—	0.1 $\pm$ 0.00
α -terpineol	om	1191	4.9 $\pm$ 0.18	3.7 $\pm$ 0.34	2.6 $\pm$ 2.20	1.7 $\pm$ 0.02
Hexyl butyrate	nt	1193	—	—	—	0.4 $\pm$ 0.01
Nerol	om	1228	0.7 $\pm$ 0.08	0.5 $\pm$ 0.00	0.7 $\pm$ 0.23	—
Cumin aldehyde	om	1239	0.1 $\pm$ 0.01	—	—	—
Geraniol	om	1254	1.9 $\pm$ 0.23	1.3 $\pm$ 0.17	9.5 $\pm$ 6.72	—
Linalyl acetate	om	1257	20.1 $\pm$ 0.24	19.1 $\pm$ 0.23	10.2 $\pm$ 5.36	36.1 $\pm$ 0.03
Bornyl acetate	om	1286	0.2 $\pm$ 0.04	—	—	—
Lavandulyl acetate	om	1292	6.4 $\pm$ 0.28	3.0 $\pm$ 0.11	2.7 $\pm$ 1.69	2.9 $\pm$ 0.04
Hexyl tiglate	nt	1332	—	0.3 $\pm$ 0.05	—	—
Neryl acetate	om	1365	1.5 $\pm$ 0.04	1.1 $\pm$ 0.00	1.6 $\pm$ 0.64	0.3 $\pm$ 0.01
Geranyl acetate	om	1385	3.1 $\pm$ 0.13	2.5 $\pm$ 0.09	2.1 $\pm$ 0.10	1.5 $\pm$ 0.07
β -caryophyllene	sh	1419	5.5 $\pm$ 0.10	1.4 $\pm$ 0.07	0.6 $\pm$ 0.49	7.2 $\pm$ 0.37

(Continues)

TABLE 1 | (Continued)

Compounds	Class	Linear Retention index	Relative abundance (%) $\pm$ Standard deviation			
			<i>L. angustifolia</i>	<i>L. "Grosso"</i>	<i>L. "Super"</i>	Commercially available LEO
<i>trans</i> - α -bergamotene	sh	1436	—	—	—	0.2 $\pm$ 0.00
α -humulene	sh	1453	0.2 $\pm$ 0.01	—	—	—
(E)- β -farnesene	sh	1458	1.1 $\pm$ 0.04	0.9 $\pm$ 0.01	0.5 $\pm$ 0.12	0.5 $\pm$ 0.03
Lavandulyl butyrate	om	1460	—	—	0.1 $\pm$ 0.02	—
Germacrene D	sh	1481	0.5 $\pm$ 0.07	0.7 $\pm$ 0.00	0.7 $\pm$ 0.20	0.3 $\pm$ 0.02
Lavandulyl isovalerate	om	1508	—	0.4 $\pm$ 0.01	0.4 $\pm$ 0.05	—
<i>trans</i> - γ -cadinene	sh	1514	0.7 $\pm$ 0.04	—	0.5 $\pm$ 0.13	0.3 $\pm$ 0.04
Caryophyllene oxide	os	1582	5.3 $\pm$ 0.62	0.4 $\pm$ 0.01	0.5 $\pm$ 0.09	0.4 $\pm$ 0.05
1,10-di-epi-cubenol	os	1615	0.4 $\pm$ 0.00	—	0.3 $\pm$ 0.01	—
T-cadinol	os	1641	6.8 $\pm$ 0.41	2.2 $\pm$ 0.10	3.4 $\pm$ 0.16	0.3 $\pm$ 0.03
α -Cadinol	os	1654	—	—	0.1 $\pm$ 0.02	—
14-hydroxy-9-epi-(E)-caryophyllene	os	1670	0.3 $\pm$ 0.04	—	—	—
α -bisabolol	os	1685	—	11.5 $\pm$ 0.21	7.3 $\pm$ 1.03	—
<i>cis</i> -14-nor-murol-5-en-4-one	os	1686	0.5 $\pm$ 0.02	—	—	—
(Z,E)-farnesol	os	1689	—	0.5 $\pm$ 0.04	0.4 $\pm$ 0.03	—
Benzyl benzoate	nt	1763	0.1 $\pm$ 0.01	—	—	—
Total identified (%)			98.9	99.9	99.9	99.3
Oxygenated monoterpenes			71.0	78.2	68.7	87.1
Sesquiterpenes hydrocarbons			7.9	2.9	2.4	8.4
Monoterpenes hydrocarbons			5.7	2.9	16.9	2.0
Oxygenated sesquiterpene			13.3	14.5	11.9	0.7
Non-terpene derivatives			1.0	1.4	—	1.1

Abbreviations: mh = monoterpene hydrocarbons, nt = non-terpene derivatives, om = oxygenated monoterpenes, os = oxygenates sesquiterpenes, sh = sesquiterpene hydrocarbons.

cytotoxicity was detected for the treatment with the commercially available LEO (Figure 1D).

2.3 | LEOs Anti-Oxidative Potential

In order to test the anti-oxidative potential of the three hydrodistilled LEOs and to compare them with the commercial LEO, the SH-SY5Y cell line was initially exposed to each of the four LEOs at concentrations ranging from 0.0001% to 0.2%, for 24 h. Subsequently, cells were treated with H₂O₂ 0.2 mM, used as an anoxidative stimulus. After a further 24 h, cell viability was determined by means of an MTT assay. Pre-treatment with *L.*

angustifolia (Figure 2A), *L.* "Grosso" (Figure 2B), and *L.* "Super" (Figure 2C) EOs at concentrations ranging from 0.0001% to 0.01% was able to significantly increase cell survival compared to the H₂O₂ treated group. Interestingly, the commercial LEO did not show a significant protective effect against the H₂O₂-induced oxidative stress at any concentration (Figure 2D).

To deepen the understanding of the oxidative protection mechanism, SH-SY5Y cells were treated with *L.* "Grosso" and *L.* "Super" EOs at 0.0001%, the lowest dose that resulted in protection against the H₂O₂ insult, for 12 h. The expression levels of two genes with antioxidant functions, namely NQO1 and CYT B, were assessed. This setup reflects the cellular conditions after the treatment

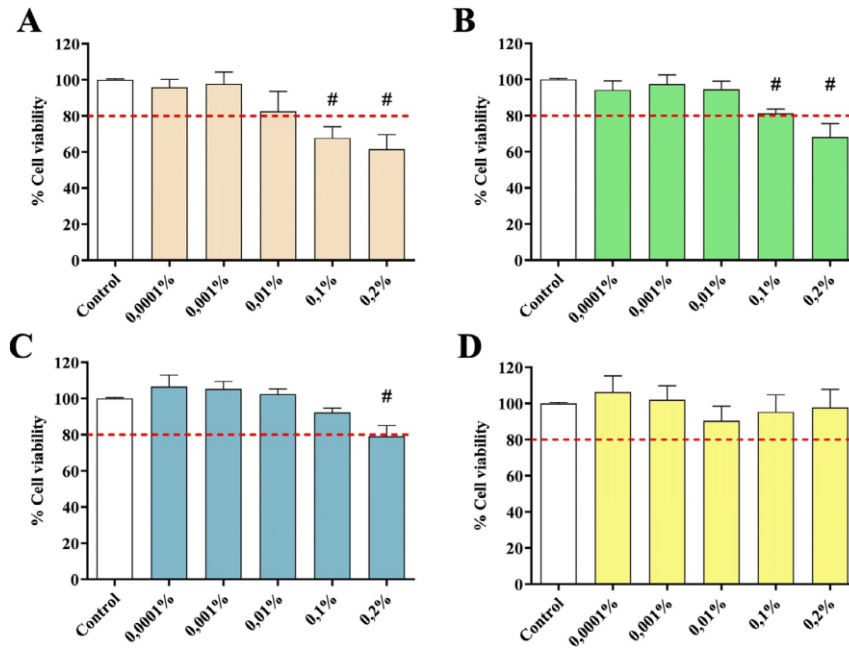


FIGURE 1 | SH-SY5Y cell line viability after treatment with *L. angustifolia* essential oil (A); *L. 'Grosso'* essential oil (B); *L. 'Super'* essential oil (C); commercial lavender essential oil (D). Data are presented as a percentage of cell viability compared to untreated cells used as a control. The red dotted line indicates the percentage of cell viability under which the treatments were considered cytotoxic. Data are shown as mean $\pm$ SEM of three replicates: # $p < 0.05$ versus the control group.

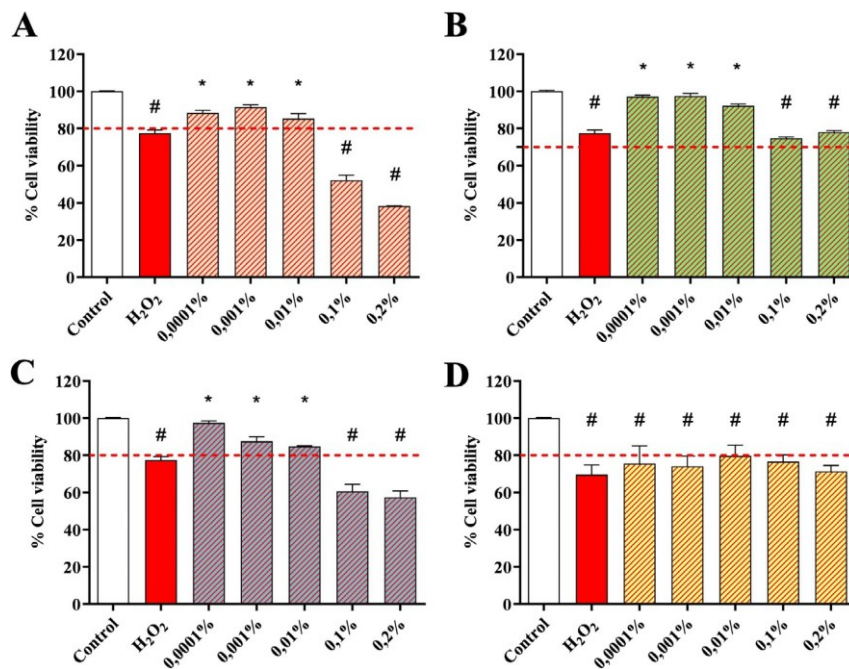


FIGURE 2 | The anti-oxidative potential of *L. angustifolia* essential oil (A), *L. 'Grosso'* essential oil (B), *L. 'Super'* essential oil (C), and commercial lavender essential oil (D) on SH-SY5Y exposed to H₂O₂, as an oxidative stimulus. Data are presented as a percentage of cell viability compared to untreated cells used as a control. The red dotted line indicates the percentage of cell viability under which the treatments were considered cytotoxic. Data are shown as mean $\pm$ SEM of three replicates: * $p < 0.05$ vs H₂O₂; # $p < 0.05$ vs. control group.

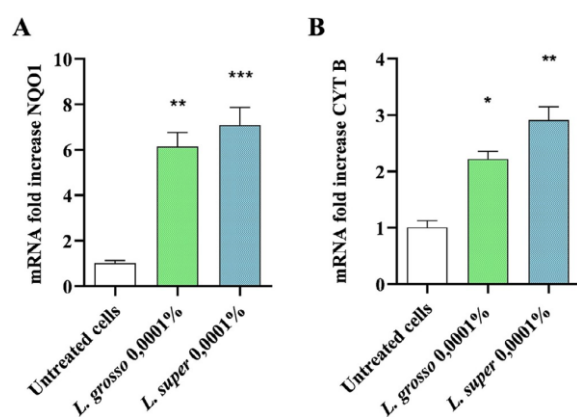


FIGURE 3 *L. "Grosso"* and *L. "Super"* essential oils increased the expression of two genes involved in antioxidant protection, NQO1 (A) and CYT B (B). SH-SY5Y cells were treated with 0.0001% *L. "Grosso"* and *L. "Super"* essential oils for 12 h. The NQO1 and CYT B mRNA expression levels were then determined by real-time polymerase chain reaction (PCR). glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normalizer. Data are representative of three replicates and shown as mean $\pm$ SEM; * p < 0.05; ** p < 0.01; *** p < 0.001 versus control.

with the LEOs and prior to H₂O₂ exposure, providing insight

2 2

into how the EOs prime the cells for oxidative stress defense. As depicted in Figure 3, NQO1 and CYT B genes were not expressed in untreated cells, whereas treatment with 0.0001% *L. "Super"* and *L. "Grosso"* EOs resulted in a significant upregulation of both NQO1 (Figure 3A) and CYT B (Figure 3B). Additionally, *L. "Super"* demonstrated a more pronounced effect compared to *L. "Grosso"*.

1 | Discussion

L. angustifolia, *L. "Grosso"*, and *L. "Super"* are plants belonging to the *Lavandula* L. genus and are mainly distributed in the Mediterranean area [16]. LEOs are characterized by unique aroma and therapeutic properties, including antimicrobial, anti-inflammatory, and antioxidant abilities. Considering these aspects, lavender extracts and EOs have been used in traditional medicine since the Middle Ages [17]. Nowadays, given their wide range of uses, many LEOs are commercially available worldwide; however, their composition and properties, which can be influenced by their production, are not always assessed. In this study, three LEOs derived from *L. angustifolia*, *L. "Grosso"*, and *L. "Super"*, obtained by hydrodistillation, as well as a commercially available LEO, were compared for their phytochemical composition and biological properties. The chemical profiles of the three LEOs extracted via hydrodistillation resulted to be similar to the commercially available LEO in terms of main constituents; linalool and linalyl acetate represented the main identified compounds, in accordance also with the literature [18], while oxygenated monoterpenes were the prevalent class of compounds [11, 19–21]. Although the commercial LEO was characterized by a higher content of linalyl acetate, linalool, and beta-caryophyllene compared to the ones obtained through hydrodistillation, it showed reduced levels of α -terpineol and

4-terpineol and the total absence of terpinolene and α -pinene. These compounds are known to be biologically active; specifically, α -terpineol has been shown to have anti-inflammatory properties [22], while terpinolene has antioxidant activity [23]; α -pinene and 4-terpineol are characterized by anti-inflammatory and antioxidant properties [24–26]. This synergistic effect among the various molecules present in EOs has been extensively studied in the literature [27]. Findings indicate that the overall effect is attributed to the entire phytocomplex and that even molecules present in small amounts play a significant role in biological activity [28]. This concept of the phytocomplex [29] helps explain why commercial EO, despite being rich in major components such as linalool and linalyl acetate, does not exhibit the same biological activity observed in hydrodistilled EOs.

Furthermore, it is worth mentioning that in the commercial LEO were detected traces of chemicals not found in any of the other three LEOs, like 2-*p*-menthen-1-ol, *cis-p*-menth-2-en-1-ol, and 1-octen-3-ol. Among these molecules, only 1-octen-3-ol has been reported in the literature and it has been associated with pro-inflammatory effects and target mitochondria, with alterations in the bioenergetic and in the levels of antioxidant enzymes in *Drosophila melanogaster* [30, 31].

To evaluate their antioxidant effect, the four LEOs were tested in vitro on a human neuroblastoma cell line (SH-SY5Y) at concentrations ranging from 0.0001% and 0.2%. The LEOs obtained through hydrodistillation did not show any cytotoxic effect, except at the highest concentrations. The commercial LEO did not reduce cell viability at any of the considered concentrations, as well. Regarding the antioxidant properties of the EOs, the hydrodistilled LEOs had a significant protective effect against the H₂O₂-induced oxidative stimulus at concentrations ranging from 0.0001% to 0.01%.

However, the commercially available LEO did not show a protective effect at any of the used dosages. To have an in-depth understanding of the antioxidant properties of the four LEOs, the expression levels of NQO1, involved in oxidative protection [32], and CYT B, a component of respiratory chain complex III [33], were evaluated in the SH-SY5Y cell line after a 12-h exposure to 0.0001% *L. "Grosso"* and *L. "Super"* EOs. These EOs were selected based on their low cytotoxicity and because only a few studies present in the literature focus on them. The results showed that both EOs significantly increased the expression levels of both genes compared to the non-treated control, with *L. "Super"* outperforming *L. "Grosso"*. The results of the antioxidant activities obtained are congruent with other studies, which show *L. angustifolia*, *L. "Grosso"*, and *L. "Super"* extracts to have a significant scavenger-like effect against free radicals, as tested with different chemical assays [5, 12, 34].

Several studies have also emphasized the antioxidant potential of *L. angustifolia* and lavandin EOs, demonstrating that their activity is influenced by variations in chemical composition as well as environmental factors, including soil composition, pH, and geographic location [35]. Specifically, Gavrić et al. [36] and Dětár et al. [37] demonstrate that the antioxidant capacity of *L. angustifolia* and lavandin EOs can vary depending on the cultivar and the content of metabolites, such as flavonoids and, more broadly, phenolic compounds.

In conclusion, the EOs of *L. angustifolia*, *L. "Grosso"*, and *L. "Super"* all showed to have significant protection against oxidative stress caused by the H₂O₂ treatment; since the main compounds found in LEOs are known for their antioxidant properties [38, 39], the effect previously mentioned could be attribute to the cooperative synergistic effects of the chemical compounds in the EOs, as also reported in the literature [40]. The ratios and interactions of the compounds enable them to act as scavengers, intercepting free radicals. However, this ability is absent in the commercially purchased LEO, which showed no protective effects at any of the tested concentrations. This lack of activity could be attributed to the different composition of the EO, as evidenced by the GS-MS results. We hypothesize that decreased levels of α -terpineol and 4-terpineol, the absence of terpinolene and α -pinene, as well as the presence of additive compounds influence the antioxidant effects of the mixture, as also reported by Hosseini et al. [41]. These chemicals that are added during the manufacturing process can improve and exalt the fragrance of the EO, as well as its conservation for longer periods of time at the expense of their biological activities and natural genuineness, resulting in the sophistication of the product.

1 | Materials and Methods

1.1 | Plant Materials and EOs

L. angustifolia, *L. "Grosso"*, and *L. "Super"* EOs were kindly provided by the University of Pisa. The EOs were obtained from plants cultivated during the summer of 2018 in Tuscany (Bibbona, Livorno, Italy; 43°27'N, 10°58'E, 60 m a.s.l.). The commercially available EO is made by the "VicTsing" company and was bought by an online retailer. A sample of each EO was preserved and cataloged at the Department of Molecular and Translational Medicine, Division of Pharmacology, University of Brescia.

1.2 | Hydrodistillation

To obtain the EOs, 50 g of air-dried aerial parts of each lavender were hydrodistilled by a Clevenger-type apparatus for 2 h, as reported in the European Pharmacopoeia. The extracted oils were then conserved at 4°C and maintained far from any light sources until analyses.

1.3 | GC-MS Analysis

The chemical analysis was performed, in triplicates, by GC-MS on EOs diluted to 5% with HPLC-grade *n*-hexane. The GC-MS analysis was carried out with an Agilent 7890B gas chromatograph paired with an Agilent 5977B single quadrupole mass detector (Agilent Technologies, Inc., Santa Clara, CA) furnished with an Agilent HP-5MS capillary column (30m x 0,25 mm; coating thickness 0,25 μ m).

