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PhD Thesis in

**Valorisation of wild edible and medicinal plants through
phytochemical analyses and evaluation of their potential
biological properties**

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Abstract

The aim of this PhD project was the valorisation of wild food and medicinal plants identified through ethnobotanical surveys and ecological data, by means of phytochemical analyses and investigations of their potential biological properties. During the first year, attention was focused on the flower extract of *Crataegus laciniata* Ucria (Rosaceae), a wild-growing species in western Sicily. LC-MS analysis identified 41 constituents in the flower extract (CLF), predominantly C- and O-flavonoid derivatives. CLF displayed strong antioxidant capacity in DPPH, ABTS, and FRAP assays. Moreover, it exhibited anti-melanogenic activity in *Danio rerio* (zebrafish) and significant inhibitory effects against tyrosinase, α -amylase, and α -glucosidase. Collectively, these findings highlight *C. laciniata* as a valuable source of bioactive compounds with potential pharmaceutical and cosmeceutical relevance, suggesting that CLF may represent a promising candidate for applications in these sectors. During the second year, fruit extracts from different *Crataegus* species were investigated. LC-MS analysis of the fruit extracts revealed 25 major constituents, primarily flavonoids, phenolic acids, and fatty acids. UHPLC-MS quantitative profiling highlighted species-specific chemical signatures: *C. laevigata* and *C. zichichianus* were particularly rich in hyperoside, *C. azarolus* showed the highest levels of cyanidin-O-glucoside, whereas *C. azarolus* subsp. *aronica* exhibited the greatest abundance of vitexin. The extracts displayed strong antioxidant activity in DPPH, ABTS, and FRAP assays and potent α -glucosidase inhibition, supporting their potential antidiabetic properties.

In parallel, the phytochemical study of stem bark extracts of *Cedrela odorata* (Meliaceae) was carried out to identify new lignan and neolignan derivatives with potential antiangiogenic activity. Two previously undescribed 8',9'-bis-nor-neolignans—9-acetyltoonin C and threo-guaiacylglycerol- β -methyl vanillate ether were isolated and structurally elucidated through spectroscopic analyses. Along with structurally related lignans from the in-house chemical library, these compounds were assessed for antiangiogenic activity using the *Danio rerio* (zebrafish) embryo model. Isolates exhibited the strong inhibition of blood vessel formation, with threo-guaiacylglycerol- β -methyl vanillate ether showing an effect comparable to the reference molecule 2-

methoxyestradiol. These findings expand current knowledge on neolignans as promising scaffolds for the development of natural antiangiogenic agents.

In addition, a comprehensive phytochemical investigation of *Lavandula pubescens*, a species traditionally used in Saudi Arabia for its antibacterial and anti-inflammatory properties, was conducted. The study of the leaf-surface extract led to the isolation of seven new diterpenoids, together with four known pimarane derivatives and three known flavonoids. Extracts, fractions, and purified compounds of *L. pubescens* were evaluated for their anti-inflammatory activity in THP-1 cells. The results indicated that 15S,16-dihydroxy-7-oxopimar-8(9)-ene and 15,16,17-trihydroxypimar-8(9)-ene significantly reduced IL-6 secretion; notably, 15,16,17-trihydroxypimar-8(9)-ene exhibited a dose-dependent inhibition of IL-6 release, thereby attenuating the LPS-induced inflammatory response.

Building on the work carried out in the second year, a further phytochemical investigation of the aerial part extract of *L. pubescens* was performed during the third year. This study resulted in the isolation of additional diterpenoids, including nine previously undescribed diterpenes and six unusual dimeric monoterpenes. Structural elucidation was achieved through comprehensive 1D and 2D NMR spectroscopy and HRESIMS analyses. The antimicrobial activity of the extracts, fractions, and isolated compounds was also assessed. A marked alteration in the growth of *Staphylococcus epidermidis* was observed among the isolates at 6 µg/mL of ent-8(9)-pimaren-15,16-diol, indicating a clear inhibitory effect. ent-8(9)-pimaren-15,16-diol displayed a dose-dependent impact on *S. epidermidis*, achieving complete growth inhibition at higher concentrations and growth modulation at lower ones. Additionally, ursolic acid and oleanolic acid exhibited significant inhibitory activity, supporting their capacity to suppress *S. epidermidis* proliferation. These findings suggest that these components contribute to the overall antimicrobial activity of the extract, potentially through membrane disruption or interference with essential metabolic pathways. Further studies will be required to fully elucidate their mechanisms of action.

Chapter 1

Introduction

**Valorisation of wild edible and medicinal plants through
phytochemical analyses and evaluation of their potential
biological properties**

