

Valorization of *Cupressus sempervirens* L. fruits as a source of bioactive compounds

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Nothing in life is to be feared, it is only to be understood.

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I would also like to thank all the friends I made in Portugal. I am deeply grateful for your companionship, the laughter, the shared moments, and the holidays we spent I

would also like to thank all the friends I made in Portugal. I am deeply grateful for your companionship, the laughter, the shared moments, and the holidays we spent together far from our families. Your presence was incredibly meaningful in moments of joy and, more importantly, in moments of sadness. You taught me that family is not always defined by blood, but by love, support, and genuine care. **Halima Khelifa**, from the bottom of my heart, thank you for being my family away from home. You gave me so much more than I could ever express. I am deeply grateful for your companionship, the laughter, the shared moments, and the holidays we spent together far from our families. You were with me in times of happiness and in moments of difficulty, and your presence gave me strength, comfort, and a sense of belonging that I will always cherish. **Souha Soulef Atig**, who has been a cherished friend since our middle school days, thank you for your precious help, your generosity, and your constant support. You were always there whenever I needed you, offering kindness, care, and sincere friendship. Your presence made my journey easier and more beautiful, and I will always be grateful for everything you did for me. **Isadora Maria Torres Melo**, thank you for your moral support in the laboratory, your positive energy, and your kindness. Your encouragement and warmth made difficult days lighter and joyful moments even brighter. To everyone who supported me, believed in me, and contributed in any way to the completion of this work, I express my sincere gratitude and appreciation.

Abstract

The valorization of underexploited plant resources represents an important strategy for promoting sustainability and the circular economy. In this context, the present work investigated *Cupressus sempervirens* L. fruits as a source of bioactive compounds, with particular emphasis on phenolic compounds, and evaluated their biological properties. Fruits collected in northeastern Portugal were subjected to hydro-methanolic extraction. Phenolic compounds were chemically characterized by ultra-high-performance liquid chromatography coupled with diode-array detection and electrospray ionization tandem mass spectrometry. In parallel, a colorimetric assay was performed to assess the presence of saponin-like reactivity. The phenolic profile was dominated by flavan-3-ols and B-type procyanidins, with catechin, epicatechin, and procyanidin oligomers as the major constituents. The colorimetric assay suggested the presence of saponin-like compounds; however, no individual saponins were confirmed by chromatographic analysis, indicating that this response is likely associated with non-saponin secondary metabolites. Biological evaluation demonstrated significant antioxidant activity and cytocompatibility in human keratinocytes and fibroblasts, while anti-inflammatory assays using THP-1-derived macrophages showed reduced interleukin-6 production. Overall, the results highlight *Cupressus sempervirens* L. fruits as a valuable source of phenolic compounds with relevant biological activities, supporting their potential application in cosmetic, pharmaceutical, and nutraceutical fields and contributing to the sustainable valorization of forest bio-residues.

Keywords: *Cupressus sempervirens* L., forest bio-residues, phenolic compounds, phytochemical profiling, antioxidant activity, anti-inflammatory activity.

Resumo

A valorização de recursos vegetais subexplorados constitui uma estratégia relevante para promover a sustentabilidade e a economia circular. Neste contexto, o presente trabalho investigou os frutos de *Cupressus sempervirens* L. como fonte de compostos bioativos, com particular enfoque nos compostos fenólicos, e avaliou as suas propriedades biológicas. Os frutos recolhidos no nordeste de Portugal foram submetidos a uma extração hidro-metanólica. Os compostos fenólicos foram caracterizados por cromatografia líquida de ultra alta eficiência com ionização eletrospray acoplada a espectroscopia de massas. Em paralelo, foi aplicado um ensaio colorimétrico para a estimativa de reatividade do tipo saponínica. O perfil fenólico foi dominado por flavan-3-óis e procianidinas do tipo B, sendo a catequina, a epicatequina e os oligómeros de procianidina os principais constituintes. O ensaio colorimétrico sugeriu a presença de compostos com reatividade semelhante às saponinas; no entanto, não foi possível confirmar a presença de saponinas individuais por análise cromatográfica, indicando que esta resposta está provavelmente associada a outros metabolitos secundários não saponínicos. A avaliação biológica demonstrou atividade antioxidante significativa e citocompatibilidade em queratinócitos e fibroblastos humanos, enquanto os ensaios anti-inflamatórios em macrófagos derivados de THP-1 evidenciaram uma redução da produção de interleucina-6. De um modo geral, os resultados obtidos destacam os frutos de *Cupressus sempervirens* L. como uma fonte relevante de compostos fenólicos com atividades biológicas promissoras, apoiando o seu potencial de aplicação nas áreas cosmética, farmacêutica e nutracêutica e contribuindo para a valorização sustentável de bio-resíduos florestais.

Palavras-chave: *Cupressus sempervirens* L., bio-resíduos florestais, compostos fenólicos, perfil fitoquímico, atividade antioxidante, atividade anti-inflamatória.

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List of Abbreviations

- ACN** – Acetonitrile
- AAPH** – 2,2'-Azobis(2-methylpropionamide) dihydrochloride
- ASE** – Accelerated Solvent Extraction
- CN** – Cellulose Nanoparticles
- CNC** – Cellulose Nanocrystals
- CNF** – Cellulose Nanofibers
- DAD** – Diode-Array Detection
- DDS** – Drug Delivery Systems
- DDA** – Data-Dependent Acquisition
- ELISA** – Enzyme-Linked Immunosorbent Assay
- ESI** – Electrospray Ionization
- EtOH** – Ethanol
- FA** – Formic Acid
- H-ESI** – Heated Electrospray Ionization
- HFF-1** – Human Foreskin Fibroblasts
- HPLC** – High-Performance Liquid Chromatography
- IBD** – Inflammatory Bowel Disease
- IL-6** – Interleukin 6
- IPR** – Intellectual Property Rights
- LC** – Liquid Chromatography
- LC/DAD/ESI-MS** – Liquid Chromatography with Diode-Array Detection and Electrospray Ionization Mass Spectrometry
- LC/DAD/ESI-MS/MS** – Liquid Chromatography with Diode-Array Detection and Electrospray Ionization Tandem Mass Spectrometry
- LPS** – Lipopolysaccharide
- LTQ** – Linear Trap Quadrupole
- MDA** – Malondialdehyde
- MS** – Mass Spectrometry
- MS/MS** – Tandem Mass Spectrometry
- ORAC** – Oxygen Radical Absorbance Capacity
- PBS** – Phosphate-Buffered Saline

ROS – Reactive Oxygen Species

SPF – Sun Protection Factor

TBA – Thiobarbituric Acid

TBARS – Thiobarbituric Acid Reactive Substances

THP-1 – Human Monocytic Cell Line

UHPLC – Ultra-High Performance Liquid Chromatography

UV-Vis – Ultraviolet-Visible

WPC – Wood-Plastic Composites

1. Introduction

Ornamental plants are renowned for their esthetic value, enhancing landscapes and enriching our living spaces with their vibrant colors and scents. However, their significance extends beyond visual beauty, as many of these plants also possess nutritional and medicinal properties that have been utilized in various cultures for centuries (Takahashi *et al.*, 2020). In ornamental plants, leaf, flower and cone development is not only a key aspect of their reproductive cycle but also a critical phase during which significant biochemical transformations occur. These transformations often result in the accumulation of valuable phytochemicals, such as flavonoids, phenolic acids, methylxanthines, and saponins (Zeng and Wang, 2024). Therefore, understanding the phytochemical composition of these plants can lead to their extended and more effective use in the food and pharmaceutical industries (Teixeira *et al.*, 2023).

Over the years, nature has been a source of food and bioactive compounds, with its resources included in folk medicine and serving as a starting point for developing bio-based ingredients for incorporation into functional foods or food supplements, nutraceuticals, and pharmaceutical and cosmetic formulations (Yu *et al.*, 2021). Therefore, plant bioactive compounds have been the subject of numerous studies aimed at developing new bio-based products. However, given the finite nature of natural resources, they should not be overexploited. Consequently, bio-residues have been identified as an excellent source of biologically active compounds, promoting the circular economy and the sustainability of processes and products (Ueda *et al.*, 2022).

In Europe, the dominant application of forest bio-residues is combustion, co-incineration, or fuel conversion (gasification, pyrolysis, torrefaction). The use of plants to produce biogas is currently at the research stage, as waste requires pre-treatment, and this reduces its attractiveness compared to agri-food waste (Thorenz *et al.*, 2018). In addition to energy and adsorbents production, forest bio-residues may be used for the production of other biomaterials, such as wood–plastic composites (WPC) and wood-based composites, as well as the production of cellulose nanoparticles (CN), including cellulose nanocrystals (CNC) and cellulose nanofibers (CNF) (Braghiroli and Passarini, 2020).

2. Objectives

Given their bioactive properties and potential industrial applications, plant secondary metabolites, such as phenolic compounds and saponins, are considered high-value compounds. Aiming to add value to forest/ornamental resources as sources of these compounds, the present work focused on the Mediterranean cypress (*Cupressus sempervirens* L.) fruit. The information regarding the phytochemical composition and bioactive potential of *C. sempervirens* fruits remains limited, and these are often discarded, generating large amounts of bio-residues.

Therefore, for this study, the following **OBJECTIVES** were proposed:

2.1. Main objective:

Valorization of *Cupressus sempervirens* L. fruits as a source of bioactive compounds, aiming to promote the utilization of this currently underexploited plant material.

2.2. Specific objectives :

- Obtain extracts from *Cupressus sempervirens* L. fruits using appropriate extraction approaches compatible with phytochemical and biological evaluation.
- Chemically characterize the obtained extracts for their phenolic compound and saponin content using spectrophotometric screening and liquid chromatography coupled with mass spectrometry (LC/DAD/ESI-MS).
- Evaluate the biological activities of *C. sempervirens* fruit extracts, including antioxidant, cytocompatibility, and anti-inflammatory effects, using relevant *in vitro* models.

3. Literature Review

3.1. *Cupressus sempervirens* L.

Cupressus sempervirens L., also known as Italian or Mediterranean cypress, is found in subtropical Asia, North America and the eastern Mediterranean region. It is found in Mediterranean bioclimates, in semi-arid or sub-humid regions, except in the variants with cold winters. It has a thermophilic temperament, is very xerophilic, and is very frugal. It is also considered a halophilic species. However, it has a long cultivation tradition dating back to ancient civilizations, and its natural range remains unclear (Caudullo and de Rigo, 2016).

C. sempervirens is a medium-sized evergreen coniferous tree with a very variable crown shape, from columnar to spread, dark green foliage, and small ovoid brown cones (Caudullo and de Rigo, 2016) (Figure 1). This tree grows up to 30 m tall. The leaves are 0.5 – 1 mm, dark green and obtuse. The male cones are 4 to 8 mm, and the female cones are 25 to 40 mm. They are elliptical-oblong (rarely globose), green when young and shining yellowish-grey when ripe, with 8 to 14 short, and obtusely spiked scales; each scale has 8 to 20 seeds (Al-Snafi, 2016).



Figure 1. *Cupressus sempervirens* L. and their fruits (Authorship: Isabel Garcia-Cabral, 2010 and Sánchez, 2025).

C. sempervirens is a multipurpose species which is utilized in the protection of the forest, soil conservation, to maintain the beauty of the landscape, decorative purposes, windbreaks, and the restoration of vegetation on derelict land, as well as being suited for producing superior-quality timber, its wood used for furniture, veneer, door frames, and window frames (Greenfield, 2024). It also has a social function in Mediterranean villages, churches, and cemeteries because it was once considered a protective and sacred tree.