The analytical conditions were set as follows: the injector and transfer line temperatures at 220 and 240°C, respectively, oven temperature progression from 60 to 240°C at a 3°C/min rate, helium as carrier gas flowing at 1 mL/min with a split ratio of 1:25. The acquisition operated in full scan mode within the

range of 30–300 *m/z* at 1,0 second scan time. The compound identification was based on the comparison of the sample's retention time and indices with authenticated samples and a series of *n*-hydrocarbons. Additionally, computer-assisted matching was used against both commercial [42] and lab-developed mass spectra libraries, comprised of pure substances, known EO constituents, and MS literature data [43–46].

1.4 | Cell Culture and Treatments

The SH-SY5Y cell line (human neuroblastoma cells) was cultured in cell culture medium composed of DMEM and Ham's F12 medium 1:1, supplemented with 10% fetal bovine serum, 0.5% L-glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin, at 37°C and 5% CO₂. Cells were seeded at a density of 2×10^4 cells/well in 96-well plates. For the cytotoxicity test, cells were treated with the four LEOs at different concentrations (0.2%, 0.1%, 0.01%, 0.001%, and 0.0001%) for 48 h. Untreated cells were used as control.

The antioxidant activity was then tested by treating SH-SY5Y cells with the four LEOs at the concentrations previously used for 24 h; cells were then exposed to the oxidant stimulus H₂O₂ 0.2 mM for a further 24 h. Untreated cells and cells treated solely with H₂O₂ 0.2 mM were used as controls.

1.5 | Cell Viability

After the treatments, the viability was assessed by incubating SH-SY5Y cells with 500 mg/mL of MTT for 90 min at 37°C; they were then lysed with DMSO and the absorbance was measured at 570 nm using an EnSight Multi-mode Plate Reader (PerkinElmer, Waltham, MA, USA).

Data were expressed as a percentage of cell viability over the control group. The results were expressed as mean $\pm$ SEM.

1.6 | Quantitative Real-Time Polymerase Chain Reaction

To deepen the antioxidant potential of the two more promising LEOs, SH-SY5Y cells were treated with *L. "Grosso"* and *L. "Super"* EOs at 0.0001% for 12 h. The expression of two genes involved in cellular protection against ROS, namely NQO1 (NAD(P)H quinone dehydrogenase 1) and CYT B (cytochrome B), was then evaluated. Specifically, cells were seeded at a density of 9×10^4 cells/well in 24-well plates, and total RNA was extracted following the TRIzol reagent protocol. Subsequently, 2 μ g of total mRNA were reverse-transcribed using a reaction mix consisting of 5 mM oligo (dT) primers, 10 U/mL M-MLV reverse transcriptase, 1 mM dNTPs, 1 U/mL RNase inhibitor, 1X RT buffer. The reverse transcription reaction was performed in three steps: 10 min at 70°C, 2 min at 4°C, and 60 min at 37°C. ViiA7 Real-Time Polymerase Chain Reaction (RT-PCR) Detection System (Applied Biosystems, Foster City, CA, USA) was used for Real-time PCR, for which were used 6 μ L of SYBR Green Master Mix, 6 pmol of forward and reverse primers, and 2 μ L of cDNA. The Real-time PCR reaction steps were 10 min at 95°C, 40 cycles

at 95°C for 15 s each, and finally at 60°C for 60 s. Primers sequences were CYT B (F: CCAGCTACCATGTCCAGAT, R: TATGCCAGCTTCCGACTCTT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (F: GAGTCAACGGATTGGTCGT, R: TTGATTTTGGAGGGA TCTCG). NQO1 QuantiTect primers were provided by Qiagen (Qiagen, Hilden, Germany). GAPDH was used as an endogenous reference. Quantitative RT-PCR was performed with the ViiA7 RT-PCR System (Applied Biosystems, Foster City, CA, USA) using the iQ SYBR Green Supermix method (Bio-Rad Laboratories, Richmond, CA, USA) according to the manufacturer's instructions. The comparative Ct method was used for relative quantification. Data were presented as the fold change in target gene expression. GAPDH was used as a calibrator. The comparative Ct method was used for relative quantification. Data were presented as the fold change in target gene expression.

1.1 | Statistical Analysis

The results are presented as mean $\pm$ standard error mean of three independent replicates. Data were analyzed using a one-way analysis of variance test, followed by Dunnett's test for multiple comparisons. A p -value < 0.05 was considered significant. Statistical analyses were performed using GraphPad Prism 9.0.0 (GraphPad Prism Software, San Diego, CA, USA).

Author Contributions

Andrea Mastinu, Giulia Abate, Luisa Pistelli, and Guido Flamini were responsible for conceptualization. Ylenia Pieracci and Gianluca Angius contributed to the data collection. Eileen Mac Sweeney, Ylenia Pieracci, Vlad Sebastian Popescu, and Gianluca Angius conducted the analysis and produced the results and figures. Eileen Mac Sweeney, Vlad Sebastian Popescu, and Gianluca Angius drafted the manuscript. All the authors have read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Both studies regarding the biological activity evaluation of *C. monspeliensis* extracts and *Lavandula* genus essential oils will be further investigated to validate their bioactivity by comparing them to reference compounds, e.g. dexamethasone and vitamin E. More analysis will be also conducted to better understand the pathways through which *C. monspeliensis* and *Lavandula* genus extracts exert their anti-inflammatory and antioxidant action. We hypothesised that one of the pathways that might be involved is NF- κ B, which is involved in both the production of pro-inflammatory cytokines and regulation of pro-oxidative genes.

Also, single molecules, thought to be responsible for the pharmacological activities, will be tested and compared to the whole extracts.

Another limitation of these studies is that natural products have not been tested *in vivo*, yet. To better determine their potential use as neuroprotective agents, they will be evaluated in a preclinical model of Alzheimer's disease. Analysis will include behavioural, biochemical and molecular tests to determine their potential in preventing or decreasing neurodegeneration.

Chapter 2

1. Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and represents the prevalent form of dementia, accounting for 60-80% of all cases, with a higher prevalence in women [102], [103], [104], [105]. The annual incidence accounts for 1275 new cases per 100.000 persons and are affected 10.8% of people aged 65 and older [106], [107]. It is estimated that the number of people affected by AD will progressively increase, reaching 13.8 million by 2060, due to population aging [104]. Clinical hallmarks include progressive cognitive decline, severe memory loss, personality or behavioural changes, and neuropsychiatric symptoms [106], [107]. Moreover, AD patients are characterized by neurons death related to brain accumulation of amyloid- β ($A\beta$) plaques, neurofibrillary tangles (NFTs) and conditions including neuroinflammation, synaptic and mitochondrial dysfunction [106].

Aging is the major risk factor for AD development [106]. The pathology is typically divided in familial early onset AD (EOAD), representing 1–5% of AD cases with patients manifesting symptoms typically in their 40s and 50s, and sporadic late onset AD (LOAD), which accounts for more than 95% of the cases and emerging after age 65, [103], [107]. Mutations in genes involved in proteolytic cleavage and $A\beta$ formation, namely Amyloid Precursor Protein (APP), Presenilin 1 (PSEN1), Presenilin 2 (PSEN2), are strongly associated with the pathophysiology of EOAD and are inherited in an autosomal dominant pattern, while the presence of the apolipoprotein E (APOE) isoform, APOE ϵ 4, increases the risk of developing LOAD [102], [103], [107]. Moreover, multiple risk factors have been associated with AD etiology, including cerebrovascular disease, hypertension, diabetes, obesity and smoking, as well as lower education levels, depression and limited social interaction [107], [108].

2. Pathogenic mechanisms and hallmarks of Alzheimer's disease

The pathogenesis of AD is complex and results from the combination of multiple mechanisms, that remain not fully understood. Several processes have been hypothesised to be implicated in AD onset, including cholinergic deficiency, accumulation of extracellular A β plaques, intracellular NFTs, neuroinflammation (microgliosis and astrogliosis), neuronal and synaptic loss (Figure 7) [102], [108], [109].

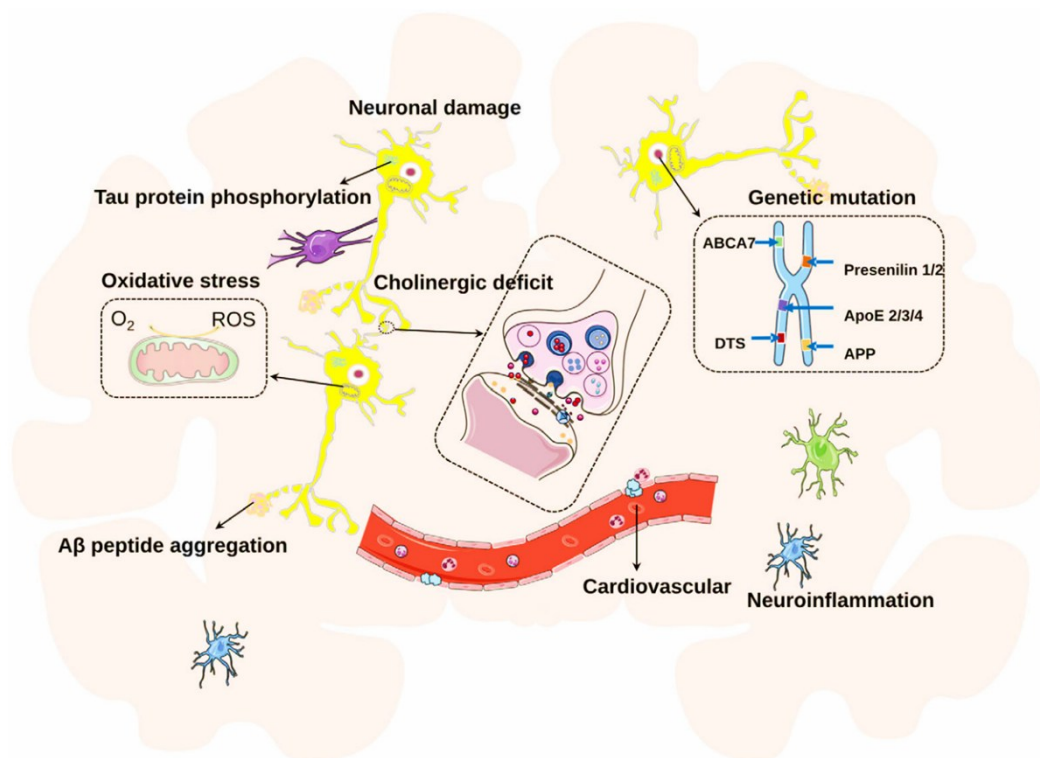


Figure 7. Schematic representation of AD pathological mechanisms. Adapted from J. Cai et al. 2025

The deposition of A β plaques is one of the main hallmarks of AD. Senile plaques consist of aggregated A β peptides formed through APP processing (Figure 8) via the amyloidogenic pathway [109], [110]. APP is a transmembrane protein mainly found in neurons and is fundamental for their plasticity and survival [103]. In physiological conditions, APP is processed by α -secretase, via the non-amyloidogenic pathway, to form soluble APP α fragments (sAPP α) and a truncated APP carboxyl-terminal fragment (α CTF or C83) [104], followed by γ -secretase cleavage of C83 to form the P3 fragment and APP intracellular domain (AICD) (Figure 8) [110]. In AD, APP follows the amyloidogenic pathway and is cleaved by β - and γ -secretase. Specifically, β -secretase leads to the production of soluble APP β fragments (sAPP β) and the carboxyl-terminal fragment (β CTF

or C99). C99 is subsequently processed by γ -secretase to obtain AICD and several A β peptides, mainly A β 40 and A β 42 [103], [111], [112], [113]. Specifically, due to its hydrophobic C-terminal [109], A β 42 is more prone to aggregate, producing oligomers, protofibrils, insoluble fibrils and, ultimately, plaques [110]. It is important to mention that neuroinflammation and oxidative stress can mediate A β plaques deposition and A β plaques themselves, besides neuronal toxicity and death, can trigger further inflammatory response, mitochondrial dysfunction and excessive ROS production, in a positive feedback loop [109], [114]. Senile plaques can be initially found in the temporobasal and frontomedial areas moving, with AD progression, to the associative neocortex, primary sensory-motor regions, medial temporal lobe and the striatum [103].

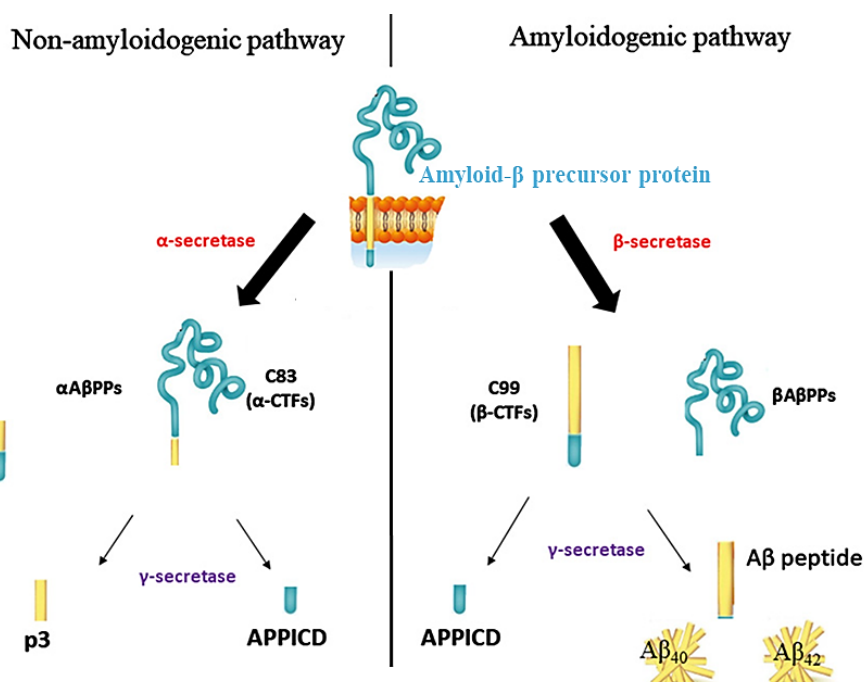


Figure 8. APP processing through the non-amyloidogenic and amyloidogenic pathways. Adapted from K. R. Kumar et al. 2022.

Tau proteins, which are microtubule-associated proteins mainly present in neurons, are responsible for microtubule stability, intracellular trafficking, as well as neuronal homeostasis and overall brain function [104], [115], [116]. In AD, Tau protein composes neurofibrillary tangles (NFTs), which are an important histopathological hallmark of the pathology. Specifically, factors including A β toxicity and neuroinflammation, cause abnormal tau phosphorylation. Hyperphosphorylated tau, in turn, aggregates to form NFTs within neurons cell body and dendrites, leading finally to neuronal death. Other

consequences of NFTs formation include the induction of further neuroinflammation, oxidative stress, and synaptic loss [104], [110].

Neuroinflammation, a chronic inflammation state in the brain, plays a central role in AD onset and progression [117]. A β and NFTs activate several immune cells, including microglia and astrocytes, and the consequent release of pro-inflammatory mediators [118]. This inflammatory response has the initial purpose of removing from the brain harmful substances [119]. In particular, activated microglia engulf senile plaques and secrete enzymes to degrade A β [120], [121]. However, when microglia are chronically exposed to A β , can become inefficient in breaking it down, leading to neuronal death and promotion of disease progression [122], [123]. Activated microglia release proinflammatory cytokines, exacerbating neuroinflammation and, in turn, accelerating A β deposition and NFTs formation [124], [125]. Astrocytes are also involved in promoting neuroinflammation and neurodegeneration due to their interaction with A β and NFTs [126].

Oxidative stress is caused by an excessive production of reactive oxygen species (ROS) due to a dysregulation in the cellular antioxidant defence mechanisms. In AD, both A β and NFTs greatly contribute to antioxidant system imbalance and, in particular, to mitochondrial dysfunction [127], [128]. Oxidative stress plays also a central role in the promotion of the amyloid pathway and in Tau hyperphosphorylation, as well as in the induction of neuroinflammation through the activation of microglia induced by ROS [129]. Thus, even oxidative stress is a central mechanism in the neurodegeneration process.

Synaptic dysfunction is closely associated with cognitive impairment in AD. In particular, the accumulation of A β plaques and NFTs lead to synaptic damage and loss due to an alteration in the release of neurotransmitters, mainly acetylcholine (ACh) and glutamate. Studies have demonstrated that the reduction of cholinergic signaling in the basal forebrain of AD patients results from a decrease in the activity of choline acetyl transferase enzyme, responsible for ACh synthesis, and contributes to memory decline and degeneration of cholinergic neurons. Glutamate, the primary excitatory neurotransmitter in the brain, acts on several post-synaptic receptors, including N-methyl-D aspartate (NMDA) receptor, which has been associated with dementia. The excessive glutamate activity, found in AD, leads to excitotoxicity mediated by neuronal calcium overload, resulting finally in neurodegeneration and memory decline [130], [131], [132], [133].

4. Current treatments for Alzheimer's disease

Despite advances in the understanding of AD pathological mechanisms, there is still no effective treatment, nor any drugs available for the prevention of this neurodegenerative disease. Currently, drugs approved by the FDA for the treatment of Alzheimer's disease (Figure 9) include the cholinesterase inhibitors donepezil, rivastigmine and galantamine; they inhibit acetylcholinesterase enzyme activity and thus increase acetylcholine levels [109], [126]. Memantine, a noncompetitive NMDA receptor antagonist, is used to block neuronal death mediated by an excessive activity of glutamate in moderate to severe AD [134], [135]. Namzaric, a combination of memantine and donepezil, is also used for the treatment of moderate to severe AD [136]. These approved drugs are effective in alleviating symptoms, however do not stop disease progression and are linked to several side effects [109]. Recently, researchers have developed novel drugs targeting other AD pathological mechanisms. In particular, Aducanumab, Lecanemab and Donanemab were approved by the FDA in 2021, 2023 and 2024, respectively. These are monoclonal antibodies selectively targeting pathogenic A β ; they are classified as disease-modifying therapies and studies have shown their efficacy in delaying cognitive decline [137], [138], [139], [140], [141]. Other innovative therapies, including drugs targeting A β accumulation (e.g. secretase inhibitors, A β aggregation inhibitors, A β vaccines), NFTs, neuroinflammation, synaptic dysfunction, are undergoing clinical trials at different stages, trying to slow down disease progression [126].