Introduction

Since ancient times of human civilization, plants as biological resources, have played extremely important roles in providing materials for nutrition, medicine, and cosmetics. Over 80% of the population also relies on traditional medicine for its health, according to the World Health Organization. These practices are based on medicinal plants, and an estimated 50,000 species are used globally, the vast majority providing contributions to traditional medicinal use (Nugraha et al., 2022). Therefore, natural resources previously studied with an interdisciplinary focus, for instance, in the pharmaceutical and the agri-food sectors, with the common objective to enhance the human health levels and to achieve an economic benefit. In particular, for the field of medicines, natural products are critical sources for the discovery and development of new drugs. Interestingly, between 1984 and 2014, approximately 37% of newly approved medicines were derived from plants, whether directly as plant derived products, unchanged natural products, or modified natural derivatives (Newman & Cragg, 2016). Despite the ever-growing reliance on synthetic medicine, the use of medicinal plants remains deeply embedded in traditional cultural approach. Even though the growing reliance on pharmaceutical commodities has decreased the salience of folk medicine in modern societies, such remedies continue to prevail among many rural communities. Indeed, traditional medicine often co-exists with, or even replaces, contemporary medicine, especially with reference to the treatment of minor illnesses. Traditionally passed down amongst members of the older generation, this knowledge is handed down orally through the generations, supplemented with personal anecdotes and practical observations. Besides the nutritional benefit, many wild edible plant species (WEPs) are rich sources of bioactive molecules with health-beneficial and therapeutic potential. Therefore, the dual capacity of plants to serve as both sustenance and medicine highlights their importance in promoting human health and merits the scientific investigation of wild edible plants as sources of phytochemicals. For decades, plant-derived natural products have inspired pharmacologists, offering immense structural diversity, new chemical scaffolds, and a precious source of drugs and potential therapeutic candidates. Wild medicinal and edible plants (WEMPs) are not adequately investigated sources of bioactive molecules. Such non-cultivated plant species thrive naturally and

independently under semi-managed ecological environments without direct human interference (Newman & Cragg, 2016). The outstanding adaptability of different plant species to persist in various ecological niches can be attributed to the synthesis of these secondary metabolites such as alkaloids, flavonoids, and phenolics. In addition, the phytochemicals enhance plant environmental tolerance and also endow them with the pharmacological and nutraceutical properties (Ashraf et al., 2018). Over the past decades, deep ethnobotanical studies have highlighted the common and extensive use of wild plant species across diverse ecological and sociocultural environments. Ethnobotany, as an interdisciplinary field of research, investigates the complex relationship that exists between human societies and plants. In addition, it has been an important part of the search for botanical resources that contributed to the creation of traditional cuisine and medicine. Edible and medicinal plants play a key role in both traditional diets and herbal medicine (Rigat et al., 2016). Studying edible plant species also helps to preserve traditional ecological knowledge and agricultural biodiversity, particularly in areas with a long history of ethnobotanical use (Motti, 2020). Hunger and malnutrition hinder sustainable development. Studies suggest wild edible plants (WEPs) often have higher levels of bioactive compounds than cultivated crops. This benefits health and physiological balance (Alam et al., 2020). Greater awareness of the nutritional and healing properties of these species has led to renewed interest in them (Mokganya & Tshisikhawe, 2019). Environmental degradation, deforestation and modern farming practices have reduced access to wild edible plants, which played an important role in food security, especially during scarcity (Balemie & Kebebew, 2006). Their value is often overlooked, despite their potential to benefit local diets and nutrition in rural areas. (Dejene et al., 2020). In addition, WEPs are known to have multiple functions; their importance extends far beyond food security. Indeed, ethnobotanical assessments have proved that WEPs have a profound nutritional value that exceeds that of conventional crops with respect to key nutrients. (Alam et al., 2020). Scientific research has shown the valuable pharmacological potential of these plants. Various species exhibit pharmacological properties such as antimicrobial, anti-inflammatory, and antioxidant activities, attributed to phenolic compounds, flavonoids and alkaloids. This highlights their significance in traditional healing and in the discovery of new drugs. Whereas ethnobotanical research provides strong evidence for the traditional uses

of wild medicinal and edible plants, systematic phytochemical investigations are essential to fully understand their chemical composition. The phytochemical profiles of these plants play a key role in determining their biological activities, emphasizing that identifying and characterizing bioactive compounds are fundamental steps toward exploring their biological potential. To achieve this purpose, the most recent advances in analytical technologies, including LC-MS, NMR, and metabolomics, along with the establishment of green extraction protocols, have provided powerful tools for the elucidation of phytochemicals and their biological effects. Such approaches support both scientific accuracy and sustainability in natural product research. Since in recent years a remarkable rise in the prevalence of chronic non-communicable diseases has been observed worldwide (Branca et al., 2019; Qiao et al., 2019), several studies have reported that consuming these plants may help mitigate these ailments (Lyons et al., 2020). The disease prevention capabilities are ascribed to high levels of phytochemical content, which possess therapeutic benefits and represent a key feature in disease prevention for major ailments, including heart disease and cancer (De Carvalho et al., 2021).

Phytochemicals are secondary metabolites produced by plants, encompassing a wide variety of phenolic compounds such as tannins, flavonoids, saponins, and glycosides (Gülçin, 2020). Although the specific roles of these metabolites in plant cellular metabolism are not yet fully understood, it is widely recognized that they are synthesized as part of the plant's defense system against environmental stresses, including pathogen attacks and herbivory (Olivoto et al., 2017). Due to their diverse bioactive properties, plant-derived compounds have been extensively studied for their potential contributions to human health and well-being.

This PhD research project involves the phytochemical and biological investigation of wild edible and medicinal plant species collected from various geographical regions, including southern Italy, Venezuela and Saudi Arabia. The main aim is to identify and characterise the secondary metabolites present in these plants, and to evaluate their biological potential, paying particular attention to antioxidant and anti-inflammatory activities. This study aims to address gaps in knowledge about wild edible and medicinal species from different

ecological contexts. The thesis is organised into the following chapters, which are listed below.