Tests show that the Mediterranean cypress has low flammability, and it may be planted as a firebreak due to its dense foliage and acidic leaf litter, which completely cover the soil, eliminating the halophilic stratum from its shade and allowing the establishment of a cinephilic stratum (Rojas-Sandova, 2016).

3.1.1. *Cupressus sempervirens* L. tree's cultural relevance and utilization in folk medicine

Species of *Cupressus* have been considered very important from traditional to modern times due to their many useful characteristics (Farahmand, 2020). The Mediterranean cypress is a sacred and highly revered tree with a long and rich history in cultures across the Mediterranean basin, West Asia, Asia Minor, North Africa, and North America. It is an integral part of the landscapes, economies, histories, symbolism, traditions, religions, cultures, romances, and folklores of most countries in the Mediterranean and Middle Eastern regions (Farahmand, 2020). The tree has religious significance, e.g., to the Zoroastrians of Iran, who have a story that Zoroaster brought a sapling from Paradise. Many huge individual trees are protected in Iran, perhaps partly because of this belief (Lewis, 2023).

Known as the ‘mournful tree’ by the Greeks and Romans, the tree was sacred to the Fates and Furies, as well as to the rulers of the underworld. These cultural and symbolic uses mainly refer to the whole tree rather than a specific plant organ. Cypress trees were commonly planted near graves or houses as symbols of mourning and protection. Romans would carry branches of cypress as a sign of respect, and the bodies of the respected were placed upon cypress branches prior to interment. It is for reasons such as these that the tree still survives in the Muslim world and in Western culture; the cypress symbolizes hope, pointing to the heavens. Here, there is a strong continuity of use for the tree, as, despite cultural interchange, it remains understood for the same purposes in detail (Mourning, 2011).

Some pharmacological investigations showed that *C. sempervirens* also has biological properties, including aromatherapeutic, antiseptic, astringent, balsamic, anti-inflammatory, antiperspirant, diuretic, and antispasmodic (Batiha et al., 2023). These activities have been primarily attributed to extracts obtained from the leaves, cones, and essential oils of the plant. It has been used for centuries in traditional medicine for its expectorant and antiseptic properties, particularly through preparations of leaves and cones, to treat coughs, bronchitis, diabetes, boils, and laryngitis, as well as inflammation

and toothache (Nehdi, 2013). In addition, extracts derived mainly from leaves and cones have shown antioxidant, antimicrobial, and insecticidal effects (Hasaballah *et al.*, 2018). This evidence confirms the plant's long history of use in traditional medicine across many countries, primarily for cough and flu treatment. Moreover, *C. sempervirens* leaves and cones have been used as folk remedies in different parts of the world for antiseptic, antipyretic, anthelmintic, astringent, antirheumatic, antihemorrhoidal, antidiarrhoeic, and vasoconstrictive purposes. More recent research has shown that *C. sempervirens* exhibits antimicrobial, antiviral, insecticidal, antihyperlipidemic, cytotoxic, antioxidant, antiplatelet, hepatoprotective, and neurobiological activities to varying extents (Orhan & Tumen, 2015).

A decoction of *C. sempervirens* leaves and cones was used in a sitz bath 3 times a day for 1 week for hemorrhoids. The cones and leaves were used orally for internal illnesses. Externally, formulations prepared from leaf and cone extracts (ointments and suppositories) were applied to treat hemorrhoids, venous circulation disorders, and varicose veins. The essential oil was utilized as an antispasmodic for persistent coughs and as an antiseptic (Rawat *et al.*, 2010). Cypress has also been used as a diuretic to increase kidney and venous circulation, improve bladder tone and treat urinary incontinence and enuresis (Mahmood *et al.*, 2013). The parts of the plant utilized for medicinal purposes were the cones and leaves (Rawat *et al.*, 2010).

3.1.2. Bioactive compounds present in *Cupressus sempervirens* L.

Studies on the phytochemical composition of *C. sempervirens* remain scarce. Preliminary analyses of *C. sempervirens* revealed that it is mainly constituted by alkaloids (0.7%), saponins (1.9%), flavonoids (0.22%), phenols 0.067%, tannins (0.31%), terpenes (42.70 – 43.21%), among other bioactive compounds (Al-Snafi, 2016). The leaves, fruits, and cones of *C. sempervirens* contain essential oils. Selim *et al.*, (2014) determined 20 compounds in the oil of Mediterranean cypress, including tricyclene, α -thujene, α -pinene, camphene, sabinene, β -pinene, myrcene, δ -3-carene, p-cymene, limonene, γ - γ -terpinene, α -terpinolene, camphor, bronyl acetate, carvacrol, β -caryophyllene, α -humulene, germacrene-D, δ -cadinene and α -cedrol. However, the major components were α -pinene (48.6%), δ -3-carene (22.1%), limonene (4.6%), and α -terpinolene (4.5%).

According to Al-Rajhi *et al.* (2023) the HPLC analysis revealed the existence of 18 phenolic and flavonoid compounds in the *C. sempervirens* extract. Hesperetin has the

highest concentration (25,579.57 $\mu\text{g/mL}$). At the same time, other compounds, such as pyro catechol, rutin, gallic acid, chlorogenic acid, naringenin, and quercetin, were recognized in concentrations of 2922.53 $\mu\text{g/mL}$, 1313.26 $\mu\text{g/mL}$, 1107.26 $\mu\text{g/mL}$, 389.09 $\mu\text{g/mL}$, 156.53 $\mu\text{g/mL}$, and 97.56 $\mu\text{g/mL}$, respectively.

No research on saponins in *C. sempervirens* was found, underscoring the need for further studies to investigate this compound class in the plant.

3.2. Phenolic compounds

Phenolic compounds are the most widely distributed secondary metabolites, ubiquitously present in the plant kingdom, with immense structures and functions. They are among the most abundant classes of phytochemicals with health-promoting properties (Swallah *et al.*, 2020). Phenolic compounds, or polyphenols, constitute one of the most numerous and widely distributed groups of substances in the plant kingdom, with more than 8.000 phenolic structures currently known (Urquiaga and Leighton, 2000). They are important secondary metabolites in fruits, vegetables, tea, coffee, chocolates, legumes, cereals, and beverages (Ganesan and Xu, 2017). They are present in all organs of the most diverse plants (fruits, seeds, roots, bark, wood, and leaves) (Teplova *et al.*, 2018). In terms of structure, they have a common element, which is the presence of at least one hydroxyl group on an aromatic ring. It can contain a free hydroxyl or another functional group (ether, ester, or heteroside) (García-Pérez, M., Kasangana, P. B., and Stevanovic, T., 2017; Elazzouzi *et al.*, 2019).

3.2.1. Chemical structure and classification of phenolic compounds

The most widely adopted classification in the literature divides phenolics into two main groups: flavonoids and non-flavonoids (Kabera, 2014; De La Rosa *et al.*, 2019; Durazzo *et al.*, 2019). Furthermore, in another classification (Figure 2), phenolic compounds are divided into several groups based on the phenolic rings and the elements that connect them, including flavonoids, phenolic acids, stilbenes, and lignans (Di Lorenzo *et al.*, 2021).

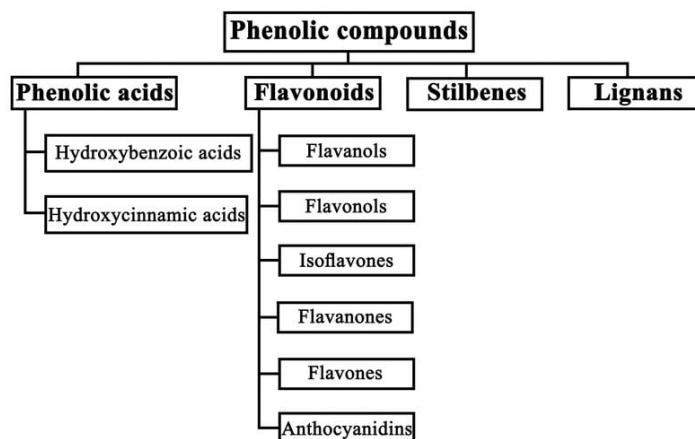


Figure 2. Classification of phenolics based on the phenolic rings and their connecting elements (Authorship: Bachir & Mohamed, 2021).

Phenolic acids and their derivatives are secondary metabolites widely spread in fruits, vegetables, and cereal grains (Das, Goud and Das, 2019). The primary sources are berries, cherries, apples, citrus fruits, rice bran, passion fruit, mangoes, coffee, kiwis, tea, wheat, corn, and oat flours (Klensporf-Pawlik and Aladedunye, 2017). Phenolic acids are mainly divided into two sub-groups: hydroxybenzoic and hydroxycinnamic acids (Bachir and Mohamed, 2021). In plants, phenolic acids play roles in growth, reproduction, and defense against environmental stress and microorganisms. Most of them are an integral part of the human diet and are also consumed as medicinal preparations (Shahidi and Ambigaipalan, 2015). Many of the health-protective effects of phenolic acids have been ascribed to their antioxidant, antiulcer, antidiabetic, cardioprotective, antimutagenic, anticarcinogenic, anti-inflammatory, neuroprotective, hepatoprotective, antiaging and antimicrobial properties (Saibabu *et al.*, 2015).

Flavonoids are a group of more than 4.000 phenolic compounds that occur naturally in fruits, vegetables, and beverages (tea, coffee, and fruit drinks) (Raguindin *et al.*, 2021). Their general structure consists of two phenyl rings (A and B) and a heterocyclic ring (C), with the carbon structure abbreviated as C₆-C₃-C₆. Due to the hydroxylation pattern and variations in the chromane ring (Ring C), flavonoids can be further divided into different sub-groups such as anthocyanins, flavan-3-ols, flavanones, and flavonols. Among these subgroups, flavan-3-ols and their oligomeric/polymeric forms, known as procyanidin, represent an important and biologically active subclass of flavonoids. Flavan-3-ols, such as catechin and epicatechin, are commonly found in many plant-derived foods and beverages and are the main dietary forms of flavonoids in human

nutrition. procyanidin are formed by the polymerization of flavan-3-ol units and include dimers, trimers, and higher oligomers (Dobani *et al.*, 2025). Flavonoids are crucial for normal growth and development, as well as for plant defense systems. Some flavonoids are responsible for imparting color, flavor, odor to flowers, fruits, and leaves (Albishi, 2018). In addition to their antioxidant properties, flavonoids exhibit diverse biological activities that contribute to human health. These include anti-inflammatory, antihyperlipidemic, hepatoprotective, antiulcer, antiviral, anti-cancer, anti-diabetic and cytotoxic effects (Durazzo *et al.*, 2019; karak, 2019).

Stilbenes are phenolics with a small molecular weight (200–300 g/mol). They are naturally found in a wide variety of dietary sources, such as grapes, blueberries, and other plants (Tsai *et al.*, 2017). Structurally, stilbenes have a basic carbon skeleton, C₆–C₂–C₆, that contains two benzene rings linked by an ethanol or ethylene group. Stilbenes and their analogues hold enormous potential due to their diverse spectrum of biological applications, such as anticancer, antiproliferative, antiangiogenesis, antimicrobial, antileukemic, antioxidant, anti-inflammatory, anti-HIV, anti-herpes simplex virus, and tyrosine kinase inhibitors.

The lignans belong to a group of polyphenolic compounds containing the 2,3-dibenzylbutane skeleton. They are present in seeds, vegetable oils, cereals, legumes, fruits, and vegetables as aglycones, glycosides, esterified glycosides, or as bio-oligomers (Gerstenmeyer *et al.*, 2013; Nabavi *et al.*, 2013). Structurally, they are formed of two phenylpropane units joined together by at least one carbon-carbon bond between the two central β -carbons of the C₃ chains (lignans) or by bonds other than the β - β' carbon-carbon bond (neolignans) (Ferrazzano *et al.*, 2011). The lignans have exhibited several biological activities, including anticancer, antimicrobial, antiviral, immunosuppressive, anti-inflammatory, antioxidant, and hepatoprotective actions, as well as osteoporosis-prevention properties (Pilkington, 2018).