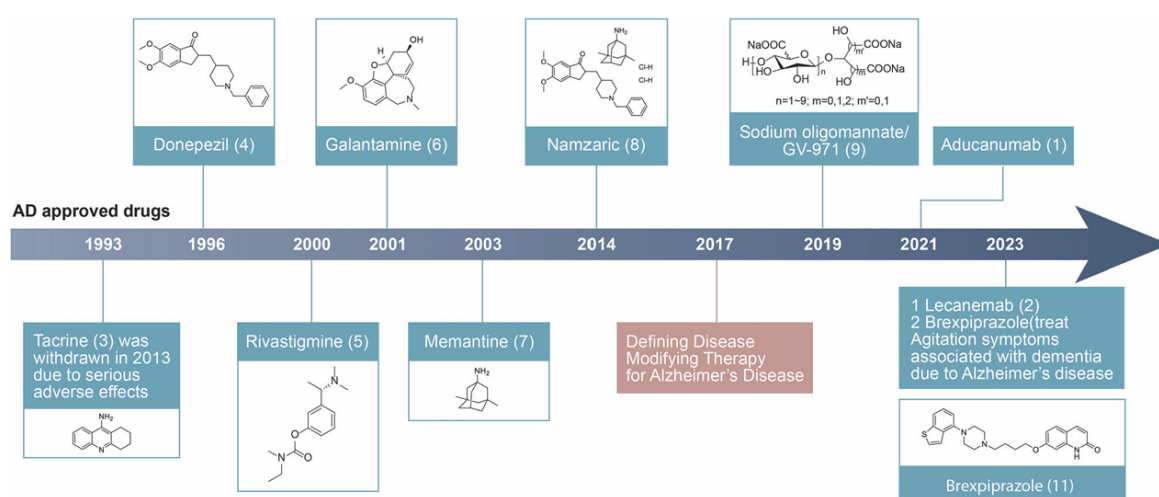


Figure 9. FDA approved drugs for Alzheimer's disease. Adapted from J. Zhang et al. 2024.

5. Senescence and SASP related markers

Senescence is one of the major drivers of aging and thus, has a central role in age-related diseases, including cancer, type 2 diabetes, cardiovascular and neurodegenerative diseases [142], [143]. Despite its importance in normal development, later in life is associated with genomic instability and telomere shortening, other factors involved in aging [142], [143], [144]. Several studies indicated that senescent cell burden increases with age [142] and, despite they represent only 4% to 15% of the total number of cells present in a tissue, significantly contribute to age-related diseases progression [145].

Specifically, cellular senescence is defined as an irreversible cell cycle arrest in G1 phase due to various stressors, including DNA damage, oxidative stress and chronic inflammation [146]. Senescence cells are also characterized by resistance to apoptosis, alteration of metabolic functions (such as increased glycolysis, reduced fatty acid oxidation) and secretion of an array of pathogenically active molecules, known as senescence-associated secretory phenotype or SASP [145], which contributes to tissue damage and chronic cell death [142]. SASP composition is heterogeneous and differ based on cell type, tissue and stressor [146]. In general, senescent cells are characterized by: increased levels of p16^{INK4}, p21 and p53 (for cell cycle arrest), increased levels of Senescence-associated β -galactosidase (SA β -gal), nuclear alterations, telomere shortening, up-regulation of pro-inflammatory cytokines (e.g. IL-1 α , IL-6, IL-1 β , TNF- α), increased ROS production and oxidative stress, reduced phagocytosis and dystrophic morphology [147], [148]. SASP main functions include: i) induction of paracrine senescence in neighboring cells and ii) autocrine maintenance of cellular senescence through a positive feedback loop mediated by growth factors, proteases, chemokines, and pro-inflammatory cytokines [145].

In particular, p16^{INK4}, p21, p53, pro-inflammatory cytokines levels and β -galactosidase activity are considered some of the main markers for senescence state characterization.

Cell cycle arrest during senescence is regulated by the activation of p53/p21 and p16^{INK4a}/Rb pathways as a DNA damage response (DDR) to factors including telomere attrition, oxidative stress, chronic inflammation and chemotherapeutic drugs. Senescence can also occur in terminally differentiated cells (e.g. neurons). Studies also suggested that p21 might be involved in the onset of senescence, while p16^{INK4a} is necessary for

maintenance of cell cycle arrest. Also, the activation and interplay of these two pathways can be cell-type specific [149], [150].

In particular, DNA damage activates the serine/threonine kinases ATM, which phosphorylates p53, leading to downstream activation of p21, hypophosphorylation of Rb and consequent cell cycle arrest. Prolonged stress can induce p16^{INK4a} activation, which inhibits cyclin-dependent Kinases 4 and 6 (CDK4 and CDK6) and, as a result, hypophosphorylated Rb sustains cell cycle arrest (Figure 10) [150].

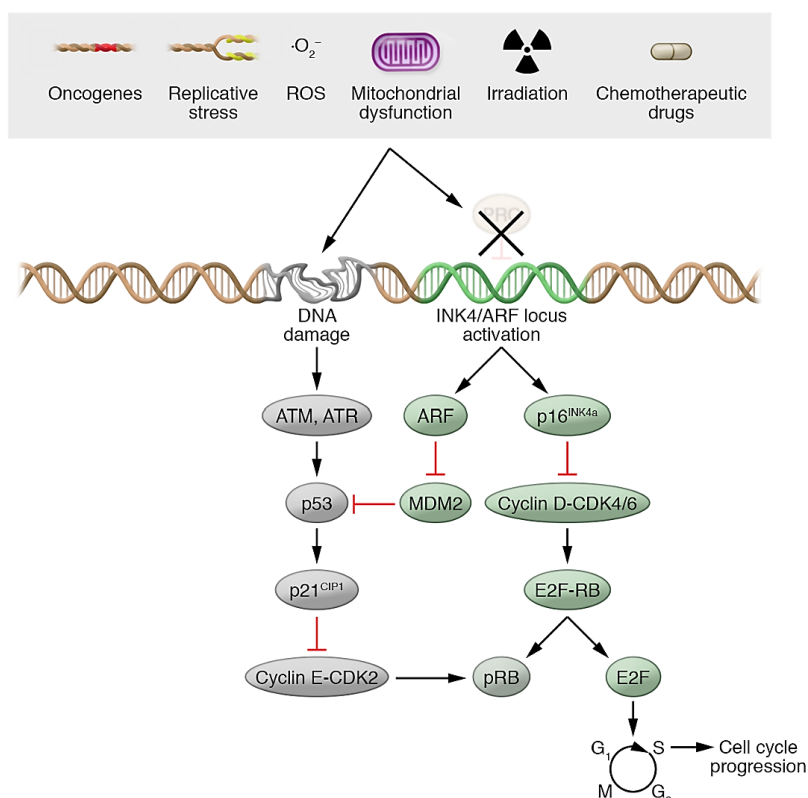


Figure 10. Molecular pathways involved in senescence cell cycle arrest. Adapted from N. Herranz and J. Gil, 2018.

As mentioned, SASP main functions include the support of autocrine senescence and induction of paracrine senescence. In addition, it also induces chronic inflammation, tumorigenesis and aging-related organ dysfunction [151]. Cytokines and chemokines belonging to SASP are mainly produced through the activation of the transcription factor NF- κ B. DDR activates associated proteins, including ATM, CHK2, ATR, GATA4 and poly(ADP-ribose) polymerase 1 (PARP1), which subsequently interact with NF- κ B for the production of SASP molecules, e.g. IL-1 α , IL-1 β , IL-6, IL-8, CXCL1, CCL2, CCL5 (Figure

11) [152]. Persistent DDR, necessary to induce senescence, can be induced by carcinogenesis, amyloid plaques and oxidative stress [148]

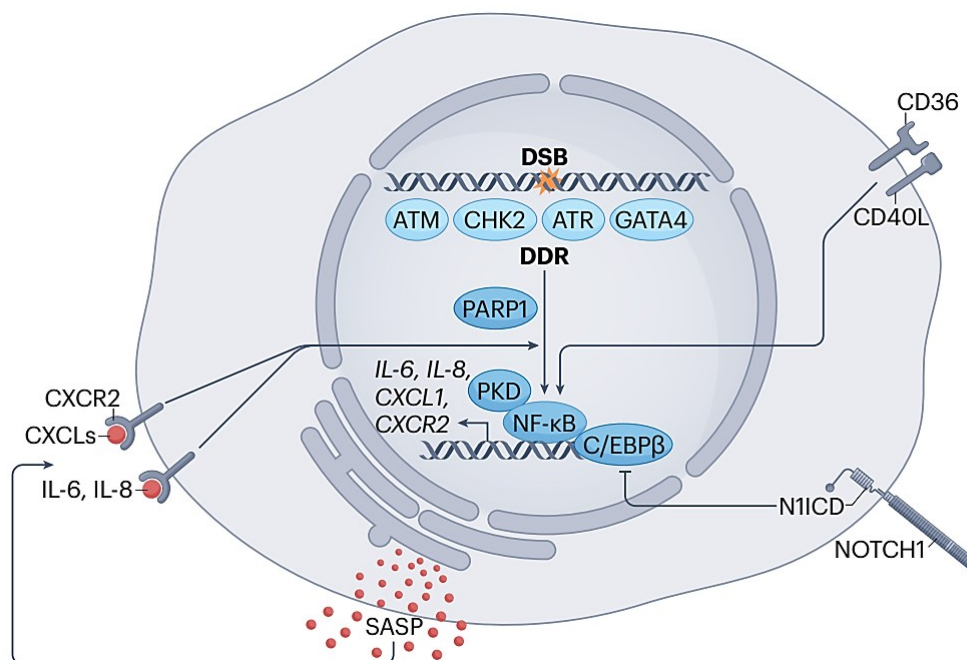


Figure 11. DDR and NF- κ B mechanism for SASP production. Adapted from B. Wang et al. 2024.

β -galactosidase is lysosomal enzyme encoded by GLB1 present in normal cells and its activity can be detected at pH 4. Since, increase of lysosomal size, number, pH neutralization and upregulation of lysosomal enzymes are well recognized characteristics of senescent cells, senescence-associated β -galactosidase (SA β -gal) is one of the main markers for senescence state evaluation. Its activity is significantly increased in senescence cells and can be specifically detected at pH 6 [150], [153].

5.1 Implications of Senescence in Alzheimer's disease

As mentioned, aging is one of the major risk factors for AD and is also tightly connected with senescence. Despite the interplay between neurodegeneration and senescence has been known for years, the implications of cellular senescence in AD development and progression have not been fully understood yet. It is known that AD brains present typical factors for senescence induction, including telomere shortening, cells hyperproliferation around plaques, mitochondrial dysfunction and chronic inflammation, leading to DNA double-strand breaks (DSBs) [148]. On one hand, increasing evidence have

showed that A β and NFTs are potent inducers of cell senescence [154][155], [156]. On the other hand, aging-induced senescence can promote a pro-inflammatory environment for AD development and progression [148].

It is hypothesised that, pro-inflammatory molecules can enter the brain thanks to leakage of the blood–brain barrier (BBB) caused by aged senescent vascular cells (endothelial cells and pericytes). Moreover, the interaction of A β with neurons and oligodendrocyte progenitor cells (OPCs) can trigger a senescence-like phenotype, which in turn promote further inflammation in the brain. In neurons, oxidative stress, inflammation, and DNA damage induce tau hyperphosphorylation. Further, senescent neurons, OPCs and chronic inflammation can trigger paracrine senescence in microglia and astrocytes, exacerbating AD pathology. In particular, senescent microglia become unable to phagocytose hp-tau neurons and A β aggregates, promoting paracrine senescence, senile plaques formation and neurodegeneration (Figure 12) [148].

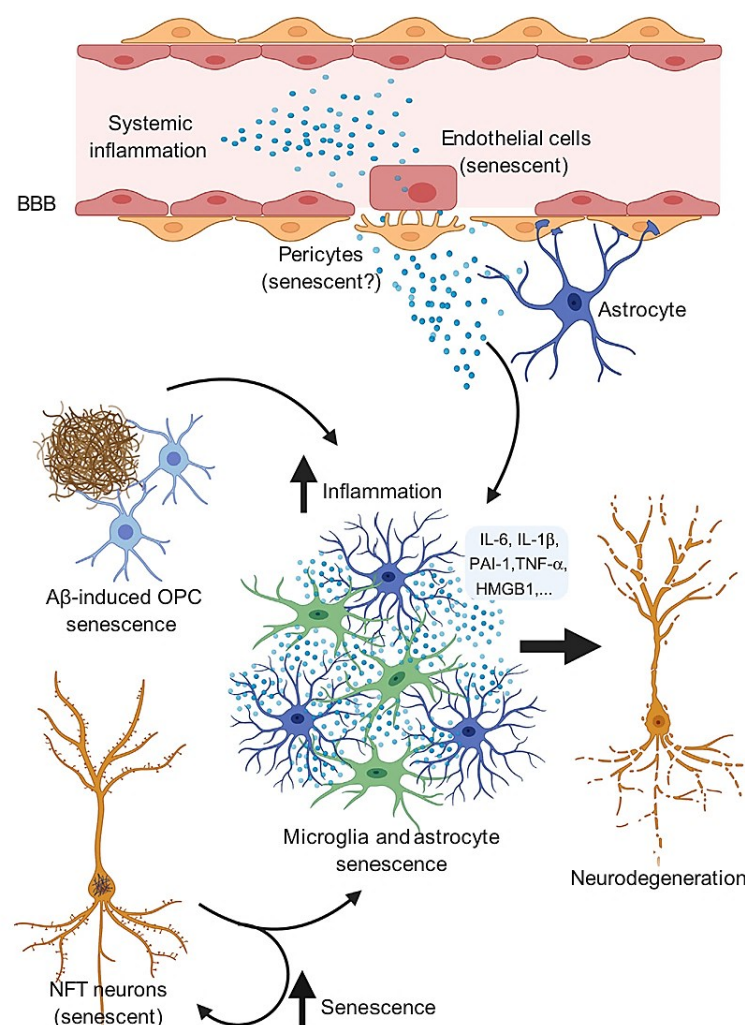


Figure 12. Senescence role in Alzheimer's disease (AD). Adapted from A. Guerrero et al. 2021.

6. Our contribution: characterization of senescence in the mouse model of Alzheimer's Disease *App*^{NL-G-F}

Alzheimer's disease (AD) is the fifth-leading cause of death for people over 65 years of age. It has been estimated that more than 70 million individuals worldwide will be diagnosed with dementia by 2030, of which AD represents 60-80% of cases [148]. This increasing incidence will represent a major challenge for health care systems and families [145]. To date, AD therapies act on symptoms and no drugs are available for disease prevention and progression [109] [126]. In this context, senescence can be a novel potential target for AD treatment [142]. Senotherapeutics target both senescent cells and the SASP and can be used to support healthy aging and to modulate disease progression [142]. To develop potential molecules targeting senescence, there is the need of preclinical AD models that recapitulate the interplay between the pathology and senescence [148].

During the second year of my PhD, I had the opportunity to carry out a 12-month research internship at Karolinska Institutet (Department of Neurobiology, Care Sciences and Society, Division of Neurogeriatrics), Stockholm, Sweden, under the supervision of Dr. Simone Tambaro (Per Nilsson group). The project focused on the characterization of senescence in the Alzheimer's disease murine model *App*^{NL-G-F}. To the best of our knowledge, no previous studies investigated senescence in this mouse model. In particular, female mice were evaluated at three different time points, 4, 12 and 24 months of age, and the expression of markers belonging to the SASP (p16, p21, p53, SA- β -gal, IL-1 β , IL-6 and cell morphology) were analysed considering both hippocampus and cortex. Also, the specific expression of p16, p21, p53 in neurons, microglia and astrocytes was further explored.

The *App*^{NL-G-F} is a quite novel mouse model, generated in 2014 by Saito and colleagues and brought to Karolinska Institutet by Dr. Nilsson, who participated to the model development. This mouse model presents a humanized amyloid precursor protein (APP) sequence, whose expression is regulated by an endogenous promoter, thus better reflecting AD progression and avoiding the artifacts obtained with murine models overexpressing APP. In the *App*^{NL-G-F} mice, APP is knocked-in and presents the Swedish (NL), Iberian (F), and Arctic (G) mutations, resulting in increased amount of total A β , augmented A β ₄₂/A β ₄₀ ratio and aggressive A β amyloidosis, respectively. These mice are characterized by A β

deposition starting at 2 months, memory impairment by 6 months, strong microgliosis and astrogliosis [157].

This research is currently under revision (major revision) in *Aging cell*:

Mac Sweeney E, Abate G, Bakker BRV, Mastinu A, Lai Y, Uberti D, Nilsson P, Tambaro S. Cell Type-Specific Expression of p16, p21, and p53 Reveals Age-Dependent Glial Senescence in the *App^{NL-G-F}* Mouse Model of Alzheimer's Disease. Under review, *Aging Cell*.

6.1 Introduction

Senescent cells, often referred to as "zombie cells", are characterized by an irreversible state of cell cycle arrest and various biological events, including telomere shortening, DNA damage, oxidative stress, mitochondrial dysfunction, and oncogene activation [158], [159]. Initially identified as a tumor-suppressive mechanism to prevent the proliferation of damaged or potentially cancerous cells, senescence also plays critical roles in processes such as tissue remodeling during development and wound healing [160], [161].

The growth arrest observed in senescent cells is associated with an upregulation of the tumour suppressors p16 and p21. p16 is a well-characterized cyclin-dependent kinase inhibitor that plays a crucial role in inducing and maintaining cell cycle arrest, and its expression is known to increase with age in various tissues, including the brain, and has been consistently observed in neurodegenerative diseases [162], [163]. Similarly, p21 is another cyclin-dependent kinase inhibitor involved in cell cycle regulation and in the establishment of senescence, often acting downstream of the tumour suppressor protein p53, which is involved in regulating several genes that play crucial roles in arresting the cell cycle and repairing damaged DNA [163]. Numerous studies have demonstrated that p53 is vital for protecting the genome in response to DNA damage [164]. Senescence process may or may not involve the activation of p53 [165]. In senescent cells, the activation of p21 and p53 can be temporary, as their protein levels generally decline once growth arrest is firmly established [149], [166], [167]. While p21 expression might decrease after its initial peak, p16 expression may increase, contributing to the maintenance of growth arrest in certain, though not all, senescent cells [149]. Senescent cells exhibit characteristic phenotypical changes, including an increase in cellular volume and heightened lysosomal activity, which is prominently detected through the expression of senescence-associated β -galactosidase

(SA- β -gal) [168]. Telomere shortening and nuclear alterations also emerge as hallmarks of cellular senescence, including persistent nuclear localization of DNA damage response proteins, such as reduction in the nuclear lamina protein Lamin B1 (LMNB1) [169] [170]. Despite these protective roles, senescence cells can have a harmful impact haltering the surrounding homeostasis, particularly in the context of aging and chronic diseases [171]. Furthermore, senescent cells secrete a pro-inflammatory cocktail of cytokines, chemokines, growth factors, and proteases collectively referred to as the senescence-associated secretory phenotype (SASP) [172] which are involved in various processes such as tissue repair while chronic secretion of SASP results in tissues damage and cell death [173].

The accumulation of senescent cells has been observed in the pathophysiology of various age-related diseases, including cardiovascular diseases, diabetes, and neurodegenerative disorders [174], [175], [176], [177]. In particular, senescence in the central nervous system (CNS) is associated with several neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease, and Huntington's disease [176]. In AD, emerging evidence suggests that senescent glial cells contribute significantly to the onset of the pathology [178]. In the brain, cellular senescence affects both dividing cells, such as glial cells (including astrocytes and microglia), and post-mitotic cells, like neurons [179]. Microglia, the resident immune cells of the brain, undergo a senescence-like state characterized by impaired phagocytic activity and an increased pro-inflammatory SASP, which can exacerbate amyloid plaque deposition [178]. Similarly, senescent astrocytes lose their neuroprotective functions, contributing to synaptic dysfunction and neuronal degeneration [180]. Neurons can also exhibit features of senescence or “neurescence”, including DNA damage and the activation of cell cycle regulators like p16 and p21 [178]. Neurescence can be associated with a loss of neuronal identity, including reduced expression of canonical neuronal genes, simplified neuritic arborization, and the retraction or loss of dendritic spines [181].