Chapter 2 focuses on the phytochemical and biological study of *Crataegus laciniata* flowers. The first part of the study examines the phytochemical composition of flower extract, assessing antioxidant properties and the inhibitory effects on the enzymes α -amylase and α -glucosidase, which are closely associated with diabetes management. The tyrosinase-inhibiting potential of the extract and its influence on skin pigmentation were examined using the zebrafish (*Danio rerio*) model. This chapter also analyzes the phytochemical composition and bioactivity of fruits from several *Crataegus* spp. (Rosaceae) variety collected in Western Sicily. Using UHPLC-HRMS analysis, the secondary metabolites were identified, and their antioxidant activity was evaluated through DPPH, FRAP, and ABTS assays. Additionally, **chapter 3** focuses on the ability of the fruit extracts to inhibit α -amylase and α -glucosidase to determine their potential antidiabetic effects. Statistical analyses were conducted to explore the relationships between the chemical composition and biological activities, thereby highlighting the chemotaxonomic significance and nutraceutical potential of *Crataegus* fruits.

Chapter 4, the focus is on investigating *Cedrela odorata* (Family Meliaceae), with significant pharmaceutical and agricultural interest because of its unique phytochemical composition. This particular chapter characterizes the bioactive compounds found in stem bark and evaluates their antioxidant and anti-inflammatory activities.

Chapter 5 specifically focuses on *Lavandula pubescens*, a species of the Lamiaceae family that has been traditionally employed in Saudi Arabia for its anti-inflammatory properties. In this chapter, the isolation and characterization of leaf-surface diterpenoids were carried out, and their effects on the modulation of inflammatory cytokine particularly IL-6 were analyzed. On the other hand, the study of its aerial parts led to the isolation of nine new diterpenes, six new dimeric monoterpenes, together with the known *ent*-pimar-8(14)-15,16 diol (Hang et al 2005), 15(*S*)-isopimar-7-en-15,16 diol. Their structures were established using comprehensive spectroscopic data analysis, including 1D and 2D-NMR, high-resolution electrospray ionization mass spectrometry, electronic circular dichroism,

and comparison with the literature data. Furthermore, the antimicrobial activity of the extracts, their fractions, and isolated compounds was assessed. In general, this PhD study combines advanced analytical techniques including high-performance liquid chromatography (HPLC), mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, and in vitro bioassays to systematically characterize the phytochemical composition and biological activities of the investigated plant species, providing a comprehensive and scientific assessment of their biological potential.

This PhD project aims to:

- To investigate the main extracts and active components of selected wild edible and medicinal plants that have potential applications in herbal medicine.
- To identify and characterize the bioactive compounds responsible for their pharmacological properties.
- To assess the antioxidant, antimicrobial, anti-inflammatory and antidiabetic activities of these phytochemicals through integrated phytochemical and biological analyses.
- To explore the potential of these plants as sources for the development of health products, dietary supplements, and pharmaceutical agents.

The project will also collaborate with local communities and knowledge holders to document traditional uses and promote the sustainable management of wild edible and medicinal plant resources.

Chapter 2

Chemical Profile and Health-Promoting Activities of *Crataegus laciniata* (Rosaceae) Flowers

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Chemical Profile and Health-Promoting Activities of *Crataegus laciniata* (Rosaceae) Flowers (Mirabile et al., 2024).

2.1 Introduction

The genus *Crataegus* (Rosaceae), also known as hawthorn, comprises a taxonomically complex group of shrubs and small trees widely distributed across *Europe, North America, and Asia* and include about 200 species; however, there are differing opinions on the exact number of species because of hybridization and a high level of diversity (Spadaro et al., 2024). Hawthorn is also known by several vernacular names such as English hawthorn, Maybush, and whitethorn. Morphologically, the plants are characterized by thorny branches, three to five-lobed deciduous leaves, and clusters of white flowers blooming in spring. The berry-like fruits (haws) range from yellow or orange to bright red, most commonly the latter, and typically contain one to three pyrenes, depending on the species (Venskutonis, 2018). Because of their extensive traditional application and well-documented safety, the Committee on Herbal Medicinal Products of the European Medicines Agency classified *Crataegus* plants as “traditional herbal medicinal products” in 2016. This classification underscores the long-standing role of *Crataegus* in traditional medicine and its ongoing importance in contemporary medical practice. Phytochemical studies showed that various *Crataegus* species produce a wide variation in specialized metabolites, including flavonoids, pro-anthocyanidins, phenolic acids, and triterpenoids, which are associated with their pharmacological potential. Notably, within the diverse polyphenolic profile of *Crataegus*, the flavonoids hyperoside and vitexin have been consistently reported as major determinants of its therapeutic efficacy (Edwards et al., 2012). Owing to this remarkable phytochemical richness, hawthorn has attracted considerable scientific attention and is increasingly regarded as a valuable candidate for the development of nutraceuticals and phytopharmaceuticals, especially for the prevention and management of cardiovascular and inflammatory disorders (Nazhand et al., 2020). Hawthorn has been historically known and documented for its uses as medicinal plants across different civilizations. Since the European Middle Ages, its berries and leaves have been prescribed for digestive ailments such as diarrhea and vomiting, whereas in Traditional Chinese Medicine the hawthorn fruits were commonly used to improve digestion, alleviate food stagnation, and treat hyperlipidemia and dyspnea. The first