3.2.2. Bioactive properties of phenolic compounds

The evaluation of the bioactive properties of phenolic compounds from different plant sources has become a rapidly growing area of research. This growing interest is mainly attributed to structural diversity and their ability to interact with multiple biological targets, resulting in a wide range of biological activities.

Reactive Oxygen Species (ROS) are radicals that entail more than one unpaired electron in the outer orbit or highly reactive non-radicals. They may damage the protective mechanisms of cells and destroy the structure of DNA, proteins and lipids in biological systems (Hogervorst Cvejić *et al.*, 2017). The human body needs endogenous and exogenous antioxidants to minimize the effects of ROS. In this respect, phenolic compounds are highly crucial for human health (Güneş Bayir, Aksoy and Koçyiğit, 2019). The antioxidant activity of different phenolic compounds varies according to their chemical structures (Ahmaruzzaman *et al.*, 2024). The antioxidant property of phenolic compounds stems from their hydroxyl groups and conjugated aromatic system, and it depends on the ability of phenolic compounds to give electrons to trap free radicals by forming stable phenoxyl compounds (Reddy *et al.*, 2020). In cases where the concentration of phenolic compounds is high, due to increased numbers of hydroxyl groups in the reaction medium and the greater likelihood of hydrogen donation to free radicals, the radical-scavenging ability is noticeable (Ebrahimi and Lante, 2021). The radical-scavenging ability largely depends on the position and number of hydroxyl groups, as well as the molecular weight of phenolic compounds. In phenolic compounds with lower molecular weight, hydroxyl groups are more readily available. Therefore, it should be noted that the type and quality of phenolic compounds are more responsible for the amount of antioxidant activity than their quantity (Dessalegn *et al.*, 2025).

Inflammation is a natural protective mechanism that occurs when biotic, physical, or chemical agents disrupt the body's tissue homeostatic mechanisms. The immune response generates pro-inflammatory mediators, but excessive output, such as chronic inflammation, contributes to many persistent diseases. Some phenolic compounds work in tandem with nonsteroidal anti-inflammatory drugs to inhibit the activity or gene expression of pro-inflammatory mediators.

The antimicrobial activity of phenolic compounds is strongly influenced by their molecular structure. The presence of hydroxyl groups (-OH) enables substances to interact with charged molecules such as RNA, DNA, and proteins, thereby triggering an antimicrobial effect. The biocidal activity of phenolic compounds results from a combination of membrane disruption, enzyme inhibition and oxidative damage, making them promising antimicrobial agents in food preservation and agriculture (Dembińska *et al.*, 2025).

Phenolic compounds are also known for their antidiabetic properties. Cytotoxic human amylin [hA] accumulates, provokes cytotoxicity in pancreatic islet β cells, and

disrupts these cells. Additionally, their role in oxidative stress and inflammation is pronounced. Phenolics exhibit significant antidiabetic effects by inhibiting hA accumulation and modulate ROS and inflammation, thereby protecting β -cells. Recent research suggests that they exerts their effects by reducing ROS, inflammation, and cellular pathways; this may have benefit β -cell survival and insulin sensitivity (Nie and Cooper, 2021).

Phenolic compounds have also emerged as potential neuroprotective agents due to their ability to reduce neuroinflammation and enhance neuronal survival. They can cross the blood-brain barrier and exert effects on neuronal health, mitigating neurodegenerative diseases, including Alzheimer's and Parkinson's, by reducing oxidative stress and inflammation and enhancing neuronal communication (Hossain *et al.*, 2019; Rahman *et al.*, 2020).

3.2.3. Applications of phenolic compounds

Phenolic compounds have several biotechnological applications in different industries. Their exploitation is due to their antioxidant, antimicrobial, and colouring properties, among others, especially by the food industry for food preservation and packaging, and by the cosmetics and textile industries.

In agriculture, phenolic compounds have emerged as promising agents for controlling a wide range of phytopathogens and food spoilage microorganisms. Most crops are susceptible to fungal diseases, including those of significant economic importance, such as rice and wheat (Zhou *et al.*, 2024). The antimicrobial potential of phenolic compounds has been extensively demonstrated through various *in vitro* and *in vivo* studies, highlighting their capacity to inhibit fungal growth, disrupt cell membranes, and interfere with toxin biosynthesis (Dembińska *et al.*, 2025).

In food packaging, phenolic compounds can be used to improve food safety and extend freshness and shelf life by reducing microbial growth. This approach is particularly relevant in the context of foodborne pathogen contamination (Aladhadh, 2023).

Phenolics have also shown potential for use as bioactive ingredients in cosmeceutical products (Soto *et al.*, 2015). They can absorb ultraviolet radiation due to the presence of chromophores in their structure, avoiding the penetration of solar radiation into the skin. UV protection has been reported for some phenolics, which have

shown a sun protection factor (SPF) ranging from 7 to 30 (De Lima Cherubim et al., 2020). As is well known, free radicals can accelerate ageing by interacting with proteins in the skin, leading to the degradation of collagen and elastin. In a study on these skin lesions, a body cream formulated with the phenolic extract of *Nymphaea rubra Roxb* was effective in reducing skin wrinkles and improving skin whitening, with no irritation, in healthy volunteers after 60 days of use (Kamma and Leelapornpisid, 2019).

The textile industry is one of the highest polluters, as large amounts of water are contaminated with heavy loads of chemicals. On the other hand, allergic reactions have also been associated with synthetic dyes. In this scenario, the interest in the use of phenolics as natural dyes with high biodegradability has grown (Bonet-Aracil *et al.*, 2016; Jia *et al.*, 2017). In addition to being less aggressive to the environment, natural dyes obtained from red, black and green tea extracts, showed UV protection when applied in Tussah silk and cotton, respectively. Antimicrobial activity against *E. coli* and *S. aureus* was also demonstrated using a natural dye from oak bark (Jia *et al.*, 2017).

Overall, phenolic compounds have attracted considerable attention due to their wide range of health-promoting and therapeutic properties, as reflected in the growing number of technological and pharmaceutical innovations. Some of the patent applications involve the use of phenolic compounds for the development of therapeutics for the treatment and prevention of disorders such as cancer, cardiac diseases, tumors, and obesity. They are also exploited for the control of microorganisms and mycotoxins, as well as for the production of rigid foams, anti-adhesive coatings and metal nanoparticles (De Araújo *et al.*, 2021). Similarly, a balanced combination containing nutrients, phenolic compounds, anti-oxidative elements, and essential micronutrients can be used to prevent cardiovascular disease, Alzheimer's disease, and diabetes, and to regulate and reduce blood sugar and insulin resistance (Koynova and Tenchov, 2018).

3.3. Saponins

Saponins are widely distributed in nature and are found in several plants and some marine animals (Decroo *et al.*, 2017). The primary dietary source of saponins is legumes. Saponin content, structure, and composition may vary even among the same species due to differences in cultivars, growing locations, irrigation conditions, soil types, and climatic conditions (Shi *et al.*, 2004).

Structurally, saponins are a diverse group of compounds with extensive functional diversity (Moses, Papadopoulou and Osbourn, 2014). As schematically presented in Figure. 3, there are generally two functional groups in the saponin structure: an aglycone (a 30-carbon skeleton molecule) and a sugar chain (with one or more sugar units) (Ashour, El Aziz and Gomha Melad, 2019). Aglycone components are triterpenoids or steroids. The glycone or sugar moiety generally consists of a monosaccharide or an oligosaccharide covalently linked to the skeleton molecule at the C3 position (K M and Naik, 2019). Saponins have been traditionally used as natural detergents. The name ‘saponin’ comes from the Latin word ‘sapo,’ which means ‘soap’ as saponins show the unique properties of foaming and emulsifying agents (Najjar, 2017). They consist of a hydrophilic sugar moiety linked to a lipophilic aglycone (Figure 3), conferring amphiphilic properties and unique functional properties. Their amphiphilic structures enable saponins to exhibit surface-active properties, resulting in stable foams and complexes with various molecules (Timilsena et al., 2023).

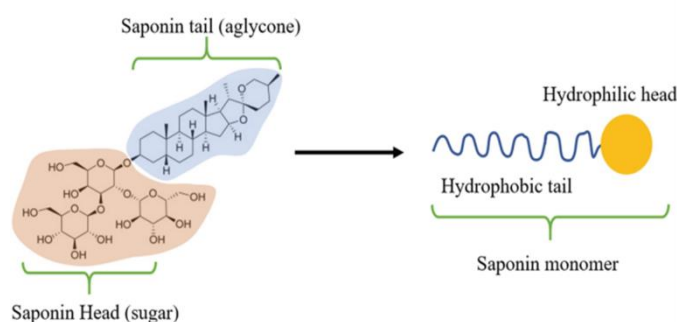


Figure 3. Representation of the hydrophilic and hydrophobic parts of saponins (Author: Akbari et al., 2023).

3.3.1. Bioactive properties of saponins

Saponins have been reported to exhibit a wide variety of biological activities. They demonstrate efficacy in combating inflammation and acting as potent antimicrobial agents. They can inhibit the production of pro-inflammatory cytokines and enzymes, thereby reducing inflammation and alleviating symptoms in conditions like arthritis, inflammatory bowel disease (IBD), and other inflammatory disorders (Dong *et al.*, 2019).

Various saponins serve as adjuvants and immunostimulants; they enhance the immune response to infections and diseases and can activate immune cells, such as macrophages and natural killer cells, which are crucial in combating infections. Additionally, saponins are used as adjuvants in vaccines to boost the immune response

and improve vaccine efficacy (Güçlü-Üstündağ and Mazza, 2007; Timilsena, Phosanam and Stockmann, 2023).

Saponins possess antioxidant activity, helping neutralize free radicals and oxidative stress in the body. By scavenging these free radicals, saponins help protect cells from damage and promote overall health. Saponins can form insoluble complexes with cholesterol and other sterols, as well as bile acids. They can trap total cholesterol, LDL, and bile salts in the intestines, inhibiting their absorption, without affecting HDL levels (Kim *et al.*, 2003). By lowering LDL cholesterol, saponins contribute to cardiovascular health and reduce the risk of heart disease (Timilsena, Phosanam and Stockmann, 2023).

Saponins exhibit antimicrobial effects against a wide range of pathogens by disrupting the microbial cell membranes and interfering with their replication, making them potential candidates for developing new antimicrobial agents and improving existing treatments. Oleanolic acid, derived from the root bark of *Newbouldia laevis*, exhibited wide-ranging antimicrobial properties when tested against six Gram-positive, twelve Gram-negative bacterial species, and three *Candida* species (Patra, 2012).

The cytotoxic effects of most of the reviewed saponins were due to their ability to stimulate the apoptotic process in tumor cells, usually through the intrinsic pathway. Moreover, non-apoptotic processes were also involved in saponin cytotoxicity, including cell cycle arrest, stimulation of autophagic cell death, inhibition of metastasis, and cytoskeleton disintegration (Podolak, Galanty and Sobolewska, 2010).

3.3.2. Saponins applications

Saponins serve as natural emulsifiers, foaming agents, and stabilizers, thereby enhancing the texture and stability of food products. Similarly, saponins' antimicrobial properties could be explored for their potential as natural preservatives in food products, as they may also be incorporated into (edible) packaging as antimicrobial agents, and help address the challenge of inhibiting the growth of spoilage-causing microorganisms and extend extending the shelf-life (Timilsena, Phosanam and Stockmann, 2023).