The removal of senescent cells is an emerging promising therapeutic strategy to target the aging process and help prevent or reduce aging-related diseases, such as AD [142], [156]. This therapeutic strategy consists of compounds able to eliminate senescent cells (senolytics) or modulate the SASP (senomorphics) in order to reduce neuroinflammation and restore tissue homeostasis [156]. However, for these therapeutic strategies to be effective, appropriate pre-clinical models of senescence related to AD with high translational value are required.

Furthermore, a significant gap remains in understanding the interplay between senescence and AD pathology. AD is primarily characterized by the early and abnormal accumulation

of amyloid-beta (A β) peptides, which leads to the formation of toxic oligomers and plaques. This initiates a pathological cascade that ultimately results in the development of neurofibrillary tangles formed from hyperphosphorylated Tau proteins [182]. This cascade contributes to widespread synaptic dysfunction, neuroinflammation, and neurodegeneration. While a link between tauopathy and the induction of senescence has been demonstrated *in vivo*, the effect of A β pathology on this process remains unclear [154] [183]. Furthermore, the precise involvement of different brain cell types in the progression of amyloid-induced senescence is poorly investigated. Addressing these knowledge gaps is critical, as it could clarify the contribution of different brain cell types to disease progression and facilitate the design of effective senescence-targeting therapies for AD.

Taking this into consideration, this study aimed to investigate the alterations in senescence markers across different stages of disease progression in the amyloidogenic amyloid precursor protein (*App*) knock-in AD mouse model *App*^{NL-G-F}, focusing on the expression of key senescence-associated markers p16, p21, and p53 in neurons, microglia, and astrocytes. The *App*^{NL-G-F} mice carry a humanized A β 42 sequence and feature the Swedish (NL), Iberian (F), and Arctic (G) mutations in the amyloid precursor protein (APP). These mutations lead to significant A β pathology, gliosis, and memory impairment, starting at 7 months of age, making them a widely accepted model for studying AD-related mechanisms [157]. We examined the expression levels of p16, p21, and p53, at early (4 months), advanced (12 months), and late (20–24 months) stages of A β pathology. Additionally, we investigated lysosomal dysfunction markers such as β -galactosidase and pro-inflammatory cytokines, including interleukin-1 β (IL-1 β) and interleukin-6 (IL-6). We also assessed the distribution and morphology of senescent microglia.

Our results show the accumulation of senescent glial cells in the *App*^{NL-G-F} mouse model of AD and reveal distinct, stage-dependent changes in senescence-associated markers. Notably, we did not detect any pathology-related senescence in neurons. These findings emphasize the potential of the *App*^{NL-G-F} mouse model for targeting glial senescence as a therapeutic strategy to counteract A β -driven pathology in AD.

6.2 Materials and Methods

6.2.1 Mouse models

All mice used in this study were bred in the animal facility Comparative Medicine Biomedicum (KM-B), Solna campus, Karolinska Institutet. *App*^{NL-G-F} mice (Apptm3.1Tcs/Apptm3.1Tcs) and C57BL/6J mice of the same genetic background were used as controls. Mice were caged in groups of three to five individuals, and the light-dark condition was 12-h:12-h (lights on at 7:00). The ethical permission for described animal experiments has been obtained from Stockholm animal ethical board (15758–2019 and 12570–2021). Animal housing and care were conducted under the supervision of licensed veterinarians, with technical support provided by trained personnel. All measures were taken to minimize the use of animals in accordance with ethical guidelines. Female Wilde Type (WT) and *App*^{NL-G-F} mice aged 4, 12, and 22/24 months were anesthetized with 2% isoflurane and subjected to intracardiac perfusion with PBS to remove blood. Brains were promptly extracted and divided into two hemispheres. One hemisphere was fixed in 10% formalin for subsequent morphological and immunohistochemical analyses, while the other hemisphere was dissected to isolate the hippocampus and cortex for biochemical investigations.

6.2.2 Immunofluorescence analysis

Brain tissue sections (5 μ m) of WT and *App*^{NL-G-F} mice at 4, 12 and 24 months of age were put on glass slides and deparaffinized by washing in Xylene and decreasing concentrations of ethanol (99–70%). For antigen retrieval, slides were pressure boiled in citrate buffer solution (0.1 M citric acid and 0.1 M sodium citrate) at 110°C for 5 min and then washed with tap water followed by 3x washes in Phosphate-Buffered Saline, Tween 0.05% (PBS-T) for 5 minutes each. Sections were then incubated with normal goat serum in PBS-T (Vector Laboratories, USA) for 30 min at room temperature. Samples were washed 3x in PBS-T for 5 min each with slow agitation and then incubated with the primary antibody at 4°C, overnight (Supplementary Table 1). Thereafter, sections were washed 3x in PBS-T for 5 min each with slow agitation and incubated with secondary antibodies (Supplementary Table 1) for 2 hours at room temperature. After the washing step, samples were incubated for 15 min with slow agitation with Hoechst solution, 1:500 in PBS-T. For amyloid plaque visualization, brain sections were incubated with FSB (1-Fluoro-2,5-bis(3-carboxy-4-hydroxystyryl)benzene) for 30 minutes. After the samples were washed 3x in PBS-T for 5 min each followed by mounting with PermaFluor Aqueous Mounting Medium

(ThermoScientific, USA) and kept for drying overnight. Samples were analyzed with a Zeiss AXIO Observer.Z1 Inverted Fluorescence Microscope (Carl Zeiss, Jena, Germany) in 4x, 10x and 20x magnifications and image reconstruction was performed with an LSM Zen Blue Image Examiner (Carl Zeiss S.p.A., Milan, Italy). Quantification of the immunoreactive signals was performed using ImageJ software (National Institutes of Health, MD) by counting the total number of neuronal or glial cells positive to the senescence markers in the hippocampus and in the retro splenial cortex normalized to the total number of cells.

6.2.3 *Beta-galactosidase staining*

SA- β -gal staining was performed on brain sections of WT and *App*^{NL-G-F} mouse tissues at 12 months of age. After perfusion with PBS, the mouse brains were removed, embedded with OCT solution and immediately frozen in cold 2-methylbutane for cryopreservation; tissues were then stored at -80°C. The brains were sectioned using a cryostat (Leica CM1860) with 10 μ m section thickness. SA- β -gal staining was performed according to the protocol of the Senescence- β -Galactosidase Staining Kit (#9860, Cell Signaling Technology, Danvers, MA, USA). For the double staining with Iba1, tissues were further incubated overnight with the primary antibody and 2 hours at room temperature with the secondary antibody. Images were acquired with Nikon Eclipse E800 light microscope in 10x and 20x magnifications. Quantification was performed using ImageJ software by determining the percentage of SA- β -gal positivity per ROI.

6.2.4 *RNA isolation, and qPCR*

RNA was extracted from hippocampus and cortex of WT and *App*^{NL-G-F} mouse tissues at 12 months of age using RNeasy Mini Kit (74104, Qiagen), according to the manufacturer's instructions. RNA quality (RNA integrity number) and quantity were measured by NanoDrop ND1000 Spectrophotometer (ThermoFisher). 1 μ g of RNA for each sample was reverse transcribed to cDNA. The RNA was mixed with 20 μ l of master mix and amplified by using a cycler (S1000 Thermal cycler Bio-Rad). For the RT-qPCR a total of 10 μ L reactions were run in duplicates using TaqMan Fast advanced Mastermix (ThermoFisher, Waltham, MA, USA) and StepOne Plus real-time PCR Detection System (Applied Biosystem, Waltham, MA, USA). The primers (ThermoFisher) used in this study were: p16 (Mm0049449_m1), p21 (Mm00432448_m1), p53 (Mm01731290_g1), IL-1 β (Mm00434228_m1), IL-6 (Mm00446190_m1) and β -Actin (Mm02619580_g1). The

expression levels were normalized against β -Actin. Relative quantification (RQ) for mRNA was calculated using the $\Delta\Delta$ cycle threshold ($\Delta\Delta CT$) method, with fold changes using the formula, $\Delta\Delta Ct = \Delta Ct T2 - \Delta Ct T1$.

6.2.5 ELISA

IL-1 β and IL-6 protein levels in the cortex of 12-months old WT and *App*^{NL-G-F} mice were determined by an enzyme-linked immunosorbent assay, according to the instructions provided by the manufacturer (DuoSet kits, R&D Systems, Minneapolis, USA). The results were expressed in pg/mL.

6.2.6 Skeletal and Fractal analysis

Morphological changes in microglia were analyzed using skeletal and fractal analyses, following the method established by Young and Morrison [184] on hippocampus and cortex microglia of 12-months-old WT and *App*^{NL-G-F} mice. Images at 20x were analyzed using ImageJ software, with AnalyzeSkeleton (2D/3D) and FracLac plugins. Specifically, for the Skeletal analysis three mice per genotype were used and cells Iba1⁺ were analyzed. The number of microglia endpoints/cell and microglia process length/cell were then calculated. For the Fractal analysis, four mice per genotype were analyzed, considering 10 cells p21⁺ and 10 cells p21⁻ per mice. Fractal dimension and lacunarity were then calculated.

6.2.7 Statistical analysis

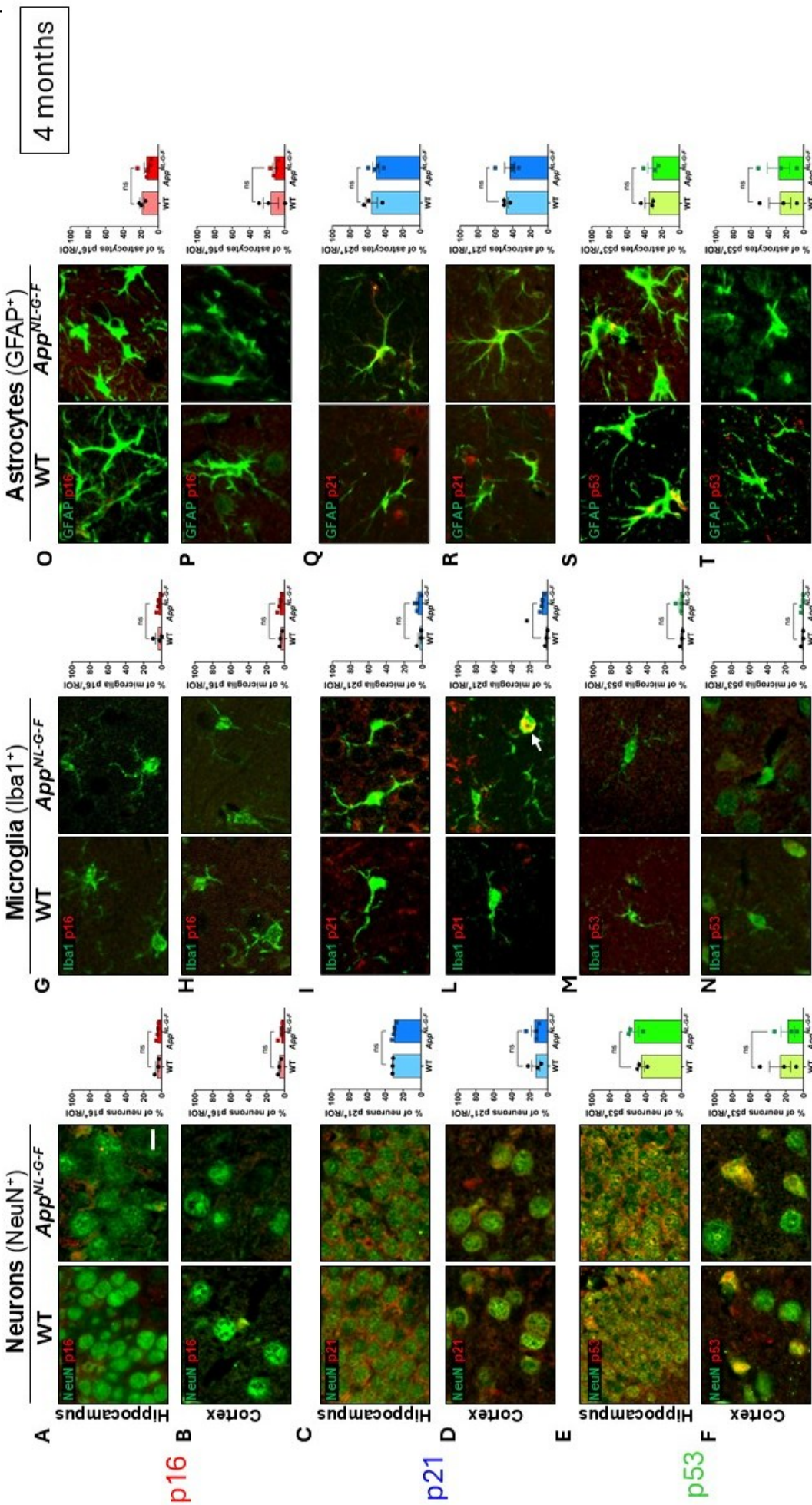
Data were expressed as mean $\pm$ standard error of the mean (SEM) and analyzed with the GraphPad Prism 10 software (GraphPad Software Inc., CA, USA). Results were analyzed by multiple t-tests or unpaired Student's t-test. For comparisons among more than two groups, one-way ANOVA or two-way ANOVA test was used followed by Tukey's multiple comparison test. Variability of the estimates was reported as the standard error of the mean (SEM). A heatmap was generated in GraphPad Prism to visualize the age-dependent changes between genotypes and cell type.

6.3 Results

6.3.1 Cell-Type-Specific Analysis Reveals Early Microglial Senescence in *App*^{NL-G-F} Mice.

To investigate cellular senescence in the context of both normal aging and AD-like pathology, we analyzed the expression of senescence markers in female WT and *App*^{NL-G-F} mice at 4, 12, and 24 months of age. We first focused on the early stage of pathology by examining 4-month-old *App*^{NL-G-F} mice, a time point at which initial A β plaque deposition has been previously reported [185]. To determine the cellular localization of senescence markers, we performed co-immunostaining for p16, p21, and p53, together with cell-type-specific markers for neurons (NeuN), microglia (Iba1), and astrocytes (GFAP) (Figure 13). No significant differences were observed between WT and *App*^{NL-G-F} mice in the number of neuronal, microglial, or astrocytic cells expressing p16 or p53 in the hippocampus nor the cortex (Figure 13). However, despite the overall low abundance of p21⁺ (see also Suppl. Table 2), a significant increase in p21⁺ microglial cells was detected in the cortex of *App*^{NL-G-F} mice compared to WT controls (Figure 13L), suggesting an early onset of microglial senescence associated with AD-like pathology. In contrast, p21 expression levels did not differ in hippocampal microglia or in neurons and astrocytes located in either the hippocampus or cortex.

Figure 13. Cell-specific immunofluorescence analysis of senescence markers p16, p21 and p53 at 4 months of age. Immunofluorescence co-labeling with neuronal (NeuN), microglial (Iba1), and astrocytic (GFAP) markers was performed to evaluate cellular senescence status. Quantification of NeuN⁺ (A-F), Iba1⁺ (G-N), GFAP⁺ (O-T) cells co-expressing p16, p21 and p53 in the hippocampus and cortex of WT and *App*^{NL-G-F} mice at 4 months of age. (L) A significant increase in p21⁺ microglial cells was observed in the cortex of *App*^{NL-G-F} mice compared to WT. Scale bars: 50 μ m. Data are presented as mean $\pm$ SEM ($n = 4-5$ per group). Statistical analysis: unpaired *t*-test. * $p < 0.05$ WT group.



6.3.2 Glial Cells Exhibit Pronounced Senescence in 12-Month-Old *App^{NL-G-F}* Mice with AD-like Pathology.

By 12 months of age, pathology in *App^{NL-G-F}* mice is markedly progressed, with extensive amyloid plaque accumulation in both the cortex and hippocampus, accompanied by pronounced microgliosis and astrogliosis [185]. To assess whether these pathological alterations are associated with cellular senescence, we next analyzed the expression and cell-type distribution of the senescence markers p16, p21, and p53 by immunofluorescence, using NeuN, Iba1, and GFAP to identify neurons, microglia, and astrocytes, respectively.

In neurons, p16 immunoreactivity was detected only in a small subset of cells, and its expression did not differ between WT and *App^{NL-G-F}* mice (Figure 14A-B, Suppl. Table 2). Similarly, although p21 and p53 were present in higher levels in neurons (40–60%), no genotype-dependent differences were observed in the hippocampus nor in the cortex (Figure 14C–F, Suppl. Table 2). These findings suggest that, at this stage, neuronal populations are not affected by senescence-related changes, despite the presence of amyloid pathology. In contrast, microglial cells exhibited a clear senescence-associated profile. The percentage of p16⁺ and p21⁺ microglia was significantly higher in both the hippocampus and cortex of *App^{NL-G-F}* mice compared to WT controls (Figure 14G–L), indicating that microglial senescence develops alongside amyloid pathology and neuroinflammation. Notably, p53 expression remained unchanged between groups (Figure 14M–N), suggesting that p16/p21 pathways, rather than p53, may predominantly drive microglial senescence in this context. Among astrocytes, p16 expression remained very low across genotypes (Figure 14O–P), whereas p21 positivity was markedly increased, rising from approximately 20% in WT to 80% in *App^{NL-G-F}* mice (Figure 14Q–R). This robust induction of p21 suggests that astrocytes undergo strong senescence-like changes in response to chronic amyloid burden and gliosis. Interestingly, the proportion of p53⁺ astrocytes was also significantly elevated in the hippocampus but not in the cortex (Figure 14S–T), indicating region-specific susceptibility of astrocytic populations to senescence signaling under AD-like conditions.