recorded application of its diuretic effect was in the eighteenth century, followed by its use in treatment of kidney stones. Later, in the nineteenth century, hawthorn was introduced in Europe and the US as an effective drug for cardiovascular diseases (Edwards et al., 2012, Bahri et al., 2009). Among *Crataegus* specialized metabolites, flavonoids and procyanidins are regarded as the contributors to the principal cardioprotective actions, including antioxidant, anti-inflammatory, vasodilatory, and hypolipidemic effects. In addition, triterpenic acids, such as crataegolic acid identified in hawthorn fruits, have also been implicated in coronary vasodilation (Degenring et al., 2003; Long et al., 2006). Pharmacological studies have confirmed that hawthorn fruit/leaf extracts can exert hypotensive, hypocholesterolemic, and hypolipidemic effects. The positive effects of *Crataegus* extracts in mitigating diabetic complications have been mainly attributed to the reduction of blood glucose levels, displaying hypolipidemic properties, and controlling the release of insulin (Aierken et al., 2017). In addition to their medicinal uses, *Crataegus* species are valued as non-cultivated or wild fruits for culinary purposes, as their fruits are often processed into jams, jellies, and teas. For centuries, *Crataegus* fruits have been part of culinary heritage, most notably in Sicily, where their transformation into preserves and herbal teas reflects their enduring role in regional dietary culture.

Despite the substantial body of research devoted to the fruits and leaves of *Crataegus* species, the flowers have received comparatively little attention, even though they are known to contain high levels of phenolic compounds and may contribute significantly to the overall medicinal value of the genus. This gap is particularly pronounced in the case of *Crataegus laciniata*, a Mediterranean species characterized by a limited distribution and still insufficient phytochemical and pharmacological documentation relative to more extensively studied species such as *C. monogyna* or *C. pinnatifida* (Issaadi, et al., 2021, Mirabile et al., 2024).

Addressing this gap, this project aims to provide a detailed chemical characterization of *C. laciniata* flowers and to evaluate their biological properties, thereby contributing to the valorization of this underutilized medicinal and edible plants. *Crataegus laciniata* can be distinguished by its slightly lanate twigs, leaves, inflorescences, hypanthia, and fruits. Considering its restricted distribution and the scarcity of phytochemical studies, the flowers of *C. laciniata* represent a promising yet underexplored reservoir of bioactive compounds

for further investigation (Calvo et al., 2015; Mirabile et al., 2023). Accordingly, this part of the study focused on characterizing the chemical composition of a 70% (v/v) ethanolic extract of *Crataegus laciniata* flowers (CLF) using LC-HRMS analysis, and evaluating its *in vitro* health-promoting potential through antioxidant tests and the inhibition of key enzymes (α -amylase and α -glucosidase) relevant to diabetes management. The effects of CLF on tyrosinase, a major enzyme for the catalysis of two rate limiting reactions of melanin pigment biosynthesis were also investigated. Finally, the toxicity and whitening properties of CLF on zebrafish (*Danio rerio*) embryos, which provided fresh perspectives on the use of *C. laciniata* flowers as natural resources of bioactive molecules for metabolic/skin pigmentation disorders were studied.

2.2 Materials and Methods

2.2.1. Sample Preparation and Extraction

Flowers of *Crataegus laciniata*, *monogyna*, *Crataegus aff. Insengae*, were collected in June 2022 from wild populations growing in Canna and Castelbuono (Madonie, Sicily) at an altitude of 1610 m a.s.l. The botanical identification was performed by Prof. F. M. Raimondo, and a voucher specimen was deposited at the PAL-Gr herbarium. For extraction, 5 g of air-dried flowers (shade-dried) were macerated in 50 mL of 70% (v/v) ethanol under magnetic stirring for 10 min, followed by ultrasound-assisted extraction for 30 min at 25 °C. The solvent was subsequently evaporated to dryness, affording 0.638 gr of extract (yield 12.76%).

2.2.2 Chemicals and Reagents

All solvents, analytical-grade chemicals, and reagents used in this study were purchased from Merck (Milan, Italy). The enzymatic assays were carried out using α -amylase (EC 3.2.1.1), α -glucosidase (EC 3.2.1.20), and mushroom tyrosinase (EC 1.14.18.1), all supplied by Merck.

2.2.3 Determination of Total Phenolic Content (TPC)

TPC was determined using the Folin-Ciocalteu reagent with small modifications (Singleton et al., 1999): 100 μ L of sample solution (1 mg/mL) was initially diluted with 2000 μ L of distilled water and subsequently mixed with 200 μ L of the Folin-Ciocalteu reagent. After 3 min, 1000 μ L of aqueous Na_2CO_3 (15% w/v) were added. The reaction mixture was then incubated in the dark at room temperature for 1 h. Finally, the absorbance of the sample was read at 765 nm using a spectrophotometer (UV-Spectrophotometer Cary 60, Agilent Technology, Milan, Italy). TPC was expressed in mg gallic acid equivalents (GAE) per 100 g of dry weight (DW) using a calibration curve of this compound.

2.2.4 Determination of Total Flavonoid Content (TFC)

TFC was measured via the method of Xiong et al., albeit with some modifications (Xiong et al., 2014). 100 μ L of *C. laciniata* flower extract (1 mg/mL) was initially diluted with 400 μ L of distilled water and then mixed with 30 μ L of NaNO_2 (5%). After 5 min, 30 μ L of AlCl_3 (10%) were incorporated with the reaction mixture, and 6 min later, 200 μ L of NaOH (1 M) and 240 μ L of distilled water were finally added. The absorbance of the reaction mixture was then read by spectrophotometry at 510 nm. The results were expressed as mg rutin equivalents (RE) per 100 g DW.