Natural surfactants made from plants and microorganisms are an alternative that has been thoroughly researched and is even used commercially by some businesses worldwide (Jahan *et al.*, 2020). Saponins have demonstrated significant promise for use in cosmetics, including shampoos, conditioners, and skincare items (Tsai and Lin, 2019). The use of saponins in shampoo formulations with cleaning, anti-dandruff, and anti-itch

properties has been commercialized and protected by IPR, demonstrating the importance of saponins to the cosmetic industry (Bezerra *et al.*, 2018).

The pharmaceutical industry may use saponin surfactants in drug delivery systems (DDS) (Zhang *et al.*, 2020). Saponins' biological activity and surface activity enable them to interact with medications, thereby improving their efficacy compared to conventional carrier materials. This is accomplished through mechanisms or pathways that are absent in common carrier materials (Peng *et al.*, 2018; Kaur *et al.*, 2024).

The derivatives of these compounds have been tested and found to be beneficial for inhibiting the production of nitric oxide by macrophages, and thus can be employed in the development of chemopreventive and anti-cancer drugs (Moses, Papadopoulou and Osbourn, 2014). The clinical significance of triterpenoid saponins in the prevention and treatment of metabolic and vascular disease is noteworthy (Waheed *et al.*, 2012). Zhang *et al.* (2023) suggested that saponins have the potential to ameliorate Alzheimer's disease by reducing amyloid beta peptide deposition, inhibiting tau phosphorylation, modulating oxidative stress, reducing inflammation, and promoting antiapoptosis.

In agriculture, livestock and pet industries, saponins are employed in animal nutrition and as natural biocontrol agents (Hussain *et al.*, 2019). The exogenous application of saponins affects various biological processes; for instance, in the presence of saponins, more rapid germination of corn and pea seeds is observed due to increased water absorption. Biosurfactant tea saponins have significant potential in the removal of heavy metal ions such as cadmium, copper and lead (Mulligan, 2021).

4. Material and Methods

4.1. Sampling of *Cupressus sempervirens* L. fruits

The fruits of *Cupressus sempervirens* L. were collected in public areas of the city of Bragança, in the Northeast region of Portugal, during April 2024. Afterwards, the fruits were washed, air-dried at room temperature, and ground into a fine powder using an electric grinder, and stored in a place protected from light and temperature (to prevent the loss of active components) for subsequent analyses.

4.2. Extraction

Maceration extraction was employed as a standard procedure for the recovery of bioactive compounds from the fruits of *Cupressus sempervirens* L. and for subsequent bioactivity evaluation. 25 g of the powder were macerated in 150 mL of 80% (v/v) methanol (hydro-methanol). The mixture was maintained under continuous agitation at room temperature for 16 h, protected from light. Following extraction, the suspension was centrifuged at 5000 rpm for 10 min, and the supernatant was carefully collected. The solvent was subsequently removed under reduced pressure at 35 °C using a rotavapor (Rotary Evaporator model Hei-VAP from Heidolph, Schwabach, Germany), yielding a concentrated crude extract.

4.3. Liquid Chromatography with Diode-Array Detection and Electrospray Ionization Tandem Mass Spectrometry (LC/DAD/ESI-MS²) phenolic compounds Analysis

For the analysis of phenolic compounds in *Cupressus sempervirens* L. fruits, first, 10 mg of dry extract was weighed and placed in 1.5 mL microcentrifuge tubes, then dissolved in EtOH/H₂O (80:20, v/v). This is because 10 mg of extract is used for each 1 mL of ethanol. After dissolving the extract completely, the samples were filtered through a 0.2 µm disposable nylon disk filter LC and transferred to an injection vial for HPLC-DAD analysis.

Phenolic compounds were analyzed using a UHPLC–DAD–Orbitrap MS (Thermo Scientific, Jan Jose, CA, USA). Chromatographic separation was carried out on a reversed-phase C18 column using water containing 0.1% formic acid (solvent A) and

acetonitrile (solvent B) as mobile phases. The elution gradient was programmed as follows: 0–3 min, 10% B; 3–37 min, linear increase from 10 to 14.5% B; 37–40 min, increase to 20% B; 40–55 min, increase to 35% B; 55–60 min, increase to 60% B; followed by a return to initial conditions (60 to 0% B) between 63 and 66 min and a re-equilibration period at 0% B from 66 to 70 min. Mass spectrometric detection was performed using a heated electrospray ionization source (H-ESI) operating in negative ionization mode, with a spray voltage of 2.5 kV. Nitrogen was used as the nebulizing gas at a flow rate of 50 arbitrary units, while auxiliary and sweep gas flows were set at 10 and 1 arbitrary units, respectively. The ion transfer tube and vaporizer temperatures were maintained at 325 °C and 350 °C, respectively. Data acquisition was carried out in data-dependent acquisition (DDA) mode, employing a normalized collision energy of 30%. Full-scan mass spectra were acquired over an m/z range of 110–1100. Compounds identification was carried out by comparison of retention time, UV-Vis spectra and fragmentation patterns (MS/MS) with authentic standard, or with data from literature. Quantification was performed using external calibration curves obtained with catechin standard ($y = 5156.7x - 5176.7$; $R^2=0.9902$).



Figure 4. Vanquish UHPLC System

4.4. Quantification of total saponins

Total saponin content was determined by using the vanillin-sulphuric acid spectrophotometric method, according to Le et al. (2018). An aliquot of 250 μL of the extract solution was transferred into test tubes, followed by the addition of 250 μL of an 8% (w/v) vanillin solution and 250 μL of sulfuric acid 72% (v/v). The reaction mixture

was vortex-mixed and subsequently incubated at 60 °C for 10 min to allow chromogen formation. After incubation, the reaction was terminated by cooling the tubes in a cold bath. The absorbance of the developed color (Figure 5) was measured at 540 nm using a UV–visible spectrophotometer against a reagent blank, using the solvent of extraction prepared and treated under identical conditions. Quantification was performed using the soyasaponin I standard calibration curve. Results were expressed as saponin concentration (mg/mL) and reported as mean \pm standard deviation from replicate measurements.

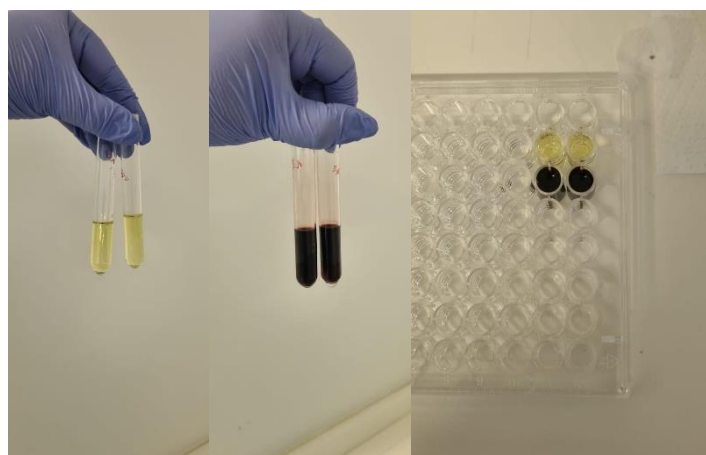


Figure 5. Visual appearance of the chromogenic reaction obtained during total saponin quantification of *Cupressus sempervirens* L. fruit extract using the vanillin–sulfuric acid method.

4.5. Liquid Chromatography with Diode-Array Detection and Electrospray Ionization Tandem Mass Spectrometry (LC/DAD/ESI-MS²) saponins analysis

For saponin analysis, the same procedure used for phenolic compounds was followed, except that the dry extract was dissolved in ACN/H₂O (20:80, v/v) instead of EtOH/H₂O. The solutions were filtered through a 0.2 μ m nylon LC filter and transferred to injection vials for HPLC-DAD analysis. Saponin analysis was performed using a UHPLC Vanquish system (Thermo Scientific, San Jose, CA, USA) equipped with a diode array detector (DAD) and a C18 reversed-phase column (Spherisorb S3 ODS-2, 3 μ m, 4.6 \times 150 mm). The chromatographic separation was achieved using 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) as mobile phases. The elution program consisted of an optimized gradient previously validated for saponin analysis, with a flow rate of 0.5 mL/min. Detection was carried out at 205 nm, a wavelength commonly used for saponin monitoring.

For compound identification, chromatographic profiles were compared with reference data reported in the literature. When applicable, confirmation was performed by mass spectrometric detection using a linear ion trap mass spectrometer (LTQ XL, ThermoFinnigan) operating in positive ionization mode, following conditions described by Dias et al. (2022).

4.6. Bioactivity evaluation

The bioactivity of the extracts was evaluated using different *in vitro* assays. The extracts were obtained by maceration, as previously described in Section 4.2, and the resulting dry extracts were used for all subsequent bioactivity evaluations. For antioxidant assays, dry extracts were weighed (20 mg) and dissolved to a final concentration of 20 mg/mL in the appropriate buffers: potassium phosphate buffer (pH 7.4) for the ORAC assay and Tris–HCl buffer (pH 7.4) for the TBARS assay. For cytocompatibility and anti-inflammatory assays, extract stock solutions were prepared at 80 mg/mL by dissolving the dry extracts in phosphate-buffered saline (PBS). These stock solutions were subsequently diluted in the respective cell culture media to obtain the desired working concentrations for each assay.

4.6.1. Antioxidant activity

The antioxidant capacity of extracts from *Cupressus sempervirens* L. fruits was evaluated using the oxygen radical absorbance capacity (ORAC) and thiobarbituric acid reactive substances (TBARS) assays.

Oxygen radical absorbance capacity (ORAC)

The tests were performed according to the method described by Dávalos et al. (2004) using a microplate reader FLUOstar Omega (BMG LABTECH, Ortenberg, Germany). Fluorescein was used as the fluorescent probe and potassium phosphate buffer (pH 7.4, 75 mM) as the reaction medium. The microplates containing 20 μL of the sample or different concentrations of Trolox, 120 μL of fluorescein (0.4 $\mu\text{g}/\text{mL}$), and 60 μL of radical AAPH (2,2'-azobis (2-methylpropionamidine) dihydrochloride) (108 $\text{mg}\cdot\text{mL}^{-1}$) was subjected to reading every 1 min for a total of 80 min (emission and excitation wavelength of 485 nm and 520 nm, respectively), under controlled temperature at 37 °C. ORAC results were determined using a regression equation relating Trolox

concentrations to the net area under the fluorescein kinetic decay curve. They were expressed in μmol Trolox Equivalent per 100mL of sample.

Lipid peroxidation inhibition (TBARS)

TBARS assay was performed according to the procedure described by Sarmiento et al., 2015. In this assay, lipid peroxidation is assessed by the products of oxidation that react with thiobarbituric acid (TBA), namely malondialdehyde, which give rise to pink compounds that are known as thiobarbituric acid reactive species (TBARS). Porcine brains were obtained from official slaughter animals, dissected, and homogenized in ice-cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 w/v brain tissue homogenate, which was then centrifuged at 3500g for 10 min. An aliquot (100 μL) of the supernatant was incubated in a 48-well microplate with different concentrations of the extract ranging from 10 mg/mL to 0.039 mg/mL, prepared by serial dilution, in the presence of FeSO_4 (10 mM; 100 μL) and ascorbic acid (0.1 mM; 100 μL). The microplate was incubated at 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 500 μL), followed by the addition of thiobarbituric acid (TBA, 2%, w/v, 350 μL) (Figure 6). The plate was then heated at 80 °C for 20 min, and the contents of each well were transferred to 2 mL tubes that were centrifuged (3000g, 5 min). After centrifugation, 300 μL of the supernatant from each tube was transferred to a 96-well plate, and the absorbance of the malondialdehyde (MDA) TBA complexes formed was measured at 532 nm. A blank using the solvent extraction was prepared under the same conditions. The inhibition ratio (%) was calculated using the following equation:

$$\text{Inhibition ratio (\%)} = \frac{(A-B)}{A} \times 100$$

Equation 1. Percentage of lipid peroxidation inhibition

Where A and B were the absorbance of the control and the sample solution, respectively. All results for antioxidant activity were expressed as EC_{50} values, which correspond to the extract concentration that inhibits lipid peroxidation by 50%.