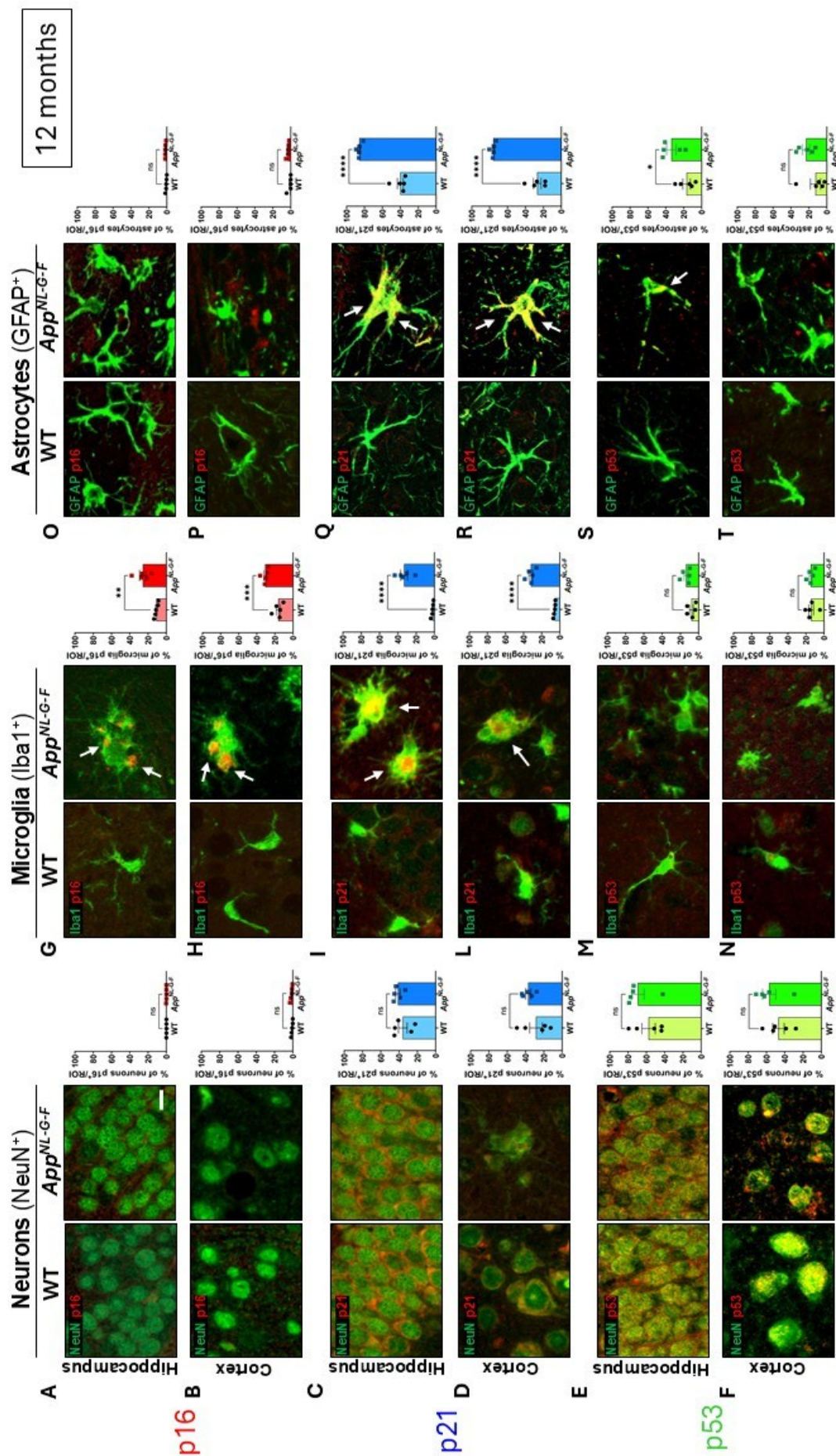


Figure 14. Cell-specific immunofluorescence analysis of senescence markers p16, p21 and p53 at 12 months of age. Immunofluorescence co-labeling with neuronal (NeuN), microglial (Iba1), and astrocytic (GFAP) markers was performed to evaluate cellular senescence status. Quantification of NeuN⁺ (A-F), Iba1⁺ (G-N), GFAP⁺ (O-T) cells co-expressing p16, p21 and p53 in the hippocampus and cortex of WT and *App*^{NL-G-F} mice at 12 months of age. Significant increases in microglia positive for p16 (G-H) and microglia and astrocytes positive for p21 (I-L, Q-R) were observed in *App*^{NL-G-F} mice. p53 was elevated in GFAP⁺ astrocytes in the hippocampus only (S). Scale bars: 50 μ m. Data are presented as mean $\pm$ SEM ($n = 4-5$ per group). Statistical analysis: unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; **** $p < 0.0001$ vs WT group.

6.3.3 Sustained Microglial and Astrocytic Senescence in 24-Month-Old *App*^{NL-G-F} Mice.

The presence of a senescence state in *App*^{NL-G-F} mice at 24 months of age was also assessed. Immunofluorescence analysis, as previously conducted at 4 and 12-months of age, showed no differences in p16⁺ neural cells in either the hippocampus (Figure 15A) nor cortex (Figure 15B) of *App*^{NL-G-F} mice compared to WT mice, with both groups showing low marker positivity, around 10% of neurons. Data showed that p21 (Figure 15C-D) and p53 (Figure 15E-F) were expressed in approximately 50% of neurons, however, their expression levels did not differ significantly between genotypes (Suppl. Table 2).

In microglia, all three senescence markers, p16, p21, and p53, were significantly higher in *App*^{NL-G-F} mice compared to WT (Figure 15G-N). In astrocytes, the percentage of p21⁺ cells were significantly higher in both the cortex and hippocampus of *App*^{NL-G-F} compared to WT mice (Figure 15Q-R), while no significant differences were detected in the percentage of p16- (Figure 15O-P) and p53- (Figure 15S-T) positive astrocytes between the two genotype.

Summarizing the immuno expression data across the three markers demonstrate that the pathology in *App*^{NL-G-F} mice induces a severe, age-dependent cellular response primarily characterized by the accumulation of positive microglia and astrocytes (Supplementary Figure 1A). This strongly suggests that glial senescence and/or cell-cycle-related stress mechanisms are key components of the pathology in this model. Neurons, while positive for the senescent makers, showed less pronounced relative changes compared to the dramatic activation observed in the microglia and astrocytes.

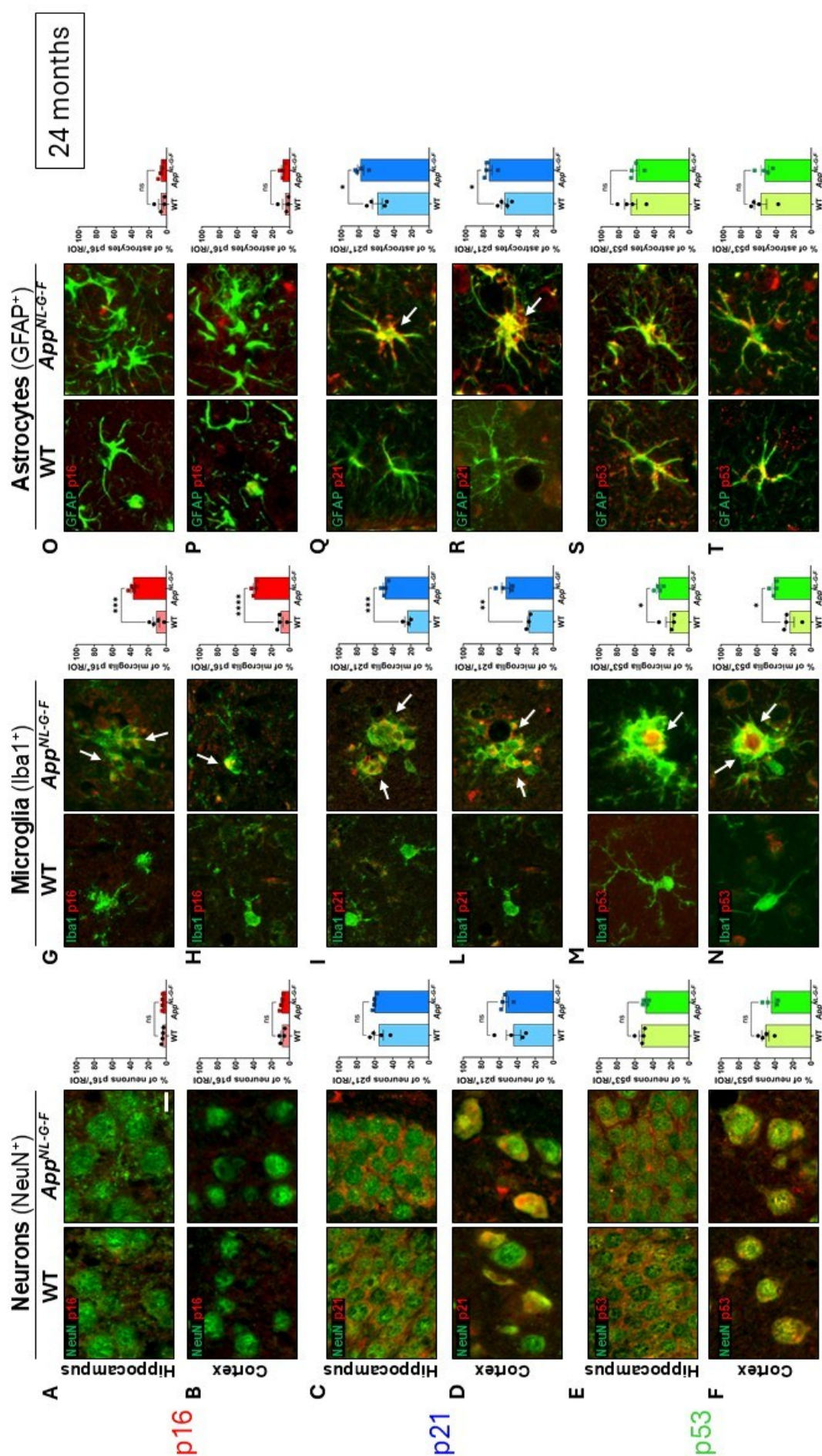


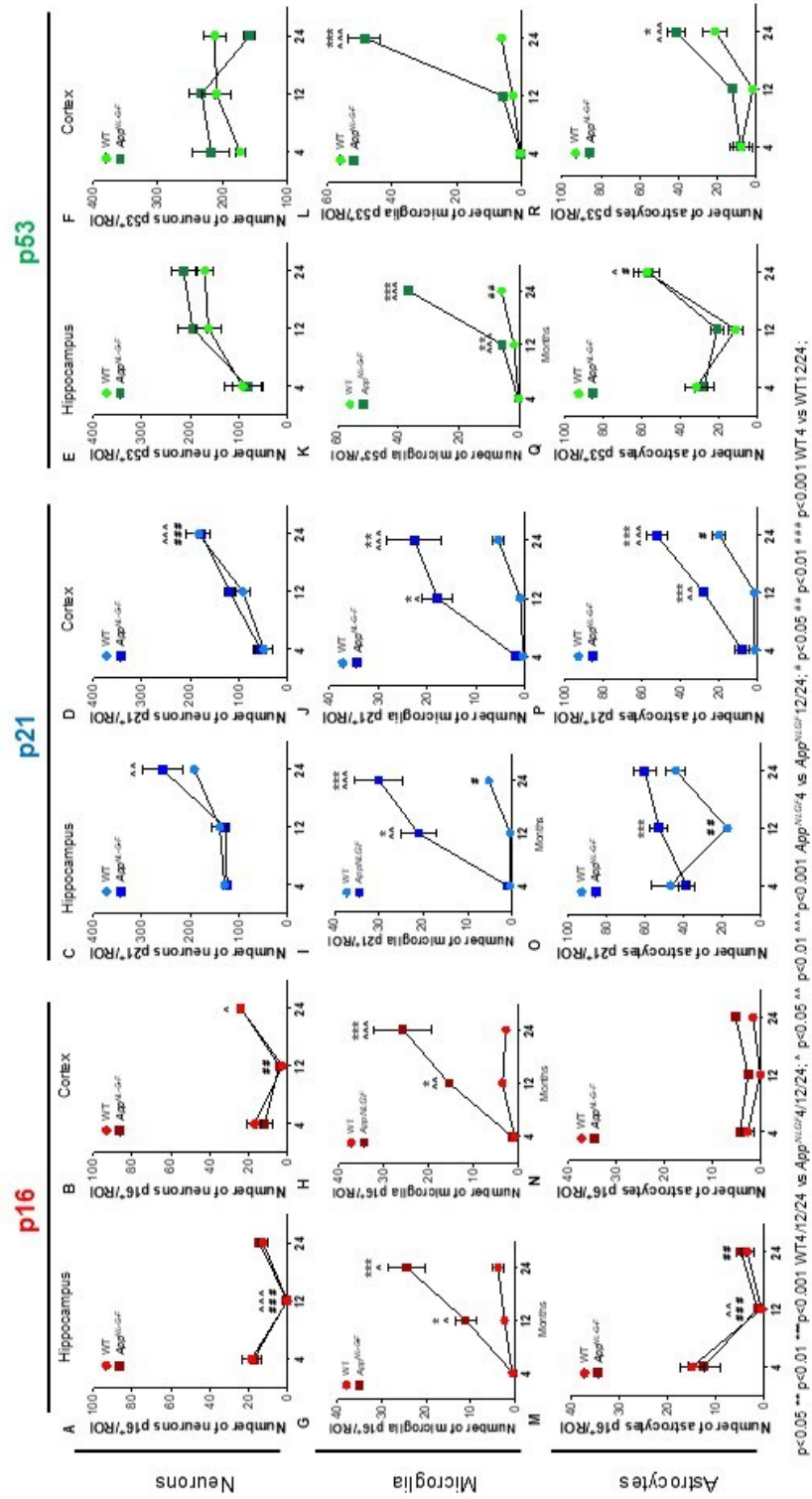
Figure 15. Cell-specific immunofluorescence analysis of senescence markers p16, p21 and p53 at 24 months of age. Immunofluorescence co-labeling with neuronal (NeuN), microglial (Iba1), and astrocytic (GFAP) markers was performed to evaluate cellular senescence status. Quantification of NeuN⁺ (A-F), Iba1⁺ (G-N), GFAP⁺ (O-T) cells co-expressing p16, p21 and p53 in the hippocampus and cortex of WT and *App*^{NL-G-F} mice at 24 months of age. Significant increases in p16 (G-H), p21 (I-L) and p53 (M-N) in microglia and p21 (Q-R) in astrocytes of *App*^{NL-G-F} mice. Scale bars: 50 μ m. Data are presented as mean $\pm$ SEM ($n = 4-5$ per group). Statistical analysis: unpaired *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; **** $p < 0.0001$ vs WT group.

6.3.4 Total Number per Area of p16, p21, and p53-Positive Neurons, Microglia, and Astrocytes in the Hippocampus and Cortex of WT and *App*^{NL-G-F} Mice Across Aging

To complement our previous analysis, we next performed a quantitative assessment of the total number of p16-, p21-, and p53-positive neurons, microglia, and astrocytes per defined area in both the hippocampus and cortex in WT and *App*^{NL-G-F} mice across the examined ages (4, 12, and 24 months of age) to capture age-dependent changes across brain regions and genotypes. By reporting the total cell counts normalized per tissue area, we aimed to evaluate the accumulation of senescent cells and their potential contribution to AD progression and aging-related neurobiology. This analysis indicates that the neuronal expression levels of p16, p21, and p53 are not affected by genotype, as no significant differences were observed between WT and *App*^{NL-G-F} mice (Figure 16A–F). In contrast, an age-dependent variation in neurons was noted for both p16 and p21. Interestingly, the number of p16⁺ neurons in hippocampus were significantly lower at 12 months of age, compared to 4 months, in both WT and *App*^{NL-G-F} mice (Figure 16A). In cortex, at 12 months of age WT mice showed, similarly to the hippocampus, a significantly lower number of p16⁺ neurons, compared to 4 months (Figure 16B). The number of p21⁺ neurons in hippocampus were significantly higher at 24 months in *App*^{NL-G-F}, compared to 4 months (Figure 16C). In cortex both WT and *App*^{NL-G-F} mice showed a significant higher number of neurons p21⁺ at 24 months-of-age compared to 4 months (Figure 16D). The number of p53⁺ neurons did not show any significant age-dependent difference in both genotypes (Figure 16E–F). In contrast, the number of microglia expressing p16, and p21, was markedly elevated in *App*^{NL-G-F} mice compared to WT, at both 12 and 24 months-of-age (Figure 16G–J). The number of p53⁺ microglia cells in hippocampus were also significantly elevated in *App*^{NL-G-F} mice compared

to WT, at both 12 and 24 months-of-age (Figure 16K); whereas in cortex, it was only increased at 24-month-of-age in *App^{NL-G-F}* mice (Figure 16L). In WT mice, aging effects were only significant in the hippocampus at 24 months-of-age, at which an increased number of p21⁺ and p53⁺ microglia were observed (Figure 16I, K). In WT mice, the number of p16⁺ astrocytes significantly decreased at 12 and 24 months-of-age (Figure 16M), and a similar reduction was also observed in *App^{NL-G-F}* mice at 12 months compared to 4 months-of-age. No age-related changes in p16 expression were observed in cortical astrocytes (Figure 16N). A similar trend was observed for p21 in hippocampus of WT mice, where the total number of p21⁺ astrocytes cells were significantly lower at 12 months compared to 4 months-of-age (Figure 16O). Furthermore, at 12 months of age, *App^{NL-G-F}* mice displayed a significantly higher number of p21⁺ astrocytes when compared with same WT mice of same age (Figure 16O), although this trend was not maintained at 24 months-of-age. In the cortex, p21⁺ astrocytes showed an aging-dependent increase in WT mice (at 24 months-of-age) and in *App^{NL-G-F}* mice (at 12 and 24 months-of-age); furthermore, the comparison within groups showed also a significant AD-dependent increase at 12 and 24 months (Figure 16P). p53⁺ astrocytes showed a significant age-dependent increase in hippocampus of both WT and *App^{NL-G-F}* mice at 24 months-of-age (Figure 16Q). In the cortex, a significant difference between the two genotypes at 24 months-of-age was also observed, along with an age-dependent increase in *App^{NL-G-F}* mice (Figure 16R).

Figure 16. Count of cells positive to senescence markers across aging. Total number per ROI of p16⁺, p21⁺, and p53⁺ neurons (A-F), microglia (G-L) and astrocytes (M-R) in hippocampus and cortex at 4, 12, and 24 months in WT and *App^{NL-G-F}* mice. Significant age- and genotype-related differences were detected in microglia and astrocytes, while neuronal expression remained largely unchanged. Data are shown as mean $\pm$ SEM. Statistical analysis: two-way ANOVA. $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ WT4/12/24 vs *AppNLGF*4/12/24; ^ $p < 0.05$ ^^ $p < 0.01$ ^^ $p < 0.001$ *AppNLGF*4 vs *AppNLGF*12/24; # $p < 0.05$ ## $p < 0.01$ ### $p < 0.001$ WT4 vs WT12/24.