2.2.5 LC-MS/MS Qualitative Analysis

The qualitative profiling of specialized metabolites in *C. laciniata* flower extract was carried out using an Accela HPLC system (Thermo Fisher Scientific, Milan, Italy) coupled through an electrospray ionization (ESI) source to a linear ion trap and a high-resolution Orbitrap mass analyzer (LTQ-Orbitrap XL, Thermo Fisher Scientific, Milan, Italy). The instrument operated in both positive and negative ionization modes to ensure broad metabolite coverage, as phenolic compounds and flavonoids are preferentially detected in negative mode, whereas triterpenoids and other nonpolar constituents typically ionize better in positive mode. High-resolution electrospray ionization mass spectrometry (HRESIMS) data were initially acquired in full-scan and data-dependent acquisition modes, and tandem MS (MS/MS) experiments were subsequently performed to obtain structural information necessary for

metabolite identification. Chromatographic separation was carried out on a Luna C18 column (150 × 2.0 mm, 3 µm; Phenomenex®, Castel Maggiore, Bologna, Italy). The mobile phase consisted of eluent A (ultrapure water with 0.1% formic acid, v/v) and eluent B (ultrapure acetonitrile with 0.1% formic acid, v/v). A linear gradient elution was performed, starting from 5% B and increasing to 60% B within 35 min, followed by a further increase to 100% B in 15 min. The flow rate was remained at 0.200 mL/min, and the injection volume was set at 10 µL (De Leo et al., 2021). The LTQ-Orbitrap XL platform was selected because of its high mass accuracy (≤ 5 ppm) and resolving power ($> 30,000$), which are essential for the reliable annotation of isobaric or structurally related metabolites in complex plant extracts. By combining both ionization modes with MS/MS fragmentation, this analytical strategy maximizes metabolite coverage and provides detailed structural insights. Although the present study was primarily qualitative, the method establishes a robust foundation for future targeted and quantitative LC-MS/MS investigations using authentic standards, which will further strengthen the phytochemical characterization of *C. laciniata* flowers.

2.2.6 Determination of Antioxidant Activity

The antioxidant activities of *Crataegus spp* flower extracts were assessed using three assays: 1,1-diphenyl- 2-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline6-sulfonic acid) (ABTS) radical scavenging activity and Fe^{3+} reducing antioxidant power (FRAP).

DPPH Assay

DPPH scavenging activity was determined using the method of Xiong et al., albeit with minor modifications (Xiong et al., 2014). Briefly, 0.5 mL of sample (50–250 µg/mL) were added to 3.0 mL of DPPH methanolic solution (0.1 mM). After shaking, the mixture was preserved in the dark for 30 min at room temperature, and, subsequently, the absorbance was measured at 517 nm. Ascorbic acid was used as the positive control. The percentage of DPPH scavenging activity was calculated using the following formula:

$$\text{Inhibition (\%)} = ((C-S)/C) * 100$$

where C = the absorbance of the control, and S = the absorbance of the extract. Data were expressed as the concentration for 50% scavenging activity of DPPH radical (IC_{50}).

ABTS Assay

ABTS radical scavenging activity was evaluated following the procedure of Wang et al (2013). The extract solutions (12.5–125 $\mu\text{g/mL}$) were reacted with pre-formed ABTS $\bullet^+$ radicals, and the reduction in absorbance was measured at 734 nm. Trolox was used as the reference antioxidant standard. The inhibition percentage was calculated using the same formula as for DPPH. The results were expressed as IC_{50} values, corresponding to the extract concentration needed to reduce ABTS radical absorbance by 50%.

FRAP Assay

Ferric reducing antioxidant power (FRAP) was determined according to the method of Uysal et al (2017). The FRAP reagent was freshly prepared by mixing 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) with acetate buffer and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. The extract solutions (12.5–125 $\mu\text{g/mL}$) were incubated with the FRAP reagent, and the increase in absorbance due to Fe^{3+} reduction to Fe^{2+} was measured at 593 nm. Results were expressed as milligrams of Trolox equivalents (mg TE) per gram of extract. All of these assays provided complementary insights into both the radical scavenging efficiency and the reducing power of *C. laciniata* flower extracts, offering a robust evaluation of their antioxidant potential.

2.2.7 α -Amylase Inhibitory Assay

The inhibitory activity of *C. laciniata* flower extract against α -amylase was evaluated using the 3,5-dinitrosalicylic acid (DNSA) colorimetric method with slight modifications (Hawash et al., 2020). In this assay, 40 μL of the extract dissolved in DMSO (0.25–1.0 mg/mL) were combined with 160 μL of distilled water and 200 μL of α -amylase enzyme solution (4 U/mL, prepared in 0.02 M $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer containing 0.006 M NaCl, pH 6.9) and incubated at 30 $^\circ\text{C}$ for 10 min. Subsequently, 400 μL of potato starch (0.5%, w/v) were added to the mixture and re-incubated via the method previously described. Following incubation, 200 μL of the mixture were removed and added into a separate tube containing 100 μL of DNSA reagent solution (96 mM 3,5-dinitrosalicylic acid, 5.31 potassium tartrate

in 2 M NaOH) and placed into a water bath at 90 °C for 15 min to develop the characteristics. After 15 min, this mixture was diluted with 900 µL distilled water, and the absorbance was recorded at 540 nm via a UV-Visible spectrophotometer (Cary 60, Agilent Technologies, Milan, Italy). The control sample with 100% enzyme activity was prepared by replacing the extract with 40 µL of DMSO. A blank sample was similarly obtained by replacing the enzyme solution with distilled water to allow absorbance produced by the extract. Acarbose (10–100 µg/mL) was employed as a positive control. The potential of α -amylase inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = (C - S / C) \times 100$$

where C is the absorbance of the control, and S is the absorbance of the extract—blank. The results are presented as the concentration of extract inhibiting 50% of the enzymatic activity (IC_{50}).