Figure 6. Visual representation of malondialdehyde (MDA)–TBA complex formation during the evaluation of lipid peroxidation inhibition by *Cupressus sempervirens* L. fruit extract.

4.6.2. Cytotoxicity

Cell culture and seeding

The cytocompatibility of *Cupressus sempervirens* L. fruit extract with the human epidermal keratinocyte cell line (HaCaT, 300493, CLS Cell Lines Service GmbH) and the human foreskin fibroblast cell line (HFF-1, SCRC-1041, ATCC) was evaluated following the procedure described by Vieira et al. (2018), with some modifications. The HaCaT and the HFF-1 cell lines were cultured in high-glucose cDMEM at 37 °C in an atmosphere of 5% CO₂. Before seeding, the cells, at 80% confluence, were detached from the cell culture flask using Trypsin-EDTA.

HaCaT (4000 cells/well) and HFF-1 (4000 cells/well) cell lines were seeded in adherent 96-well culture plates and incubated for 24 h, at 37 °C, in a humidified atmosphere with 5% CO₂. Afterwards, the medium containing non-attached cells was removed by aspiration, and a fresh medium (500 µL) was added. Then, the different concentrations of *C. sempervirens* fruit extract, previously dried and resuspended in culture medium, were added to the cells. For HFF-1 and HaCaT cells, extract concentrations of 6.25, 12.5, 25, 50, 100, 200, and 400 µg/mL were tested.

Human keratinocytes and fibroblasts were incubated with the extract for 1, 2, and 3 days, and the cells' metabolic activity was evaluated at each time point. Cell morphology was analyzed under an inverted microscope (DMi1, Leica, Wetzlar, Germany). Cells cultured without extract were used as a negative control of toxicity. All conditions were performed in triplicate.

Metabolic activity

The metabolic activity of human keratinocytes and fibroblasts incubated with different concentrations of the *C. sempervirens* fruit extract was determined by the reduction of the resazurin (blue) to resorufin (pink) by living cells using the resazurin reduction assay. A stock solution of resazurin sodium salt (440 μM) was prepared in DPBS and further sterilized with a 0.22 μm filter under aseptic conditions (Uzarski *et al.*, 2017). Aliquots of the stock solutions were made and stored at $-20\text{ }^{\circ}\text{C}$ for no longer than 4 weeks. Resazurin working solution (44 μM) was freshly prepared in a cDMEM culture medium by adding 1/10 volume of the stock solution to the initial cell culture medium volume. After 1, 2, and 3 days of culture (Figure 7), the culture medium was removed, and the cells were gently washed three times with sterile, warm HBSS. Then, 300 μL of freshly prepared resazurin working solution was added to each well. A blank was also made (resazurin without cells). The cells were incubated at $37\text{ }^{\circ}\text{C}$ for 4 h in a humidified atmosphere containing 5% CO_2 . Thereafter, the absorbance of resazurin reduction in each sample was measured at 600 and 570 nm using a microplate reader (SpectraMax iD3 Multi-Mode, Molecular Devices).

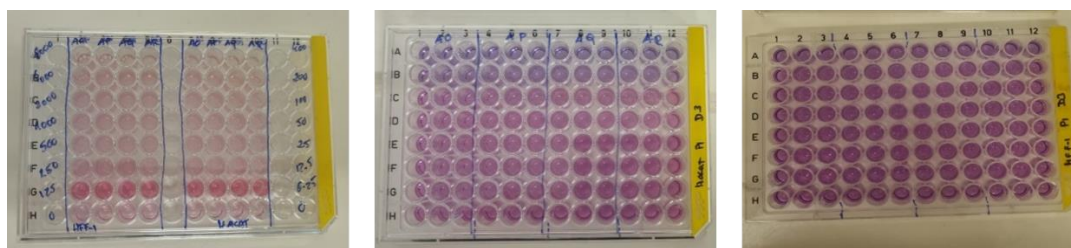


Figure 7. Representative resazurin reduction assay images illustrating cellular metabolic activity before and after treatment with *Cupressus sempervirens* L. fruit extract.

4.6.3. Anti-Inflammatory activity

In vitro evaluation of anti-inflammatory activity using THP-1–derived macrophages

The anti-inflammatory activity of the extracts obtained from *Cupressus sempervirens* L. fruits was evaluated using a macrophage cell culture model, following a procedure adapted from Vieira *et al.* (2020), employing THP-1–derived macrophages and cytokine quantification by ELISA. The human monocytic cell line THP-1 (ATCC TIB-202™) was used as an *in vitro* macrophage model. Cells were maintained in RPMI-1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS),

L-glutamine, and antibiotics, and incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

For macrophage differentiation, THP-1 cells were seeded in 96-well plates at a density of 2.0×10^5 cells/well and treated with phorbol 12-myristate 13-acetate (PMA) for 24 h to induce differentiation into adherent macrophage-like cells. After differentiation, the medium was replaced, and cells were allowed to stabilize before treatment. Differentiated macrophages were treated with different concentrations of *C. Sempervirens* fruit extracts for a defined pre-incubation period. Subsequently, an inflammatory response was induced by lipopolysaccharide (LPS) stimulation. Cells treated with LPS alone were used as positive inflammatory control, while non-stimulated cells were used as the negative control. Dexamethasone was included as a reference anti-inflammatory control.

Evaluation of cell metabolic activity

Before cytokine quantification, macrophage metabolic activity was assessed using the resazurin reduction assay, ensuring that the tested extract concentrations did not compromise cell viability. This step allowed confirmation that any observed modulation of cytokine production was not associated with cytotoxic effects.

Quantification of IL-6 production

After the treatment period, cell culture supernatants were collected for interleukin-6 (IL-6) determination. IL-6 concentrations were quantified using a commercial IL-6 ELISA kit (ELISA MAX™ Deluxe Set, BioLegend), according to the manufacturer's instructions. 96-well plates were coated with capture antibody diluted in coating buffer (100 µL/well) and incubated overnight (16–18 h) at 2–8 °C. The plates were then washed three times with wash buffer (PBS containing 0.05% Tween-20) and blocked with assay diluent (200 µL/well) for 1 h at room temperature with shaking. After washing, 100 µL of IL-6 standards or cell culture supernatant samples were added to the wells and incubated for 2 h at room temperature with shaking. The plates were rewashed, followed by the addition of the detection antibody (100 µL/well) and incubation for 1 h at room temperature with shaking. After additional washing steps, avidin–HRP (100 µL/well) was added and incubated for 30 min at room temperature with shaking. The plates were then

washed four times, with the wash buffer remaining in the wells for 30 s to 1 min during each wash to minimize background signal.

Color development was achieved by adding 100 μL /well of freshly prepared tetramethylbenzidine (TMB) substrate solution and incubating the plates in the dark for 15 min. The reaction was stopped by adding 100 μL /well of the stop solution which is sulfuric acid (Figure 8), and absorbance was measured at 450 nm with a wavelength correction at 570 nm using a microplate reader (SpectraMax iD3 Multi-Mode, Molecular Devices). IL-6 concentrations were calculated from the standard curve and expressed as $\mu\text{g}/\text{mL}$. A reduction in IL-6 production relative to LPS-stimulated control cells was considered indicative of anti-inflammatory activity.

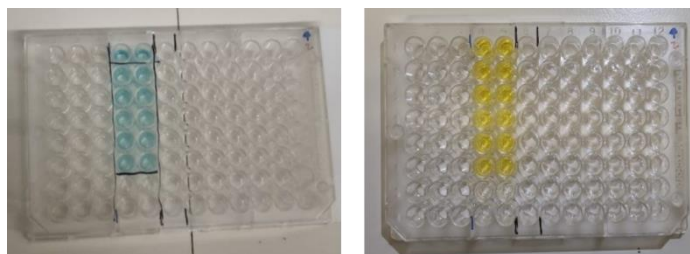


Figure 8. Representative microplate images of IL-6 ELISA assay showing colorimetric detection in THP-1–derived macrophage culture supernatants before (blue) and after (yellow) addition of stop solution.

4.7. Statistical analysis

All experiments were performed in triplicate. The results obtained throughout the different evaluation studies were analyzed using statistical tools selected according to the complexity of the data and the objectives of each assay. Quantitative results were expressed as mean \pm standard deviation (SD). Statistical analysis was applied to support comparisons between control and treated groups. Depending on the type of comparison, one-way analysis of variance (ANOVA) or Student's *t*-test was used. Statistical analyses were performed using Microsoft Excel.

5. Results and Discussion

5.1. Liquid Chromatography with Diode-Array Detection and Electrospray Ionization Tandem Mass Spectrometry (LC/DAD/ESI-MSⁿ) phenolics Analysis

The phenolic profile of *Cupressus sempervirens* L. fruit extract was characterized using UHPLC–DAD–Orbitrap MS operated in negative ionization mode. The chromatographic analysis at 280 nm revealed a complex profile dominated by flavan-3-ols and procyanidins (Figure 9). A total of 25 phenolic compounds were tentatively identified based on their retention times, UV–Vis spectra, and MS/MS fragmentation patterns, and quantified as catechin equivalents (Table 1).

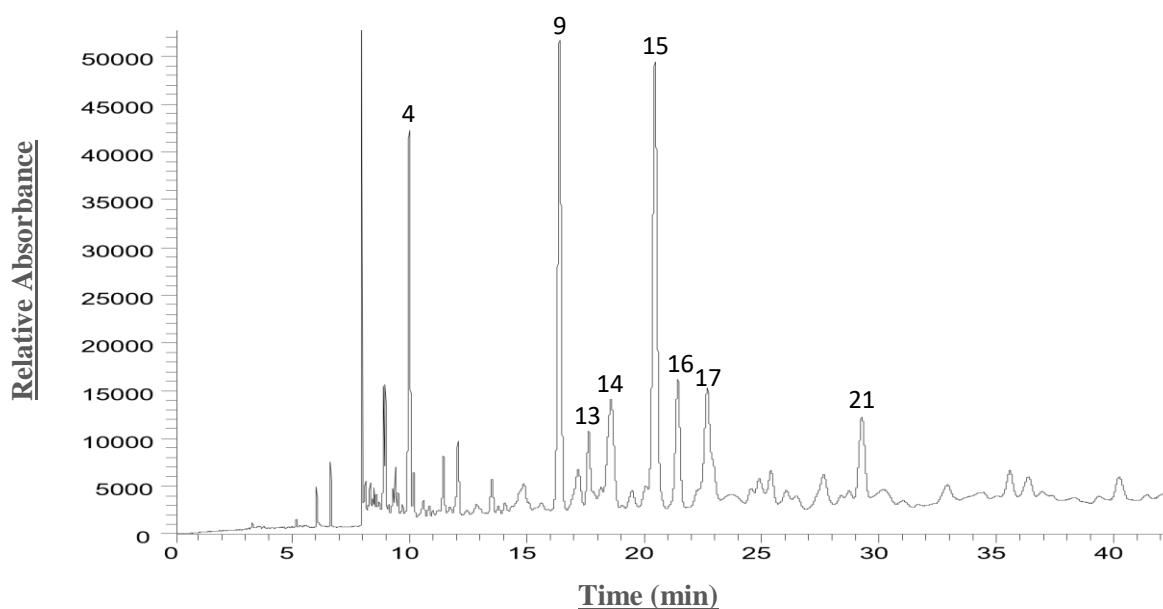


Figure 9. UHPLC–DAD chromatogram of *C. sempervirens* L. fruit extract recorded at 280 nm.