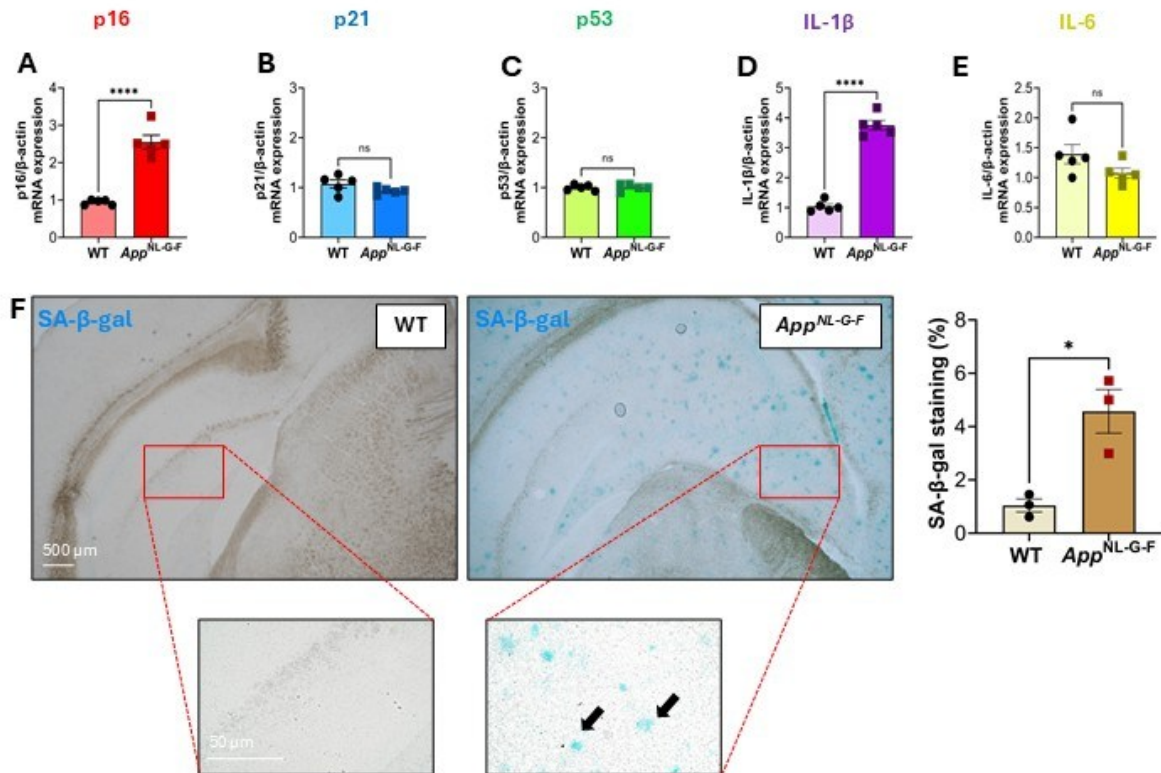
6.3.5 Gene Expression and Protein Evidence of p16-Driven Senescence, IL-1 β Upregulation, and Lysosomal Dysfunction in *App*^{NL-G-F} Mouse Brain

Furthermore, the gene expression levels of p16, p21 and p53 were evaluated by using qPCR analysis of mouse brain at 12 months of age. A significant upregulation of p16 mRNA was observed in both the hippocampus and cortex of *App*^{NL-G-F} mice compared to WT controls (Figure 17A, G). On the other hand, p21 hippocampal gene expression levels were similar between the two groups (Figure 17B), whereas a significant reduction was detected in the cortex of *App*^{NL-G-F} mice (Figure 17H). No differences in p53 gene expression were observed between groups (Figure 17C, I).

Additionally, to evaluate the presence of SASP, the gene expression and protein levels of pro-inflammatory cytokines IL-1 β and IL-6 were analysed (Figure 17D-E, L-O). IL-1 β gene expression level was significantly elevated in the *App*^{NL-G-F} mouse hippocampus (Figure 17D) and cortex (Figure 17L) compared to WT controls, as demonstrated by qPCR analyses. On the other hand, IL-6 gene expression levels were comparable between WT and *App*^{NL-G-F} mice in both hippocampus (Figure 17E) and cortex (Figure 17M). Consistent with the gene expression results, ELISA analysis showed a higher protein level of IL-1 β (Figure 17N), but not IL-6 (Figure 15O), in the *App*^{NL-G-F} mouse cortex compared to WT controls.

To evaluate lysosomal dysfunction, senescence-associated β -galactosidase (SA- β -GAL) activity was assessed. A marked increase in SA- β -GAL activity was observed in the hippocampus (Figure 17F) and cortex (Figure 17P) of *App*^{NL-G-F} mice, further supporting the presence of senescent cells in *App*^{NL-G-F} mice at 12 months of age. Furthermore, double staining of SA- β -gal+Iba1 showed a partial colocalization of SA- β -gal and microglia cells (Supplementary Figure 1B).

Hippocampus



Cortex

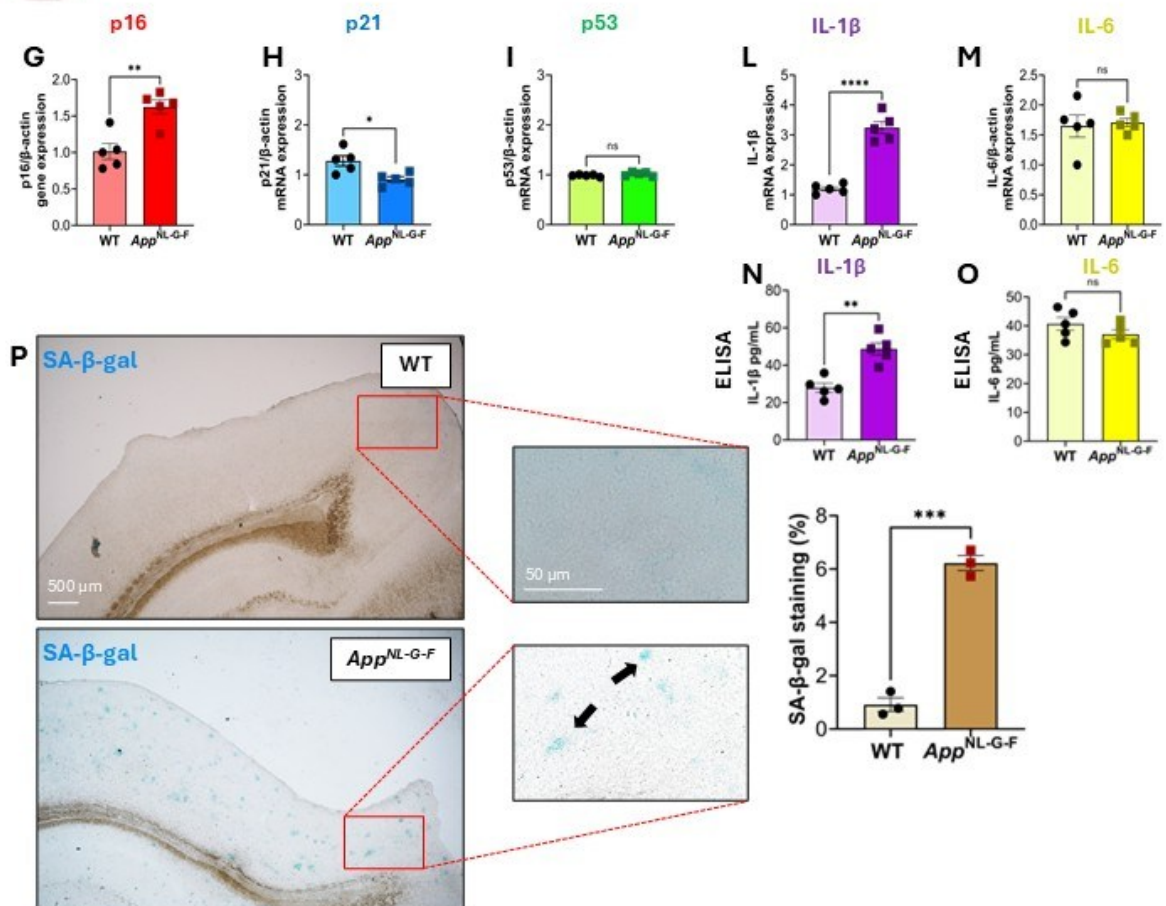


Figure 17. qPCR, ELISA analysis of senescence and inflammatory markers, and SA- β -Galactosidase staining at 12 months of age. Relative mRNA expression of p16 (A, G), p21 (B, H), and p53 (C, I) in hippocampus and cortex of WT and *App*^{NL-G-F} mice. mRNA levels of IL-1 β (D, L) and IL-6 (E, M) in hippocampus and cortex and ELISA quantification of IL-1 β (N) and IL-6 (O) in cortex of WT and *App*^{NL-G-F} mice. p16 mRNA, IL-1 β mRNA and protein levels were significantly increased in *App*^{NL-G-F} mice. (F, P) Representative images and quantification of SA- β -gal activity showing increased senescence-associated β -galactosidase staining in hippocampus (F) and cortex (P) of *App*^{NL-G-F} mice compared to WT. Scale bars: 500 μ m and 50 μ m. Statistical analysis: unpaired *t*-test. **p* < 0.05. All data are presented as mean $\pm$ SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001; *****p* < 0.0001 vs WT group.

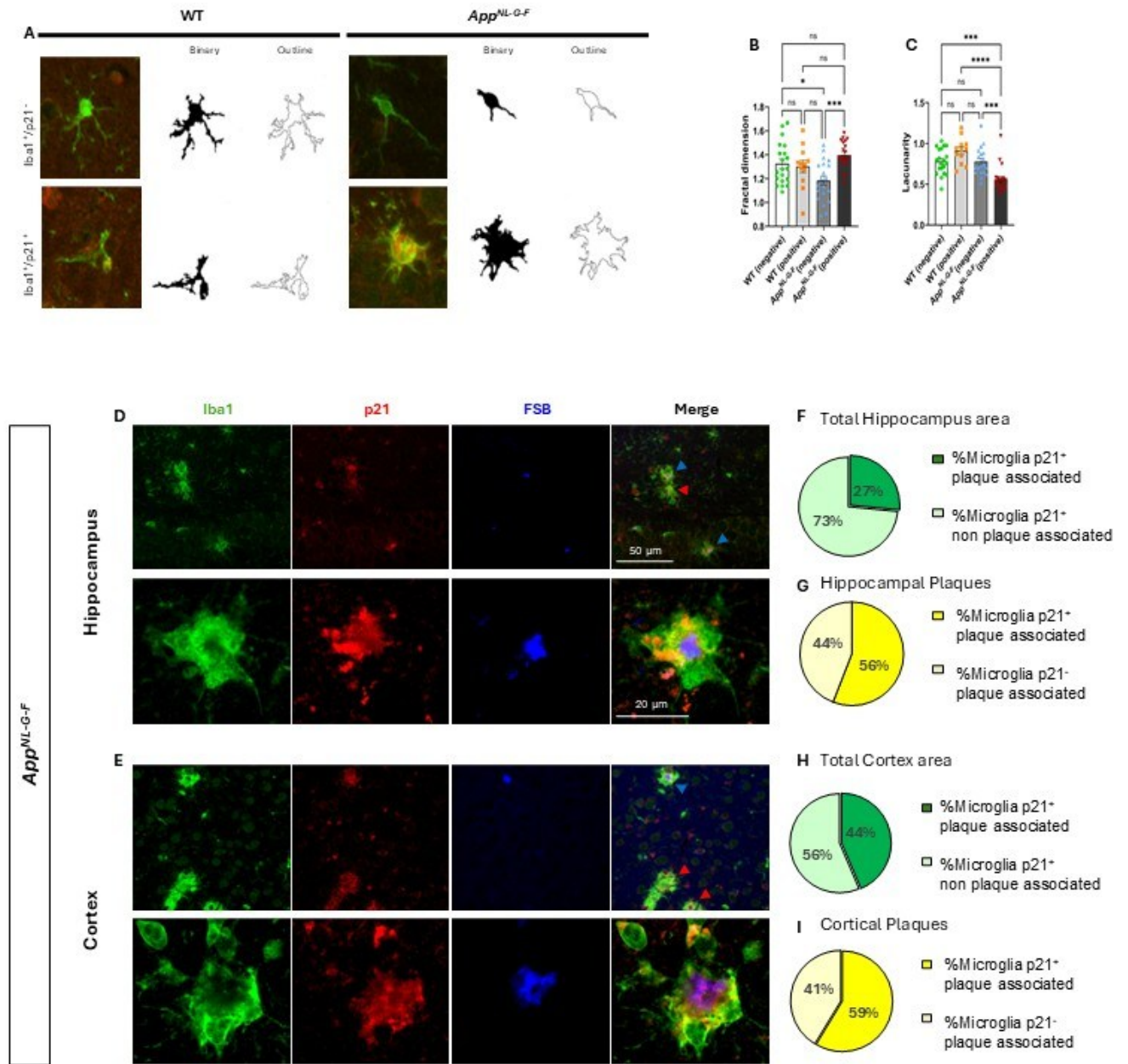
6.3.6 Morphology and Plaque-association of Senescent Microglia

The previous data indicate that microglia are one of the most significantly affected senescent cell- types in *App*^{NL-G-F} mice, along with an increase in their proliferation (Supplementary Figure 2A- B). Morphological alterations are also considered a characteristic of senescent cells. To investigate morphological differences in microglia, we analyzed microglial ramification using skeletal analysis and microglial complexity, shape, and size using fractal analysis in WT and *App*^{NL-G-F} mice at 12 months of age. Skeletal analysis, performed on Iba1⁺ cells, revealed significant differences between the two genotypes. WT mice exhibited a higher number of endpoints per cell and longer process length per cell compared to *App*^{NL-G-F} mice in both the hippocampus and cortex (Supplementary Figure 2C-D).

Fractal analysis was used to further characterize microglial morphological differences both between genotypes and within groups, specifically comparing non-senescent microglia (Iba1⁺/p21⁻) and senescent microglia (Iba1⁺/p21⁺) (Figure 18A–C). We selected p21 as a marker because it is one of the most well-established indicators of microglial senescence. In hippocampus, the fractal dimension was significantly reduced in p21⁻ microglia from *App*^{NL-G-F} mice compared to p21⁻ microglia from WT mice (Figure 18B). Notably, analysis of shape complexity revealed pronounced morphological alterations in senescent (p21⁺) microglia relative to non-senescent (p21⁻) microglia within *App*^{NL-G-F} mice (Figure 18B). Furthermore, lacunarity, a measure of spatial heterogeneity, was significantly decreased in p21⁺ microglia of *App*^{NL-G-F} mice compared to both p21⁻ microglia of *App*^{NL-G-F} mice and to p21⁺ and p21⁻ microglia of WT mice (Figure 18C).

To investigate whether microglial senescence is associated with amyloid plaques, we assessed the presence of p21⁺ microglia in 12-month-old *App*^{NL-G-F} mice. Our analysis focused on quantifying p21⁺ microglia in the vicinity of A β plaques (Figure 18D-E). We found that 56% of the microglia surrounding plaques in the hippocampus (Figure 18G) and 59% in the cortex (Figure 18I) were positive to the senescent marker p21. Notably, the plaque-associated p21⁺ microglia represented 27% of the total p21⁺ microglial population in the hippocampus (Figure 18F) and 44% in the cortex (Figure 18H). Similar results were observed at 24 months of age using p16 as a marker (Supplementary Figure 3A-D).

Figure 18. Microglial morphological analysis and association with amyloid plaques at 12 months of age. Representative images of microglial Fractal analysis in hippocampus of WT and *App*^{NL-G-F} mice (A). Microglia p21⁺ and p21⁻ from the two genotypes were considered. Fractal dimension was found to be significantly increased in p21⁺ microglia of *App*^{NL-G-F} mice compared to p21⁻ microglia of *App*^{NL-G-F} (B); Lacunarity showed a significant decrease in p21⁺ microglia of *App*^{NL-G-F} compared to all the other groups considered (C). Microglial association with amyloid plaques in hippocampus (D) and cortex (E). Data showed that 56% of the microglia surrounding plaques in the hippocampus (G) and 59% in the cortex (I) were p21⁺. The plaque-associated p21⁺ microglia represented 27% of the total p21⁺ microglial population in the hippocampus (F) and 44% in the cortex (H). Data are shown as mean $\pm$ SEM. Statistical analysis: one-way ANOVA. * $p < 0.05$, *** $p < 0.001$; **** $p < 0.0001$.



6.4 Discussion

The findings of this study reveal that cellular senescence markers increase with age in the *App*^{NL-G-F} mouse model of AD. The differences in the expression levels of p16, p21, and p53 across neurons, microglia, and astrocytes, suggest that aging may have distinct effects on each cell type. Furthermore, each type of cell may contribute distinctly to the progression of AD. Neuronal senescence has been associated with impaired synaptic function and altered gene expression [186], while senescent microglia and astrocytes are known to promote chronic neuroinflammation through the release of pro-inflammatory cytokines and SASP factors [187].

Aging, the primary risk factor for AD, is intrinsically linked to the accumulation of senescent cells throughout the body, including the brain [188]. Previous studies had identified senescent astrocytes, microglia, endothelial cells, and even neurons in the brains of both AD patients and animal models, highlighting the impact of this cellular state in the context of neurodegeneration [189], [190]. The increase in these markers with age indicates a progressive role of senescence in the disease process, which could contribute to the gradual cognitive decline that is characteristic of AD and is also extensively observed in *App*^{NL-G-F} mice [191], [192]. This study focuses on the *App*^{NL-G-F} model, recognized as one of the most relevant preclinical *in vivo* models for studying amyloid pathology [157], and supports the hypothesis that amyloid-beta exposure can directly induce cellular senescence in multiple brain cell types. In neurons the senescence markers p16, p21 and p53 did not show any significant difference between WT and *App*^{NL-G-F} mice, although an age-dependent increase of p16 and p21 expression levels was observed. These results suggest that senescence in AD is not strictly coupled to amyloid pathology. Although A β accumulation is a central feature of AD and is well known to disrupt neuronal function, it may not be sufficient to trigger a full senescence-like phenotype in neurons. One possible explanation for the observed phenomena is that A β -related toxicity initiates early stress responses, but additional pathological factors, such as intracellular Tau aggregation, are required to drive neurons into a AD senescent state. This observation is supported by previous studies that have linked neuronal senescence to Tau pathology and the presence of neurofibrillary tangles (NFTs) in both human and mouse models [154], [193], [194]. Nonetheless, further research is required to clarify the potential role of A β pathology in neuronal senescence, including any possible indirect mechanisms.

On the other hand, the increased presence of senescent microglia in this mouse model suggests a strong correlation between the progression of amyloid pathology and enhanced microglial senescence. However, the data does not clarify whether microglial senescence is a consequence of AD pathology or contributes causally to its development. It is generally understood that senescent microglia contribute to a detrimental cycle of chronic neuroinflammation. These cells often exhibit impaired phagocytic function, limiting their ability to clear amyloid-beta plaques, tau aggregates, and cellular debris, processes essential for preserving brain homeostasis [195]. Furthermore, senescent microglia tend to secrete an increased amount of pro-inflammatory cytokines and other factors that constitute the SASP [187], [196]. This heightened pro-inflammatory state can exacerbate neuroinflammation, a well-established hallmark of AD, potentially contributing to neuronal dysfunction and degeneration.

IL-1 β and IL-6 are two of the most well-characterized interleukins associated with SASP. This study observed elevated levels of IL-1 β , supporting the idea that it is a key component of SASP and a pro-inflammatory cytokine secreted by senescent microglia, potentially contributing to neuroinflammation. Interestingly, there was no difference in IL-6 levels between WT and *App*^{NL-G-F} mice. These findings align with earlier studies conducted on adult *App*^{NL-G-F} mice, which also reported increased IL-1 β expression but no change in IL-6 levels [197].