2.2.8 α -Glucosidase Inhibitory Assay

The α -glucosidase inhibition evaluation was carried out according to the procedure described by Milella et al., albeit with minor modifications (Milella et al., 2016). The reaction mixture was prepared with 650 µL of phosphate buffer (0.1 M, pH 6.8), 200 µL of the test sample (1–100 µg/mL) and 100 of enzyme solution (0.4 U/mL in buffer) and incubated for 10 minutes at 37 °C. After that, 300 µL of 4-nitrophenyl α -D-glucopyranoside (2.5 mM in buffer) was added to the mixture and re-incubated via the method previously described. Absorbance was read at 405 nm. Control reactions representing 100% enzyme activity were prepared by replacing the extract with 200 µL of buffer, while blanks were prepared by substituting the enzyme solution with buffer to account for background absorbance from the extract. Acarbose (10–1000 µg/mL), a well-known α -glucosidase inhibitor, was employed as the positive control. The inhibition percentage was calculated according to the following formula:

$$\text{Inhibition (\%)} = ((C - S) / C) \times 100$$

Where C is the absorbance of the control and S is the absorbance of the extract (minus blank). The results are expressed as IC_{50} value.

2.2.9 Tyrosinase Enzyme Inhibitory Assay

The inhibitory effects of *C. laciniata* flower extracts on tyrosinase activity were assessed *in vitro* according to the method described by Mirabile et al., with minor modifications (Mirabile et al., 2023). Tyrosinase from *Agaricus bisporus* was employed, and both its monophenolase (L-tyrosine oxidation) and diphenolase (L-DOPA oxidation) activities were evaluated. Aliquots (50 µL) of extract (50–250 µg/mL) were added to 500 µL of substrate, L-DOPA or L-tyrosine (1.25 mM) and 900 µL of phosphate buffer (50 mM, pH 6.8) to conduct the tests. Following a 10-minute incubation at 25 °C, 50 µL of 333 U/mL enzyme solution were added to the reaction mixture. To assess the inhibitory effects, absorbance was measured at 475 nm for up to 40 min to evaluate the monophenolase activity, as well as after just 60 s for the diphenolase activity. DMSO and kojic acid (1–25 µg/mL) were utilized as negative and positive controls. The inhibition of tyrosinase enzyme activity was estimated using the following equation:

$$\text{Inhibition (\%)} = ((C-S)/C) \times 100$$

where C is tyrosinase + substrate (L-DOPA or L-tyrosine), and S is tyrosinase + substrate (L-DOPA or L-tyrosine) + sample. The results are presented as the concentration inhibiting 50% of the enzymatic activity (IC₅₀).

2.2.10 Zebrafish Embryo Maintenance and Treatment

Adult zebrafish (*Danio rerio*), both male and female, were maintained in a temperature-controlled aquarium at 28.5 °C under a 14:10 h light–dark photoperiod and regularly fed with freshly hatched *Artemia salina* larvae (Westerfield et al., 2000). Fertilized eggs were obtained through natural spawning, collected immediately after deposition, and incubated at 28.5 °C in embryo water. At 24 hours post-fertilization (hpf), eggs were examined under a stereomicroscope (SMZ-171 Series, Motic, Hong Kong, China), and only fertilized embryos were selected for subsequent experiments. The evaluation of the vitality test and whitening effects was performed *in vivo* on zebrafish embryos according to the following method (Smeriglio et al., 2019). The eggs at 24 hpf (hours post-fertilization) were manually stripped of the chorion, and the embryos were separated into 96-well plates (1 embryo per well),

randomly divided into four experimental groups (20 embryos per three replicates for each group), and subjected to treatment with CLF extract at specific concentrations (25, 50, and 100 µg/mL) before being solubilized in DMSO (0.2%). The control embryo groups were incubated with embryo water (as a negative control). Following 48 h of incubation at 28 °C (72 hpf), embryos were observed under a stereomicroscope (SMZ-171 Series, Motic) equipped with a digital camera (Moticam® 5 Plus) for image acquisition. Mortality and abnormal phenotypes were documented in all groups. The abnormal phenotypes and mortality of each treated group were recorded. With regard to the whitening effects, the Pillow library for the Python programming language was employed to process the acquired images of the CLF treatment (25–100 µg/mL), PTU (30 µg/mL) and controls. Images were converted into grayscale, and areas of pigmentation were quantified using pixel density analysis. Data were expressed as percentage pigmentation relative to the untreated control group, which was set as 100%. All experimental procedures were conducted in accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes and complied with the ethical guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2.11 Statistical Analysis

The statistical significance was determined through one-way analysis of variance (ANOVA). Data were deemed statistically significant for $p < 0.05$ and $p < 0.01$.

2.3 Results and discussion

2.3.1 Total Phenolic (TPC) and Flavonoid (TFC) Content

The total phenolic content (TPC) and the total flavonoid content (TFC) were determined via the Folin–Ciocalteu colorimetric method and the aluminum chloride assay, respectively. The total content of polyphenols and flavonoids of *C. laciniata* flower extract was shown in Figure 1. It was determined that CLF extract possesses a high phenolic content (2910.8 mg GAE/100 g DW), mainly consisting of flavonoids (2531.4 mg RE/100 g DW).