The identified compounds mainly belonged to the flavan-3-ol class, particularly catechin and epicatechin derivatives, as well as B-type procyanidin oligomers (dimers, trimers, and tetramers). Phenolic acids and their glycosides, such as protocatechuic acid hexoside, vanillic acid glucoside, and ferulic acid glucoside, were also detected.

Among individual compounds, procyanidin dimers and oligomers were the predominant constituents, representing the highest concentrations in the extract. In particular, procyanidin dimers detected at retention times around 16–19 min exhibited the highest abundance, with concentrations reaching approximately 9–10 mg/g extract, indicating that condensed tannins are the major phenolic constituents of *C. sempervirens*

fruits. Catechin and epicatechin were also present in appreciable amounts, confirming the relevance of flavan-3-ols as key contributors to the phenolic fraction.

Table 1. Identification and quantification of phenolic compounds in *Cupressus sempervirens* L. fruit extract by UHPLC–DAD–Orbitrap MS.

PEAKS	Rt (min)	λ (nm)	[M-H] ⁻ m/z	MS/MS	Assignment	mg/g extract
1	9.09	280	1027	407, 289, 125	Proanthocyanidin oligomer	0.135 ± 0.0003
2	9.25	280	881	407, 289, 125	B-type proanthocyanidin trimer	0.217 ± 0.0001
3	9.47	280	315	153, 109	Protocatechuic acid hexoside	NQ
4	9.95	280	865	407, 289, 125	Procyanidin trimer	4.341 ± 0.285
5	11.41	280	329	167	Vanillic acid glucoside	NQ
6	12.03	280	451	289, 245	Catechin- <i>O</i> -glucoside	0.793 ± 0.083
7	13.48	280	451	289, 245	Catechin- <i>O</i> -glucoside	0.632 ± 0.027
8	12.03	280	305	125, 179, 167, 219	Epigallocatechin	0.793 ± 0.083
9	16.31	280	577	289, 407, 125	Procyanidin dimer	9.127 ± 0.668
10	16.31	280	1155	407, 289	Proanthocyanidin oligomer	9.127 ± 0.668
11	17.16	280	1153	407, 289	B-type Procyanidin tetramer	0.899 ± 0.034
12	17.58	280	355	193, 149, 134	Ferulic acid glucoside	NQ
13	17.58	280	577	289, 407, 125	B-type Procyanidin dimer	1.216 ± 0.096
14	18.56	280	577	407, 289, 125	B-type Procyanidin dimer	3.716 ± 0.125
15	20.43	280	289	245	Catechin	10.278 ± 1.671
16	21.4	280	865	125, 407, 289	B-type procyanidin trimer	3.301 ± 0.184
17	22.66	280	865	125, 407, 289	B-type procyanidin trimer	2.318 ± 0.163

18	22.66	280	1153	407, 287	B-type procyanidin tetramer	2.318 ± 0.163
19	25.38	280	577	407, 289, 125	B-type procyanidin dimer	0.862 ± 0.101
20	25.38	280	865	125, 407, 289	B-type procyanidin trimer	0.862 ± 0.101
21	29.25	280	289	245	Epicatechin	2.570 ± 0.416
22	35.56	280	865	125, 407, 289	B-type procyanidin trimer	0.996 ± 0.069
23	36.35	280	1153	407, 287	B-type procyanidin tetramer	0.974 ± 0.005
24	36.35	280	865	125, 407, 289	B-type procyanidin trimer	0.974 ± 0.005
25	40.23	280	577	407, 289, 125	B-type procyanidin dimer	1.054 ± 0.055

NQ: identified but not quantified compound.

The predominance of B-type procyanidin, characterized by typical MS/MS fragments at m/z 289 and 407, is consistent with previously reported phenolic profiles of *C. sempervirens* extracts from other plant organs such as leaves. Similar phenolic patterns have been reported. For instance, Rguez et al. (2019) and Rahmani et al. (2024) identified catechin, epicatechin, and procyanidin oligomers as dominant phenolics in leaf and cone extracts of *C. sempervirens*. Likewise, Al-Rajhi et al. (2023) reported that flavan-3-ols and condensed tannins were the main contributors to the antioxidant activity of *C. sempervirens* extracts.

The present results extend these findings by demonstrating that the fruits, often underutilized, also constitute a rich source of flavan-3-ols and B-type procyanidin. While quantitative differences between organs are expected due to physiological and developmental factors, the qualitative similarity in phenolic classes suggests a conserved pattern of phenolic biosynthesis within the species.

Flavan-3-ols and their oligomeric forms, procyanidins, are among the most biologically active subclasses of flavonoids and are widely recognized for their strong antioxidant and anti-inflammatory properties. Monomeric compounds such as catechin and epicatechin, together with B-type procyanidin dimers and oligomers, exhibit potent radical-scavenging capacity, metal-chelating activity, and inhibition of lipid peroxidation, thereby contributing to cellular protection against oxidative stress (Fraga *et al.*, 2019). In

addition to antioxidant effects, these compounds are known to modulate inflammatory responses by suppressing pro-inflammatory cytokines, including IL-6 and TNF- α , and by inhibiting key signaling pathways such as NF- κ B and MAPKs in activated macrophages. Recent studies further indicate that oligomeric procyanidins often display stronger bioactivity than monomeric flavan-3-ols, highlighting a structure–activity relationship linked to the degree of polymerization (Mithul Aravind *et al.*, 2021; Kanon, Carroll and Lomiwes, 2023; Jomova *et al.*, 2025). These well documented bioactivities provide a clear mechanistic basis for the antioxidant and anti-inflammatory effects observed for the flavan-3-ol- and procyanidin- rich *Cupressus sempervirens* fruit extract in the present study.

In summary, UHPLC–DAD–Orbitrap MS analysis confirms that *C. sempervirens* fruit extract is particularly rich in condensed tannins, with proanthocyanidin dimers and oligomers as the predominant compounds. This composition supports the strong antioxidant capacity and reinforces the potential valorization of fruits as a source of bioactive phenolics for food, pharmaceutical, and nutraceutical applications.

5.2. Quantification of total saponins

The total saponin content of *Cupressus sempervirens* L. fruit extract was determined using the vanillin–sulfuric acid colorimetric method and quantified by a calibration curve ($R^2 = 0.991$) with soyasaponin I as the standard. The extract exhibited a total saponin concentration of 264.4 ± 16.0 mg/L, normalizing this value to the amount of starting plant material corresponds approximately to 1.6 mg of saponin-like compounds per gram of dry powder, which can be considered moderate, indicating the presence of saponin-type compounds at measurable levels in the analyzed sample. It is well established that total saponin levels in plant extracts may vary considerably depending on several factors, including the plant part analyzed, the extraction solvent and conditions, the geographical origin, and the analytical methodology. In this context, the vanillin–sulfuric acid assay is widely used as a reliable screening method for estimating total saponin content in complex plant matrices.

Comparable spectrophotometric approaches using the vanillin–sulfuric acid method have been reported in the literature for various medicinal plants, with total saponin values showing substantial variation across species and studies. For instance, Le *et al.* (2018) demonstrated that saponin concentrations determined by vanillin-based assays are strongly influenced by extraction and experimental parameters. Similarly,

studies on *Ilex paraguariensis* confirmed the applicability of this method for assessing total saponins in plant extracts while highlighting the inherent variability associated with botanical and methodological differences (De Aguiar *et al.*, 2024).

Overall, the total saponin concentration measured in the *C. sempervirens* fruit extract falls within the range commonly reported for plant extracts analyzed using vanillin-based spectrophotometric methods. These findings contribute to the phytochemical characterization of *C. sempervirens* fruits, which is scarce in terms of saponin determination and identification, and provide complementary information to subsequent chromatographic analyses.

5.3. Liquid Chromatography with Diode-Array Detection and Electrospray Ionization Tandem Mass Spectrometry (LC/DAD/ESI-MSⁿ) saponins analysis

The presence of saponin-like compounds in *Cupressus sempervirens* fruit extract was initially suggested by the vanillin–sulfuric acid colorimetric assay. However, it is well established that this assay is non-specific and does not selectively quantify saponins. The reaction is based on the formation of colored complexes with unsaturated triterpenoid or steroidal structures, and therefore other classes of secondary metabolites, such as triterpenes, sterols, and certain phenolic compounds, may also contribute to the measured response. As a result, total saponin values obtained by this method should be interpreted as an estimate of saponin-like reactivity rather than direct evidence of individual saponin molecules (Negi *et al.*, 2011; V. Le *et al.*, 2018).

To clarify the chemical nature of these compounds, the extract was further analyzed using LC/DAD/ESI-MSⁿ. Under the applied analytical conditions, no individual saponin structures could be confidently identified based on accurate mass measurements and characteristic fragmentation patterns. This result suggests that the colorimetric response observed previously may arise from non-saponin constituents exhibiting similar chemical reactivity, rather than from true saponins present at detectable levels.

Similar analytical behavior has been documented in studies on other plant matrices where total saponin estimates from colorimetric assays were much higher than, and inconsistent with, chromatographic-based detection. For example, *Camellia sinensis* seed extracts exhibited total saponin values determined by the vanillin–sulfuric acid method that were substantially greater than those obtained using UPLC-PDA-MS/MS, and the authors attributed this discrepancy to the non-specific color reaction, which can

also respond to flavonoids, phenolics, and other secondary metabolites rather than only true saponin structures. (Vincken *et al.*, 2007; Wu *et al.*, 2019).

In addition, phytochemical investigations of *C. sempervirens* reported in the literature consistently describe diterpenes, flavonoids, flavan-3-ols, and other phenolic compounds as the dominant secondary metabolites, whereas saponins are rarely reported as characteristic constituents of this species. This observation further supports the interpretation that saponins, if present in *C. sempervirens* fruits, occur only at trace levels and do not represent a major metabolite class (Al-Rajhi *et al.*, 2023; Orhan & Tumen, 2015).

Taken together, these findings indicate that the positive response observed in the vanillin–sulfuric acid assay reflects the limited specificity of the method rather than confirmed saponin occurrence. Therefore, saponins cannot be conclusively included among the identified constituents of *C. sempervirens* fruit extract based on the present LC/DAD/ESI-MSⁿ data.

5.4. Bioactivity evaluation

5.4.1. Antioxidant activity

The antioxidant capacity of *Cupressus sempervirens* L. fruit extract was evaluated by two complementary assays: Oxygen Radical Absorbance Capacity (ORAC) and the thiobarbituric acid reactive substances (TBARS).

The extract demonstrated high antioxidant capacity in the Oxygen Radical Absorbance Capacity (ORAC) assay. The extract showed an ORAC value of 2943 ± 301 μmol Trolox equivalents per 100 mL, indicating strong peroxy radical scavenging activity. This high ORAC value reflects effective chain-breaking antioxidant potential, which is important for mitigating oxidative stress. These findings are consistent with previous studies reporting significant antioxidant activity in *C. sempervirens* extracts. Organic extracts of *C. sempervirens* have exhibited notable radical scavenging and reducing activities in the DPPH and ferric reducing antioxidant power (FRAP) assays, which correlate with high phenolic content and contribute to overall antioxidant performance (Rguez *et al.*, 2019).