The significant upregulation of p21 observed in the microglia of the *App*^{NL-G-F} mice is particularly noteworthy, as p21 is recognized as a robust marker of microglial senescence. Senescent microglia in *App*^{NL-G-F} mice, specifically the p21⁺ microglia, have been found across the tissue areas, and not solely localized around amyloid plaques, suggesting that the presence of these senescent immune cells in the *App*^{NL-G-F} mouse model is not solely dependent on direct interaction with or the immediate vicinity of amyloid plaque deposits. We hypothesize that soluble amyloid-beta, rather than plaques themselves, may directly induce senescence in microglia. The interactions involving soluble amyloid-beta could be pivotal in the observed accumulation of senescent microglia in the *App*^{NL-G-F} model; this phenomena has been shown in vitro where A β , in dose-dependent manner, induced microglial senescence, causing in particular an upregulation of p21 [198].

Furthermore, our findings, indicating a less ramified morphology (decreased endpoints/cell and process length/cell) in *App*^{NL-G-F} microglia and genotype-specific alterations in fractal

dimension and lacunarity within the p21⁺ microglial population, provide strong evidence for altered microglial function and a senescent phenotype in the context of amyloid pathology. These structural alterations contribute to the broader picture of microglial dysregulation in AD, raising important questions about how this senescent phenotype relates to other recognized microglial states, such as Disease-Associated Microglia (DAM). Although the distinction between DAM and senescent microglia remains unclear, previous studies using the 5xFAD mouse model have shown that senescent microglia possess a unique protein profile that differs from DAM, including classical senescence markers and homeostatic microglial proteins [199]. Similarly, transcriptomic analyses in P301S (PS19) tauopathy mice indicated that only a fraction of DAM exhibit features of senescence, with the overlap between these populations largely driven by shared inflammatory signatures [200]. These observations highlight the complexity and heterogeneity of microglial responses in AD models. It is possible that senescent microglia emerge as a downstream state of DAM within a common pathway, or alternatively, they may arise independently as a separate response to stress. More in-depth studies are required to better define the connection between DAM and senescent microglia.

In this study, only a small number of astrocytes were found to be positive for p16. In contrast, astrocytes positive for p21 and p53, were more prevalent. Notably, p21⁺ astrocytes showed a pronounced and significant difference between WT and *App*^{NL-G-F} mice at 12 months. Previous studies have indicated that A β 42 can induce senescence in human astrocytes, leading to an upregulation of p16 and increased activity of SA- β -GAL [201]. Moreover, an increase in senescent astrocytes has been observed in adult individuals as well as in AD patients [202].

Beyond the specific cell-type alterations observed in *App*^{NL-G-F} mice, the marked elevation of SA- β -GAL activity in both hippocampal and cortical regions of *App*^{NL-G-F} mice provides compelling evidence for widespread cellular senescence in this mouse model of A β amyloidosis. This finding is particularly significant given SA- β -GAL's established role as a biomarker of lysosomal enlargement and dysfunction, a characteristic feature of senescent cells implicated in their pro-inflammatory and proteolytic dysregulation [203].

Overall, this study provides valuable insights into the distinct patterns of expression for key senescence markers p16, p21, and p53 across different ages and cell types in the *App*^{NL-G-F} mouse model. The significant elevation of p16 and p21 levels at both 12 and 24 months-of-

age, particularly within glial cells, underscores the importance of these two markers in the context of AD-related senescence. The consistent upregulation of both p16 and p21 in the *App^{NL-G-F}* mice suggests that the observed senescence is likely mediated through pathways involving these key cell cycle regulators. In contrast, immunofluorescence data of p53, a tumor suppressor protein known for its role in DNA damage response, cell cycle arrest, apoptosis, and senescence, revealed cell-type specific increases in the marker, particularly in hippocampal astrocytes at 12 months and in microglia at 24 months-of-age. These results highlight the importance of examining senescence markers at the single-cell level and subcellular level. It suggests that while p53 might not be globally upregulated in the brain of the *App^{NL-G-F}* mice, it may still play a significant role in inducing or maintaining senescence in specific cell populations under the influence of AD pathology.

Overall, based on our data showing concurrent upregulation of p16 and p21, with largely unchanged p53 levels, and in line with current literature, we speculate that glial, and more specifically microglial, senescence in the *App^{NL-G-F}* mouse brain involves a multistep process induced by chronic stress rather than acute DNA damage [204]. This is consistent with the persistent oxidative and inflammatory environment characteristic of amyloid pathology [205]. Upstream regulators likely include the TGF- β /SMAD and NF- κ B signaling pathways, both known to drive p16 and p21 transcription and to reinforce the SASP [206], [207]. The elevated IL-1 β levels observed in our study further support NF- κ B-mediated SASP activation. In addition, sustained oxidative and lysosomal stress may activate the ATM-Chk2 and mTOR-TFEB pathways, leading to mitochondrial dysfunction, lysosomal enlargement, and increased SA- β -GAL activity [208]. Collectively, these data support a model in which chronic A β -induced oxidative stress, NF- κ B/TGF- β signaling, and mTOR dysregulation converge to sustain the p16/p21-driven senescence program in microglia, ultimately driving their proinflammatory and dystrophic phenotype in the *App^{NL-G-F}* model. Furthermore, the findings of this study highlight the potential of *App^{NL-G-F}* mice as a model for targeting cellular senescence and as a promising therapeutic strategy for AD. Senolytic drugs have shown encouraging results in early-phase clinical trials for other age-related conditions, demonstrating their feasibility and potential to reduce the burden of senescent cells in vivo [193], [209], [210]. Notably, a Phase I clinical trial investigating the use of the senolytic combination of Dasatinib and quercetin in patients with early-stage symptomatic AD showed that these drugs can penetrate the central nervous system and have an acceptable safety profile [211]. This provides critical proof-of-concept for the potential of senolytics in treating AD. Interestingly, research has also shown that senolytic compounds can target

enzymes that are linked to AD pathology, suggesting a multi-faceted therapeutic benefit. Beyond senolytics, senomorphic agents are being explored for their ability to suppress the SASP, potentially reducing the chronic inflammation associated with senescent cells in AD [212].

This study identifies microglia as a key cell type that undergoes senescence in the *App*^{NL-G-F} model. This suggests that therapeutic strategies specifically targeting senescent microglia could be particularly beneficial. Additionally, the observed elevation of pro-inflammatory cytokines like IL-1 β in this model supports the idea that senomorphics, aimed at reducing the SASP, could serve as a valuable complementary approach to senolytics in the treatment of AD. By both eliminating senescent cells and mitigating their harmful secretory products, a combined therapeutic strategy might offer a more comprehensive approach to tackling the complex pathology of AD.

Building on the findings of this study, several avenues for future research deserve attention. It is essential to investigate the specific upstream mechanisms that trigger cellular senescence in neurons, microglia, and astrocytes within the *App*^{NL-G-F} model. This could involve examining the roles of A β oligomers, oxidative stress, DNA damage accumulation, and mitochondrial dysfunction. Evaluating the efficacy of various senolytic and senomorphic compounds in the *App*^{NL-G-F} mouse model throughout different stages of disease progression would be a logical next step. Such studies could help identify the most promising therapeutic agents for targeting senescence in the context of AD pathology. Further research should focus on elucidating the functional consequences of senescence in specific brain cell types within this model. This could include assessing the impact of senescence on synaptic function, neuronal survival, and the ability of glial cells to clear amyloid-beta and other pathological proteins.

Supplementary Table 1

List of the anti-bodies used in the study.

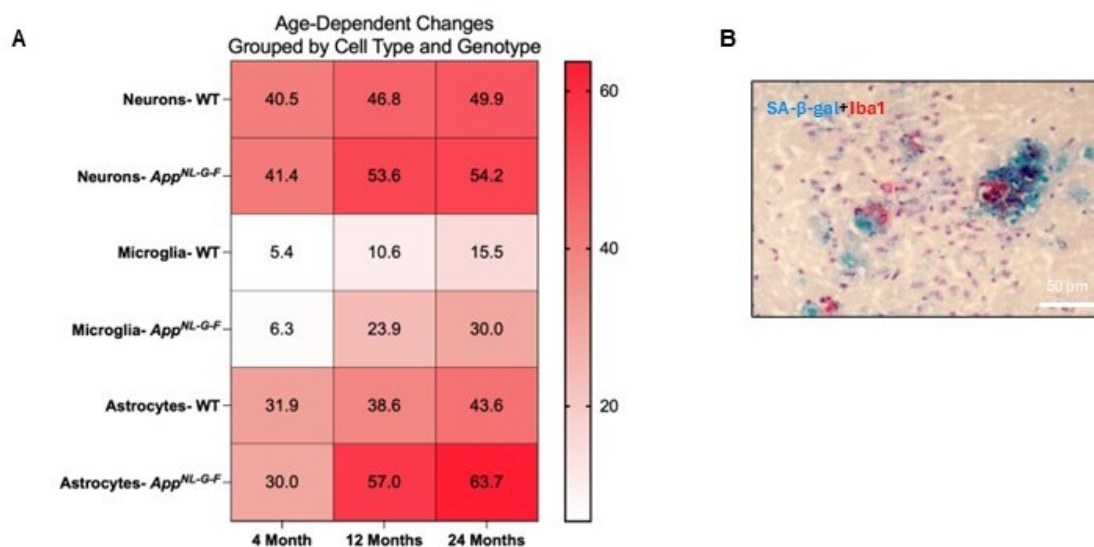
Antibody	Host and type	Dilution for IF	Product code	Source
p21	Mouse monoclonal	1:100	sc-817	Santa Cruz Biotechnology
p16	Rabbit recombinant monoclonal	1:100	ab211542	Abcam
p53	Mouse monoclonal	1:100	sc-99	Santa Cruz Biotechnology
Iba1	Mouse monoclonal	1:200	MA5-27726	Invitrogen
Iba1	Rabbit monoclonal	1:500	019-19741	FUJIFILM
NeuN	Mouse monoclonal	1:100	MAB377	Sigma
NeuN	Rabbit monoclonal	1:200	26975-1-AP	Proteintech
GFAP	Mouse monoclonal	1:150	3670	Cell Signaling
GFAP	Rabbit monoclonal	1:500	ZO334	Dako
Alexa Fluor 488 Anti-rabbit IgG	Goat	1:500 1:1000	A32731	Invitrogen
Alexa Fluor 546 Anti-mouse IgG	Goat	1:500	A-11003	Invitrogen

Supplementary Table 2

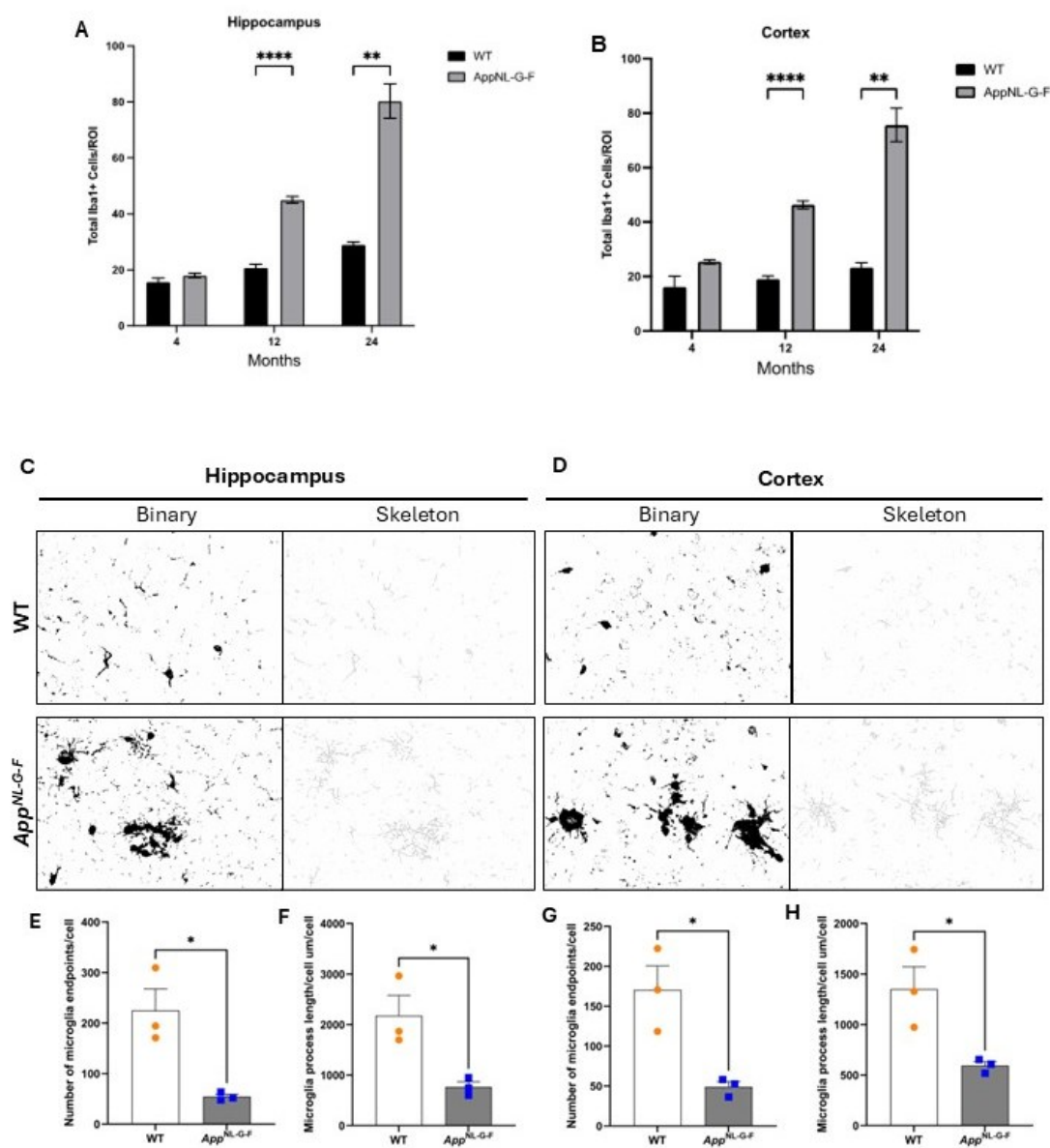
Percentage of neurons, microglia and astrocytes positive for p16, p21 and p53 in WT and *App^{NL-G-F}* mice at 4, 12 and 24 months of age. In the table are reported the percentage mean $\pm$ SEM.

	Months	Genotype	Hippocampus			Cortex		
			% of positive cells			% of positive cells		
			Neurons	Microglia	Astrocytes	Neurons	Microglia	Astrocytes
p16	4	WT	4,8 $\pm$ 1,5	4,1 $\pm$ 2,9	18,6 $\pm$ 2,6	5,6 $\pm$ 1,1	4,1 $\pm$ 0,8	16,0 $\pm$ 8,6
		<i>App^{NL-G-F}</i>	4,5 $\pm$ 0,6	3,2 $\pm$ 2,2	13,5 $\pm$ 3,6	3,6 $\pm$ 1,2	5,0 $\pm$ 1,2	11,6 $\pm$ 1,9
	12	WT	0,1 $\pm$ 0,1	11,0 $\pm$ 0,9	0,8 $\pm$ 0,3	0,9 $\pm$ 0,4	16,3 $\pm$ 2,2	0,9 $\pm$ 0,9
		<i>App^{NL-G-F}</i>	0,1 $\pm$ 0,1	25,8 $\pm$ 3,6	2,5 $\pm$ 0,4	1,5 $\pm$ 0,5	30,8 $\pm$ 1,5	4,7 $\pm$ 1,0
	24	WT	4,0 $\pm$ 0,5	11,0 $\pm$ 3,5	6,6 $\pm$ 2,7	8,0 $\pm$ 1,5	9,6 $\pm$ 2,4	5,3 $\pm$ 2,8
		<i>App^{NL-G-F}</i>	4,2 $\pm$ 0,1	37,0 $\pm$ 2,1	6,3 $\pm$ 1,2	8,5 $\pm$ 1,0	38,8 $\pm$ 1,4	8,1 $\pm$ 1,3
p21	4	WT	31,7 $\pm$ 0,2	2,8 $\pm$ 1,6	55,3 $\pm$ 6,4	13,2 $\pm$ 4,4	1,5 $\pm$ 0,9	47,6 $\pm$ 2,4
		<i>App^{NL-G-F}</i>	29,8 $\pm$ 1,1	5,1 $\pm$ 1,5	50,2 $\pm$ 3,7	14,7 $\pm$ 3,2	6,0 $\pm$ 1,1	43,5 $\pm$ 5,7
	12	WT	36,0 $\pm$ 4,7	2,4 $\pm$ 0,9	40,0 $\pm$ 3,3	28,6 $\pm$ 6,9	5,6 $\pm$ 0,4	26,9 $\pm$ 4,30
		<i>App^{NL-G-F}</i>	41,3 $\pm$ 2,4	36,13,8	85,8 $\pm$ 1,3	37,1 $\pm$ 3,0	33,4 $\pm$ 2,7	76,0 $\pm$ 1,3
	24	WT	55,6 $\pm$ 5,0	23,8 $\pm$ 2,8	58,8 $\pm$ 5,6	44,1 $\pm$ 7,9	27,4 $\pm$ 1,3	55,7 $\pm$ 3,6
		<i>App^{NL-G-F}</i>	60,3 $\pm$ 1,0	48,8 $\pm$ 1,8	77,9 $\pm$ 3,5	52,9 $\pm$ 3,0	52,7 $\pm$ 4,4	73,5 $\pm$ 3,4
p53	4	WT	44,9 $\pm$ 3,4	1,3 $\pm$ 0,9	34,9 $\pm$ 4,5	26,3 $\pm$ 11,9	0,8 $\pm$ 0,8	26,9 $\pm$ 12,4
		<i>App^{NL-G-F}</i>	52,9 $\pm$ 5,1	2,7 $\pm$ 1,8	31,0 $\pm$ 5,1	17,7 $\pm$ 7,6	1,1 $\pm$ 0,6	28,5 $\pm$ 12,7
	12	WT	57,6 $\pm$ 7,3	7,8 $\pm$ 2,1	16,9 $\pm$ 4,2	46,9 $\pm$ 6,3	14,2 $\pm$ 2,7	12,6 $\pm$ 5,7
		<i>App^{NL-G-F}</i>	69,3 $\pm$ 6,9	13,8 $\pm$ 2,1	33,8 $\pm$ 5,3	57,0 $\pm$ 7,2	14,6 $\pm$ 1,6	23,3 $\pm$ 4,1
	24	WT	52,9 $\pm$ 2,5	21,4 $\pm$ 4,0	66,4 $\pm$ 6,9	50,4 $\pm$ 3,9	23,3 $\pm$ 4,6	57,6 $\pm$ 6,9
		<i>App^{NL-G-F}</i>	48,0 $\pm$ 1,6	33,3 $\pm$ 2,3	60,6 $\pm$ 3,5	43,9 $\pm$ 4,1	40,9 $\pm$ 2,0	52,7 $\pm$ 4,4

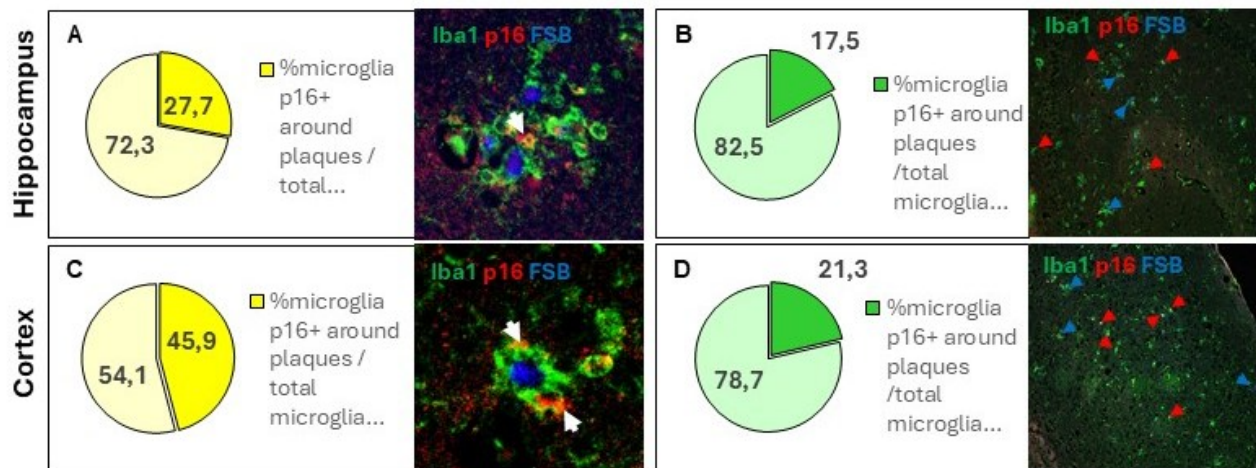
Supplementary Figures



Supplementary Figure 1. Age-Dependent Changes Grouped by Cell Type and Genotype, and SA-βGal+Iba1 staining. (A) The percentage of positive cells has been averaged across all three Markers (p16, p21 and p53) and both Regions (Hippocampus and Cortex). This visualization clearly highlights the Genotype and Age effect on the three primary cell types. Microglia - and Astrocytes show a dramatic change in color from 4 Months to 12 months, confirming the accelerated pathology in these glial cell types that is effect of the genotype. The neurons WT row is showing a relatively high percentage of senescent markers already at 4 months and stays high in older ages, reinforcing the idea that baseline neural -marker expression is high regardless of the specific disease model, making the glial changes a much clearer finding. (B) Double staining of SA-β-gal+Iba1 showed a partial colocalization of SA-β-gal and microglia cells.