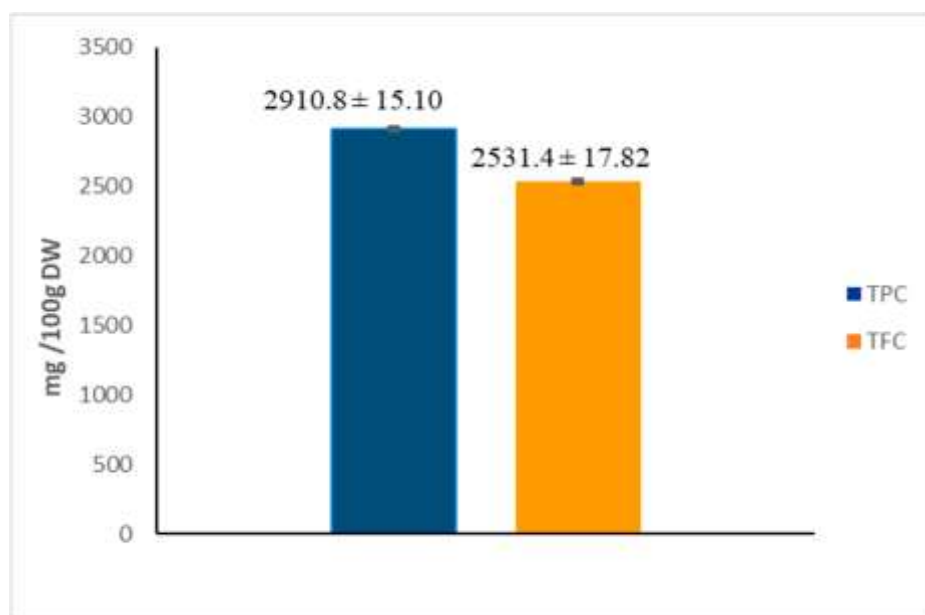


Figure 1. Total phenolic content (TPC) and total flavonoid content (TFC) of *C. laciniata* flower extract. Data are expressed as mean $\pm$ standard deviation (SD) of three independent experiments ($n = 3$). TPC values are reported as mg gallic acid equivalents (GAE) per 100 g of dry weight (DW), while TFC values are expressed as mg rutin equivalents (RE) per 100 g DW.

2.3.2 Phytochemical Characterization

The untargeted LC-MS/MS analysis of *C. laciniata* flower extract (CLF) was performed in both negative and positive ionization modes in order to maximize metabolite coverage (Figure 2). Compounds **1**, **2**, **6**, **7**, and **8** were characterized by the presence of a diagnostic fragment ion at m/z 161 $[M-H]^-$, indicative of dehydrated and deprotonated caffeic acid, together with a fragment ion at m/z 191 $[M-H]^-$, corresponding to deprotonated quinic acid. Several polyphenolic compounds were identified in the extract, such as catechin and epicatechin oligomers. In particular, compounds **10**, **11**, and **15** were identified as procyanidin derivatives, occurring as either dimers or trimers (Table 1). The most common class of polyphenols characterized in the CLF extract consisted of flavonoid glycosides, encompassing both C-glycosylated and O-glycosylated derivatives as well as the major metabolites identified in CLF, such as hyperoside (**24**) and vitexin (**21**). Several luteolin derivatives were detected, differing in their glycosylation patterns (compounds **17**, **19**, **27**, **30**, and **32**). These compounds demonstrated the fragmentation at m/z 287 $[M+H]^+$, which

corresponds to a glycone luteolin in *O*-flavonoids, whereas in *C*-flavonoids a fragmentation at m/z 431 $[M+H-H_2O]^+$ was observed, corresponding to the sugar moiety.

Quercetin was identified as one of the most abundant aglycones in the CLF extract. A diagnostic fragment ion at m/z 303 $[M+H]^+$, corresponding to the quercetin backbone, was observed in compounds **22**, **23**, **26**, **28**, **31**, and **33**. Detailed fragmentation analysis revealed that peak **26** exhibited the neutral loss of m/z 162, indicative of a hexose moiety, while compounds **22** and **23** showed sequential neutral losses of m/z 162 and 146, corresponding to hexose and deoxyhexose residues, respectively. In contrast, compounds **31** and **33** exhibited the fragmentation pattern of *C*-hexoside with fragmentation at m/z 445 $[M+H-C_2H_3O-H_2O]^+$. Anthocyanidins are abundant in the extract of CLF; in particular, compounds **16**, **18**, and **25** exhibit the same fragment ion at m/z 303 $[M]^+$, which corresponds to a delphinidin aglycone. Finally, several polyunsaturated fatty acids and hydroxylated polyunsaturated fatty acids were identified at the end of the chromatogram. The identified metabolites in CLF extract are presented in *Table 1*.