In the TBARS assay, the *C. sempervirens* fruit extract displayed a potent inhibitory effect on lipid peroxidation, with an IC₅₀ value of 0.302 ± 0.013 mg/mL. This low IC₅₀ indicates a strong capacity to reduce malondialdehyde (MDA) formation,

suggesting effective protection against lipid oxidation. The observed inhibitory activity confirms that the extract not only scavenges free radicals but also stabilizes lipid structures prone to oxidative damage. Reports on *C. sempervirens* and related Cupressaceae species have similarly demonstrated strong antioxidant effects in lipid peroxidation models and radical-scavenging assays, supporting the protective function of phenolic and flavonoid constituents present in these plant extracts (Al-Rajhi et al., 2023; Dey et al., 2009; Z. Rahmani et al., 2022).

Taken together, the ORAC and TBARS results demonstrate that *C. sempervirens* fruit extract exhibits broad antioxidant functionality, combining efficient radical scavenging with strong inhibition of lipid peroxidation. The high ORAC value and low TBARS IC₅₀ reflect the contribution of flavan-3-ols and B-type proanthocyanidins, which were identified as the predominant phenolic classes in the extract.

Although the majority of the literature on *C. sempervirens* utilizes DPPH, ABTS, and FRAP assays rather than ORAC and TBARS, the overall pattern of strong antioxidant activity remains consistent. For example, studies on organic extracts of *C. sempervirens* leaves and cones collected at different phenological stages reported significant DPPH scavenging and reducing power largely attributed to flavan-3-ols, proanthocyanidins, and other phenolic constituents (Rahmani et al., 2024; Rguez et al., 2019). These comparisons reinforce the biological relevance of the present findings. Moreover, research on the phenolic composition of *C. sempervirens* primarily in leaf and cone extracts, have demonstrated that secondary metabolites such as phenolic acids and flavonoids play a central role in antioxidant defense mechanisms (Al-Rajhi et al., 2023).

Collectively, the integrated data highlight that *C. sempervirens* fruit extract is a promising natural antioxidant source with multifaceted activity, combining radical scavenging and anti-lipid peroxidation effects. The strong antioxidant performance observed can be directly linked to the high abundance of flavan-3-ols and condensed tannins, reinforcing the potential valorization of *C. sempervirens* fruits for food, pharmaceutical, and nutraceutical applications.

5.4.2. Cytocompatibility

The cytocompatibility of *Cupressus sempervirens* L. fruit extract was assessed on human epidermal keratinocytes (HaCaT) and human foreskin fibroblasts (HFF-1) by resazurin reduction assay over 1, 2, and 3 days of exposure, and the results are presented

as metabolic activity relative to untreated control cells. In parallel, cell morphology was evaluated by optical microscopy to support the quantitative metabolic activity data. Cell morphology was evaluated by optical microscopy to support the quantitative metabolic activity data.

As shown in Figure 10, HaCaT cells exposed to low extract concentrations (6.25–50 µg/mL) maintained metabolic activity values close to 100% across all time points, indicating good cytocompatibility and preservation of cellular metabolic function. No statistically significant decrease in metabolic activity was observed at these concentrations, suggesting that the extract did not induce cytotoxicity under these conditions. At intermediate concentrations (100 µg/mL), a modest reduction in metabolic activity became evident, particularly after 2 and 3 days of incubation. However, metabolic activity remained above 80%, a threshold commonly considered indicative of acceptable cytocompatibility in in vitro studies involving natural extracts. At higher concentrations (200 and 400 µg/mL), a clear dose-dependent decrease in metabolic activity was observed, with the effect becoming more pronounced over time. This trend suggests that prolonged exposure to higher extract concentrations may negatively affect keratinocyte metabolic activity. Notably, the gradual decrease indicates a concentration- and time-dependent response, characteristic of complex plant extracts containing bioactive secondary metabolites.

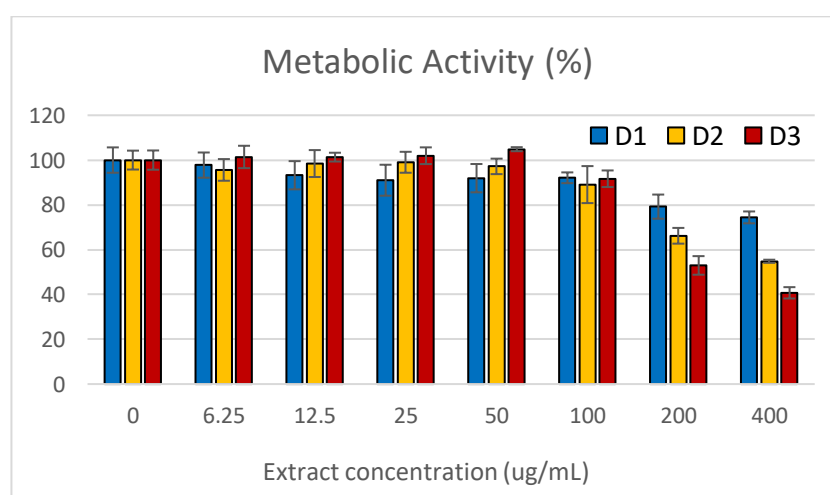


Figure 10. Metabolic activity (%) of human keratinocytes (HaCaT) cultured in the presence of increasing concentrations of *Cupressus sempervirens* L. fruit extract for 1, 2, and 3 days, assessed by the resazurin reduction assay.

A similar response pattern was observed for HFF-1 fibroblasts (Figure 11) Cells treated with extract concentrations up to 50 µg/mL exhibited metabolic activity values comparable to the control group throughout the experimental period, demonstrating good cytocompatibility. In some cases, slightly elevated metabolic activity was observed, which may reflect mild metabolic stimulation, a phenomenon previously reported for fibroblasts exposed to low concentrations of phytochemical-rich extracts. At concentrations of 100 µg/mL and above, fibroblasts showed a progressive reduction in metabolic activity, particularly after 3 days of exposure. Nevertheless, fibroblast viability remained higher than that of keratinocytes at equivalent concentrations, indicating a greater tolerance of fibroblasts to higher extract doses, a trend commonly reported in skin cell cytocompatibility studies.

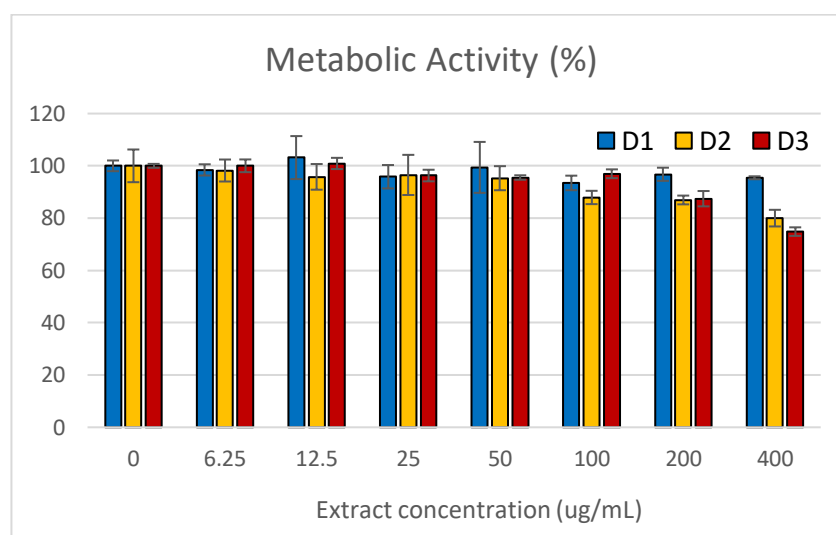


Figure 11. Metabolic activity (%) of human foreskin fibroblasts (HFF-1) cultured in the presence of increasing concentrations of *Cupressus sempervirens* L. fruit extract for 1, 2, and 3 days, assessed by the resazurin reduction assay.

The cytocompatibility profile observed for *C. sempervirens* L. fruit extract is consistent with previously published studies evaluating plant extracts on keratinocytes and fibroblasts using resazurin-based assays. Several authors have reported that low to moderate concentrations of botanical extracts maintain or slightly enhance cellular metabolic activity. In contrast, higher concentrations may induce a gradual decline in viability, attributed to increased exposure to phenolic compounds, terpenoids, or other secondary metabolites (Nizioł-Łukaszewska, Furman-Toczek and Zagórska-Dziok, 2018; Ratanachamnong *et al.*, 2023). Comparable trends have also been reported in studies on related Cupressaceae species, where extracts demonstrated differential effects

on cell viability depending on concentration and cell type. For example, hydroalcoholic extracts of *C. sempervirens* var. *horizontalis* showed no cytotoxicity against established human tumor cell lines at relevant concentrations, while essential oils from *C. sempervirens* subspecies exhibited dose-dependent cytotoxic effects in cancer models (Al-Rajhi et al., 2023; Orhan & Tumen, 2015; Rguez et al., 2019).

The metabolic activity results were further supported by optical microscopy observations (Figure 12). Both HaCaT and HFF-1 cells cultured in the presence of low extract concentrations displayed normal morphology, typical cell spreading, and preserved confluence, comparable to untreated controls. Keratinocytes retained their characteristic polygonal morphology, while fibroblasts exhibited elongated, spindle-shaped structures. At higher extract concentrations, particularly 200 and 400 µg/mL, a reduction in cell density and alterations in morphology were observed, including cell rounding and decreased adherence, especially after prolonged incubation. These morphological changes are consistent with the reduced metabolic activity detected in the resazurin assay and further support a dose-dependent effect of the extract on cell behavior.