Supplementary Figure 2. Microglial proliferation and Skeletal analysis of microglia in WT and App^{NL-G-F} mice. Quantification of total microglia (Iba1⁺) in the hippocampus (A) and cortex (B) of WT and App^{NL-G-F} mice at 4, 12, and 24 months of age. Data are expressed as total microglia per area. Data are shown as mean $\pm$ SEM. Statistical analysis: one-way ANOVA. ** $p < 0.01$; **** $p < 0.0001$ vs WT group. Quantification of the number of microglial endpoints per cell and process length per cell in the hippocampus and cortex of 12-month-old WT and App^{NL-G-F} mice. Images were acquired at 20 $\times$ magnification and analyzed using ImageJ software. Statistical analysis was performed using unpaired t-tests. Data are presented as mean $\pm$ SEM. * $p < 0.05$ vs WT group.



Supplementary Figure 3. Association of p16⁺ microglia with amyloid plaques at 24 months of age.

The number of p16⁺ microglia and microglia surrounding amyloid plaques was assessed in both the hippocampus (A-B) and cortex (C-D). Data showed that 27,7% of the microglia surrounding plaques in the hippocampus (A) and 45,9% in the cortex (C) were p16⁺. The plaque-associated p16⁺ microglia represented 17,5% of the total p16⁺ microglial population in the hippocampus (B) and 21,3% in the cortex (D). Data are expressed as total microglia per area.

7. Ongoing and future studies: evaluation of intestinal permeability and microbiota of *App*^{NL-G-F} mice

Over the past few years, there has been growing interest in the influence of gut microbiota on brain health, via the gut-brain axis. Besides its central role in this bidirectional pathway through the production and stimulation of neurotransmitters, the intestinal microbiota plays important functions in the regulation of metabolism, immunity and intestinal barrier integrity, among others [213], [214]. Increasing evidence demonstrated that alterations in the gut-brain axis may influence the development of neurodegenerative diseases, including Alzheimer's disease (AD). In particular, changes in the intestinal microbiota composition associated to age-related chronic inflammation, can lead to increased intestinal barrier permeability and release of microbial products in the blood stream. The systematic inflammation derived from this mechanism can impair the blood-brain barrier, promoting neuroinflammation and, eventually, neurodegeneration [215], [216].

In this regard, we are currently determining if the strong AD pathology present in the brain of *App*^{NL-G-F} relates to changes in the gut integrity and microbiota composition of 12-month-old female mice.

7.1 Materials and Methods

Small intestine samples were collected from the same mice (N= 8 mice/group) used in the previous study to assess the expression of markers for inflammatory state (IL-6, IL-22, IL-17a, *iba1* and GFAP) and intestinal integrity (Occludin and MUC2) (Table 1). Total RNA was extracted following the TRIzol reagent protocol (Sigma-Aldrich). RNA (2 µg) was reverse-transcribed using M-MLV reverse transcriptase (Promega). Real-time PCR was performed using ViiA7 Real-Time PCR Detection System (Applied Biosystems). The reaction mix was composed of 6 µL of SYBR Green Master Mix (BIO-RAD), 6 pmol of each forward and reverse primer and 2 µL of diluted cDNA. Samples were plated in duplicates and the reaction mix was incubated 10 min at 95 °C before 40 cycles, each one of 1 s at 95 °C and 30 s at 64 °C. β -actin was used to normalize gene expression levels. Relative quantification was performed using the comparative Ct method.

Genes	Primers sequences
B-Actin (β -Act)	For 5'-AGC CAT GTA CGT AGC CAT CC-3' Rev 5'-CTC TCA GCT GTG GTG GTG AA-3'
Interleukin-6 (IL-6)	For 5'-CCTACCCCAATTCCAATGCT-3' Rev 5'-TATTTTCTGACCACAGTGAGGAAT-3'
Interleukin-22 (IL-22)	For 5'-TCCAACCTCCAGCAGCCATA-3' Rev 5'-TTCCAGGGTGAAGTTGAGCA-3'
Interleukin-17a (IL-17a)	For 5'-TCC AGA AGG CCC TCA GAC TA-3' Rev 5'-TTC ATT GCG GTG GAG AGT C-3'
Glial fibrillary acidic protein (GFAP)	For 5'-ATTGCTGGAGGGCGAAGAA-3' Rev 5'-CGGATCTGGAGGTTGGAGGA-3'
Ionized calcium-binding adapter protein 1 (Iba1)	For 5'-GGATTTGCAGGGAGGAAAAG-3' Rev 5'-TGGGATCATCGAGGAATTG-3'
Occludin (Ocln)	For 5'-CCCCATCTGACTATGTGGAAAGA-3' Rev 5'-AAAACCGCTTGTCATTCACTTTG-3'
Mucin-2 (MUC2)	For 5'-AACTACCACTGTGATGCCAATG-3' Rev 5'-ACAATGTTGATGCCAGACTCG-3'

Table 1. Primers used for qPCR

7.1 Preliminary results

App^{NL-G-F} mice present increased inflammation and intestinal permeability

To evaluate mice intestinal inflammation and permeability, the gene expression of inflammatory state markers, IL-6, IL-22, IL-17a, GFAP and iba1, as long as for intestinal barrier integrity (Occludin and MUC2) were evaluated.

According to our results, 12-month-old *App*^{NL-G-F} showed significant increased expression of GFAP compared to WT mice (Figure 19), leading to intestinal inflammation. Despite an increased tendency in *App*^{NL-G-F}, no significant differences were found for the expression of IL-6, IL-17a and iba1 between the two genotypes. Interestingly, IL-22 expression, which contributes to intestinal barrier function, was significantly decreased in the AD mouse model. Also, *App*^{NL-G-F} mice showed a decreased expression of occludin compared to WT controls, indicating a disrupted barrier integrity. No differences were found for MUC2 expression levels.

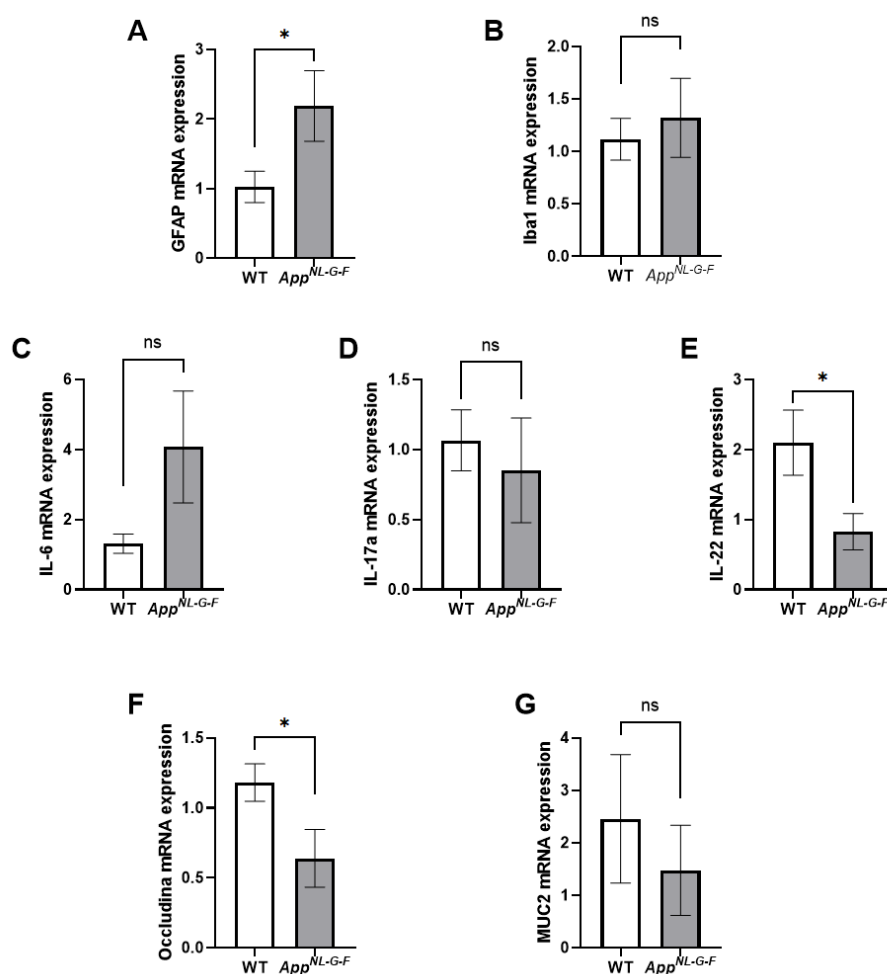


Figure 19. Intestinal gene expression of inflammatory state markers (A. GFAP, B. *iba1*, C. IL-6, D. IL-17a, E. IL-22) and intestinal barrier integrity (F. Occludin, G. MUC2) in 12-month-old WT and *App^{NL-G-F}* mice.

7.2 Preliminary conclusions and future experiments

Our preliminary results highlighted an increased inflammatory state and gut permeability in the *App^{NL-G-F}* mice compared to the control group. Specifically, enteric glial cells, which are known to express GFAP, have been associated with intestinal motility and neuroinflammation [217], while IL-22 is involved in gut barrier repair and regeneration [218]. Also, occludin plays a key role in supporting tight junctions' structural integrity, which is crucial in barrier homeostasis [219]. Our data suggest a possible interplay between AD pathology and intestinal dysfunction in *App^{NL-G-F}*, supporting the potential role of this mouse model in further investigating the gut-brain axis.

More studies are needed to better characterize these aspects. In particular, in future experiments we will further investigate intestinal barrier integrity by evaluating other proteins forming the tight junctions, namely claudins and zonula occludens (ZO)s, at both the gene expression level and protein level. Immunohistochemical analysis will be conducted to determine their localization and to better investigate intestinal inflammatory state. Moreover, since gut microbiome composition is crucial for intestinal homeostasis, we are currently analysing fecal samples of *App*^{NL-G-F} compared to WT mice through shotgun sequencing.

Conclusions

A key distinction between synthetic drugs and natural phytocomplexes lies in the nature and extent of their interactions with biological targets. Synthetic molecules are typically designed to act with high specificity and selectivity on a defined target. In contrast, phytocomplexes consist of a multitude of bioactive constituents capable of interacting with several molecular pathways simultaneously, often generating additive or synergistic effects.

Over the three years of this PhD program, I investigated in depth the relationship between the phytochemical profiles of natural compounds and their biological activities across multiple *in vitro* models. This multidisciplinary approach allowed me to explore how chemical complexity translates into functional biological outcomes.

Within the broader context of neurodegenerative diseases, including Alzheimer's disease (AD), the limited success of conventional pharmacological strategies has intensified interest in natural bioactive compounds as credible alternative or complementary options. In recent years, particular attention has been directed toward natural-derived senotherapeutics. These agents can be classified into two major groups: i) senolytics, which selectively eliminate senescent cells, and ii) senomorphics, which modulate the senescence-associated secretory phenotype (SASP), reducing the pro-inflammatory and deleterious secretome typical of senescent cells. [178]. Growing evidence highlights the potential of natural compounds in targeting cellular senescence. Notably, the combination of Dasatinib (a tyrosine kinase inhibitor) and Quercetin (a natural flavonoid with anti-inflammatory properties) has been shown to effectively clear senescent cells *in vitro* and to selectively reduce p16⁺ cells in an AD mouse model, a strategy now under clinical evaluation [156], [211]. Fisetin, another flavonoid of significant senotherapeutic interest, displays antioxidant, anti-inflammatory and neuroprotective properties and modulates key pathways such as p53 and NF- κ B. *In vivo* studies have demonstrated that fisetin attenuates neuroinflammation and improves cognition and memory in AD mouse models. [220]. Additional natural molecules—including piperlongumine and curcumin—have also exhibited senolytic activity by preferentially targeting senescent cells [142].

In this framework, the *App*^{NL-G-F} mouse model, which we validated as a tool to study senescence, represents a valuable in vivo platform for the screening and evaluation of senotherapeutic agents. Its integration into future research may support the identification of natural compounds capable of modulating senescence-related mechanisms implicated in neurodegeneration.

Overall, the work conducted during my PhD contributes to bridging the gap between phytochemical complexity and biological relevance, providing experimental evidence that supports the rationale for exploiting natural compounds in the modulation of inflammation/neuroinflammation and neurodegenerative processes. Although further studies are required, having characterized a senescence AD animal model, I hope my research can highlight new avenues for the development of senotherapeutic strategies and reinforce the potential role of natural products in addressing unmet needs in neurodegenerative disease therapy.

List of publications

During my PhD I also contributed to the publication of the following papers, determining the biological activity and potential effect on human health of several natural extracts:

1. *Cannabis Sativa* Oil Promotes Social Interaction and Ultrasonic Communication by Acting on Oxytocin Pathway

Abstract

Objective: *Cannabis sativa* is the most used recreational drug worldwide. In recent years, there has been a growing interest in the potential therapeutic benefits of medicinal cannabis to treat a variety of psychiatric and neurological conditions. In particular, cannabidiol (CBD), a nonpsychoactive cannabis constituent, has been investigated for its potential prosocial effects on behavior, although the molecular mechanisms underlying this effect are still largely unknown. The aim of this study was to investigate the effect of a *C. sativa* oil CBD rich (CS oil) on social interaction and ultrasonic communication in mice.

Study Design: Twenty-seven adult male mice (B6;129PF2) were treated daily with vehicle or CS oil for 2 weeks. At Day 14, mice were tested for behavior (social interaction test and ultrasonic communication). Forty minutes before the behavioral tests, mice were exposed to intranasal treatment with vehicle or the oxytocin receptor antagonist, L-371,257. After behavioral tests, VH- and CS oil-treated mice were sacrificed, RNA was extracted from the hypothalamus and used for quantitative Real Time-PCR experiments.

Results: We found that a 2-week treatment with the CS oil on mice exerted a prosocial effect associated with an increase in ultrasonic vocalizations. These effects were inhibited by pretreating mice with an oxytocin receptor antagonist. In addition, at the molecular level, we found that CS oil treatment caused a significant increase in oxytocin and a decrease in oxytocin receptor expression levels in the brain hypothalamus.

Conclusion: Our results suggest that CS oil promotes social behavior by acting on oxytocin pathway.

Premoli M, Carone M, Mastinu A, Maccarinelli G, Aria F, Mac Sweeney E, Memo M, Bonini SA. Cannabis Sativa Oil Promotes Social Interaction and Ultrasonic Communication by Acting on Oxytocin Pathway. Cannabis Cannabinoid Res. 2024 May 27.

2. Ecotoxicological evaluation of an aqueous phytoextract of *Melia azedarach* L.

Abstract: *Melia azedarach* L. is a Meliaceae that has shown important insecticidal activities. However, few researchers have extensively studied the toxicology of aqueous extracts of *M. azedarach* (MAE). Therefore, the main objective of this study was to characterize the phyto-eco-toxicological profile of MAE. First, a botanical and phytochemical characterization of MAE was performed using a histological, and metabolomic multi-analytical approach. Second, the toxicological effects on pollinating insects (*Apis mellifera ligustica*) and soil collembola (*Folsomia candida*) were evaluated. In addition, acute toxicity was evaluated in zebrafish (*Danio rerio*) to assess effects on aquatic fauna, and toxicity was determined in human neuroblastoma (SH-SY5Y) and fibroblast (FB-21) cell models. Finally, phytotoxic effects on germination of *Cucumis sativus* L., *Brassica rapa* L. and *Sorghum vulgare* L. were considered.

Metabolomic analyses revealed the presence of not only limonoids but also numerous alkaloids, flavonoids and terpenoids in MAE. Histological analyses allowed us to better localize the areas of leaf deposition of the identified secondary metabolites. Regarding the ecotoxicological data, no significant toxicity was observed in bees and collembola at all doses tested. In contrast, severe cardiac abnormalities were observed in zebrafish embryos at concentrations as low as 25 µg/mL. In addition, MAE showed toxicity at 1.6 µg/mL and 6.25 µg/mL in FB-21 and SH-SY5Y cells, respectively. Finally, MAE inhibited seed germination with inhibitory concentrations starting from 5.50 µg/mL in *B. rapa*, 20 µg/mL in *S. vulgare*, and 31 µg/mL in *C. sativus*.

Although *M. azedarach* extracts are considered valuable natural insecticides, their ecological impact cannot be underestimated. Even the use of an environmentally friendly solvent (an aqueous solution), for the first time, is not without side effects. Therefore, the data collected in this study show the importance of evaluating the dosages, modes of administration and production methods of *M. azedarach* phytoextracts in agricultural settings.

Popescu VS, Zhang L, Papa G, Giuliani C, Ribaud G, Abate G, Bulgari D, Mac Sweeney E, Pucci M, Bottoni M, Milani F, Zizioli D, Negri I, Gianoncelli A, Gobbi E, Uberti D, Lucini L, Memo M, Fico G, Peron G, Mastinu A. Ecotoxicological evaluation of an aqueous phytoextract of *Melia azedarach* L. *Science of the Total Environment* 950 (2024) 175314.

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