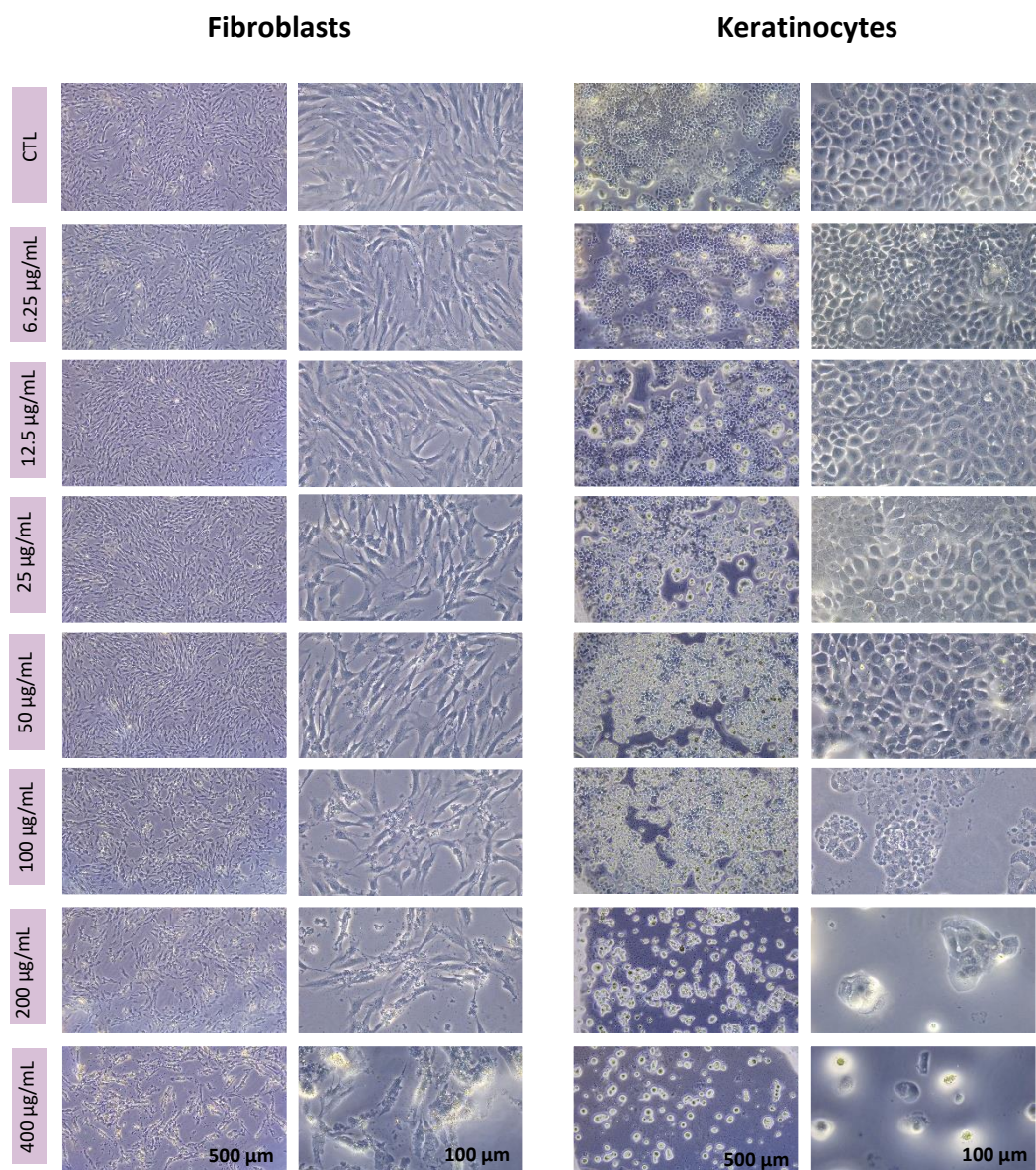


Figure 12. Optical images of fibroblasts (left) and Keratinocytes (right) cultured with increasing concentrations of *Cupressus sempervirens* L. fruit extract for 1 day.

Overall, the combined metabolic activity and morphological analyses reveal a concentration-dependent cellular response to *Cupressus sempervirens* L. fruit extract in both keratinocytes and fibroblasts. While low to moderate concentrations preserved normal metabolic function and cell morphology, higher concentrations induced a gradual reduction in cellular activity, more pronounced in keratinocytes than in fibroblasts. This differential sensitivity is consistent with the higher metabolic demand and barrier-related function of keratinocytes compared to fibroblasts, which are generally reported to exhibit

greater tolerance to phytochemical exposure. These findings support the extract's safe biological profile and provide a solid basis for further investigation in applications related to skin biology or bioactive formulations.

5.4.3. Anti-Inflammatory activity

Macrophage metabolic activity and selection of non-cytotoxic concentrations

Prior to evaluating the anti-inflammatory potential of *Cupressus sempervirens* L. fruit extract, its effect on macrophage metabolic activity was assessed using the resazurin reduction assay. Metabolic activity was monitored after 1, 2, and 3 days of exposure to the extract, following the same experimental design applied for keratinocytes and fibroblasts. The results presented correspond to day 3, which was considered the most representative time point for evaluating prolonged cellular response to the extract. As shown in Figure 13, THP-1–derived macrophages treated with extract concentrations ranging from 6.25 to 100 $\mu\text{g/mL}$ maintained metabolic activity comparable to or slightly higher than that of the untreated control, indicating good cytocompatibility under these conditions. In contrast, a decrease in metabolic activity was observed at the highest tested concentration (400 $\mu\text{g/mL}$), suggesting the onset of dose-dependent metabolic stress at elevated extract levels. Based on these findings, concentrations ≤ 100 $\mu\text{g/mL}$ were considered non-cytotoxic and were therefore selected for subsequent anti-inflammatory evaluation.

This selection strategy ensures that any modulation of inflammatory mediators reflects an actual biological response rather than effects secondary to reduced cell viability.

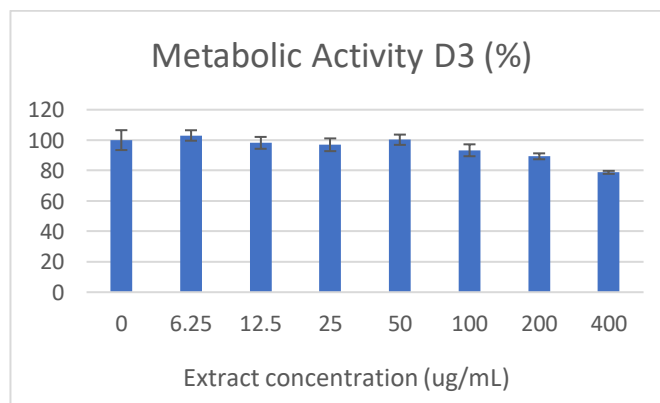


Figure 13. Metabolic activity (%) of THP-1–derived macrophage after 3 days of exposure to increasing concentrations of *Cupressus sempervirens* L. fruit extract, assessed by the resazurin reduction assay.

Effect of *Cupressus sempervirens* L. fruit extract on IL-6 production

The anti-inflammatory potential of the extract was assessed by measuring IL-6 production in LPS-stimulated THP-1–derived macrophages (Figure 14). Cells cultured in the absence of LPS (w/o LPS) exhibited low basal IL-6 levels, while LPS stimulation induced a marked increase in cytokine production, confirming successful activation of an inflammatory response. Dexamethasone, used as a positive control, strongly suppressed IL-6 production to levels comparable to non-stimulated cells, validating the responsiveness of the experimental model.

Pre-treatment with *C. sempervirens* fruit extract resulted in a concentration-dependent modulation of IL-6 production. The strongest inhibitory effect was observed at 25 µg/mL, where IL-6 levels were reduced to approximately 30% of those in LPS-stimulated controls. Higher concentrations (50 and 100 µg/mL) maintained anti-inflammatory activity but did not further enhance IL-6 suppression, respectively. Lower concentrations (6.25–12.5 µg/mL) induced only modest reductions in IL-6 production. This non-linear response suggests an optimal concentration range, with a plateau or partial attenuation of the anti-inflammatory effect at higher extract doses. Importantly, the reduction in IL-6 production at 25 µg/mL occurred under conditions of preserved macrophage metabolic activity, indicating a genuine anti-inflammatory effect rather than an indirect consequence of cytotoxicity. Taken together, these results demonstrate that *C. sempervirens* L. fruit extract exhibits moderate but significant anti-inflammatory activity in activated THP-1-derived macrophages, with maximal efficacy at intermediate concentrations.

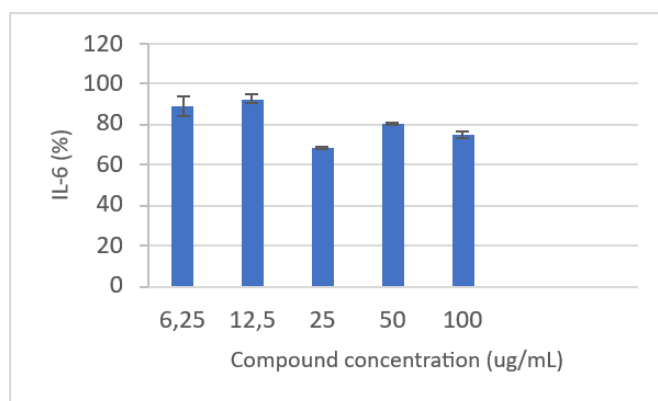


Figure 14. IL-6 production in LPS-stimulated THP-1–derived macrophage treated with increasing concentrations of *Cupressus sempervirens* L. fruit extract.

Pre-treatment with *C. sempervirens* fruit extract led to a clear attenuation of the LPS-induced IL-6 response in a concentration-modulated manner. While lower extract doses showed modest effects, intermediate concentrations produced the most pronounced reduction, and higher doses maintained this inhibition without further enhancement. This pattern is consistent with previously reported anti-inflammatory profiles of plant extracts in macrophage models, where moderate doses often yield optimal modulation of pro-inflammatory cytokines while very low or high doses may show attenuated effects (e.g., *Thymus vulgaris* essential oil on IL-6 and TNF- α in LPS-stimulated macrophages) (Khazdair *et al.*, 2021). Similar anti-inflammatory effects have been observed for extracts from *Juniperus communis* and related taxa, which also modulate macrophage cytokine production in vitro (El Jemli *et al.*, 2024).

The combined evaluation of metabolic activity and IL-6 production demonstrates that *C. sempervirens* L. fruit extract exerts anti-inflammatory activity in LPS-activated macrophages under non-cytotoxic conditions.

6. Conclusions And Future Perspectives

The present work focused on the valorization of *Cupressus sempervirens* L. fruits as an underexplored forest bio-residue and a potential source of bioactive compounds, contributing to the sustainable use of ornamental and forest resources. Overall, the results obtained successfully fulfilled the main objective and the specific objectives proposed for this study.

Maceration extraction using a hydro-methanolic solvent proved to be an effective and straightforward approach for recovering bioactive compounds from *C. sempervirens* fruits, yielding extracts suitable for both chemical characterization and biological evaluation. The phytochemical analysis performed by UHPLC–DAD–ESI–MSⁿ revealed a complex phenolic profile dominated by flavan-3-ols and B-type procyanidins, particularly catechin, epicatechin, and procyanidin oligomers. These compounds were identified as the major constituents of the extracts and are likely responsible for the observed biological activities.

In addition to phenolic compounds, the application of the vanillin–sulfuric acid assay indicated the presence of saponin-like reactivity in *C. sempervirens* fruit extracts. However, chromatographic analysis by LC/DAD/ESI–MSⁿ did not allow the confirmation of individual saponin structures. This observation reflects the non-specific nature of the colorimetric method and is consistent with the phytochemical profile reported for *C. sempervirens*, in which phenolic compounds and terpenoid derivatives are described as the dominant secondary metabolites. Although saponins cannot be conclusively included among the identified constituents based on the present data, the combined analytical strategy adopted in this work provides a robust and transparent assessment of this phytochemical class, reinforcing the scientific rigor of the study.

The biological evaluation demonstrated that *C. sempervirens* fruit extracts possess relevant bioactive properties. Antioxidant assays, namely ORAC and TBARS, showed effective capacity to scavenge reactive oxygen species and inhibit lipid peroxidation, consistent with the high content of phenolic compounds, particularly proanthocyanidins. Cytocompatibility studies using human keratinocytes (HaCaT) and fibroblasts (HFF-1) indicated that the extracts were non-toxic at low and intermediate concentrations, supporting their safe use in bio-based applications. Furthermore, the anti-inflammatory activity evaluated in THP-1–derived macrophages showed a reduction in interleukin-6

production under non-cytotoxic conditions, suggesting a genuine immunomodulatory effect.

Taken together, these findings demonstrate that *C. sempervirens* L. fruits, commonly regarded as waste material, represent a valuable natural source of phenolic compounds with antioxidant and anti-inflammatory properties. The results support their potential incorporation into high-value applications, such as functional ingredients for cosmetic, pharmaceutical, or nutraceutical formulations, while contributing to circular economy principles and sustainable resource management.

Despite the promising outcomes, several future perspectives can be considered. Further studies should focus on optimizing extraction conditions and exploring greener extraction technologies, such as ultrasound or microwave-assisted extraction, to enhance efficiency and reduce environmental impact. Advanced analytical approaches, including targeted isolation and structural elucidation of minor constituents, would allow a deeper understanding of the phytochemical composition of *C. sempervirens* fruits. In addition, *in vivo* studies and more complex biological models are required to confirm the biological effects observed *in vitro*. Finally, formulation development and stability studies would represent important steps toward the industrial exploitation of *C. sempervirens* fruit extracts.

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