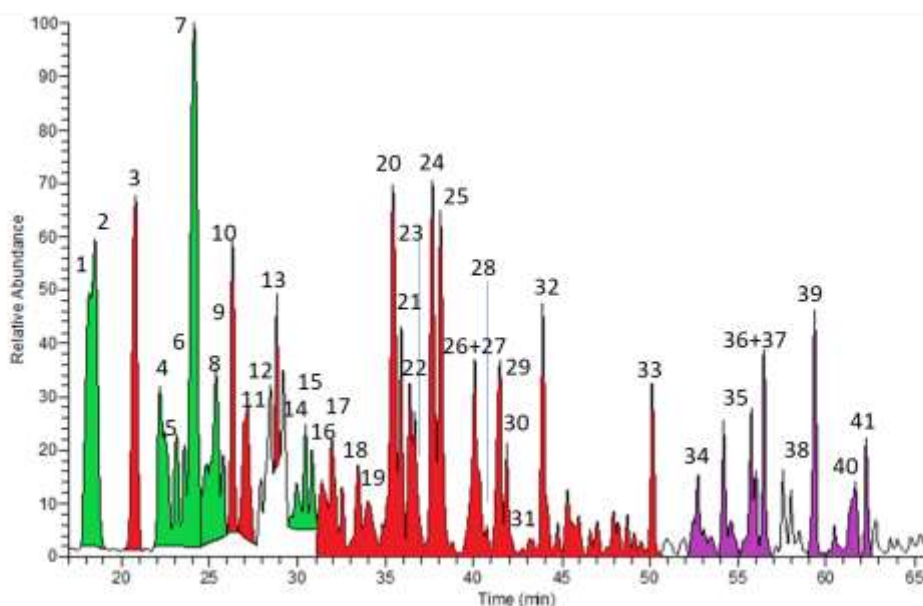


Figure 2. High-performance liquid chromatography–high-resolution electrospray ionization–mass spectrometry profile of CLF extract. Peaks (**1–41**) correspond to compounds listed in *Table 1*.

Table 1. Secondary metabolites identified in CLF extract via LC-MS/MS analysis (Rt= retention time; MSI = Metabolomics Standards Initiative).

Peaks	Rt	[M+H] ⁺	[M+H] ⁺	MS ²	Compound	MSI level ²
1	18.16	517.1550		355	Caffeoylquinic acid-O-hexoside	2
2	18.52	517.1550		355	Caffeoylquinic acid-O-hexoside isomer	2
3	20.77		477.1634	283	Cyanidin-O-hexoside	1
4	22.5		337.094	191;161	Coumarylquinic acid	2
5	22.94		325.0945	161	Coumaric acid O-hexoside	2
6	23.22		447.1528	401; 283	Dicaffeoylquinic acid	2
7	24.11		353.081	161	Caffeoylquinic acid	2
8	25.33		515.138	355;161;191	Caffeoylquinc acid O-hexoside	2
9	25.43	387.2004		369;207	Roseoside	2
10	26.29	577.1372		425;289	Procyanidin dimer	2
11	26.91	577.1372		425;290	Procyanidin dimer isomer	2
12	27.14	579.1457		433	Rutin	1
13	28.44	291.0863		273;165;151;123	Catechin	2
14	31.06	369.1180		145;117	Feruloylquinic acid	2
15	31.63	865.1974		695;577;407;287	Procyanidin trimer	2
16	32.23	627.1570		465;303	Delphinidin -O-dihexoside	2
17	32.52	449.1078		431,416,383	Luteolin-C-hexoside	2
18	32.7	627.1570		465;303	Delphinidin-O-dihexoside isomer	2
19	33.33	449.1078		431,416,383	Luteolin-C-hexoside isomer	2
20	35.4	565.1552		433;415	Apigenin-C-hexoside-O-pentoside	2
21	35.8	433.1116		415	Vitexin	1
22	36.33	611.1577		465;303	Quercetin-O-hexoside-rhamnoside	2
23	36.57	611.1577		465;303	Quercetin-O-hexoside-rhamnoside isomer	2

24	37.57	465.1010	303	Hyperoside	1
25	38.09		301	Delphinidin-O-hexoside	2
26	40.03	479.1163	317	MethoxyQuercetin-O-hexoside	2
27	40.26	449.1078	287	Luteolin-O-hexoside	2
28	40.33	625.1732	479;317	MethoxyQuercetin-O-hexoside-	2
29	41.44	449.1057	287	rhamnoside	2
30	41.46	449.1078	287	Luteolin-O-hexoside	2
31	43.4	507.2215	463;445;343;301	Luteolin-O-hexoside	2
32	45.37	535.1069	287	AcetylQuercetin-C-hexoside	2
33	50.12	507.2215	463;445	Luteolin-O-malonylhexoside	2
34	54.16		311;313;293;275	AcetylQuercetin-C-hexoside isomer	2
35	55.7	329.2310	313;295;277	Trihydroxyoctadecenoic acid	2
36	56.04	331.2465	275	Trihydroxyoctadecatrenoic acid	2
37	56.43	293.2104	293;275	Hydroxyoctadecanoic acid	2
38	57.5	333.2617	315;297;279	Dihydroxyoctadecatrenoic acid	2
39	59.31	289.2367	271;253;235	Trihydroxyoctadecatrenoic acid isomer	2
40	61.31	309.2084	291;273	Dihydroxyphenylnonanoic acid	2
41	62.28	309.2084	291;274	Dihydroxyoctadecatrenoic acid isomer	2
				Dihydroxyoctadecatrenoic acid isomer II	2

2.3.3 Antioxidant Activity

Based on the chemical composition of the *C. laciniata* flower extract, a series of *in vitro* assays were carried out to evaluate its antioxidant activities. The results, listed in Table 2, showed that CLF possessed significant antioxidant capacity, as evidenced by its performance in both the DPPH radical scavenging and FRAP assays. Furthermore, in the TEAC assay, CLF showed a strong ABTS radical scavenging activity, achieving values similar to those of Trolox, which was used as the positive control.

Table 2. Antioxidant activity of *C. laciniata* flower (CLF) extract

	DPPH (IC ₅₀ µg/mL)	TEAC (IC ₅₀ µg/mL)	FRAP (TE/g Extract)
CLF	165.25 ± 0.15	20.13 ± 0.09	75.61 ± 0.025
Ascorbic acid	16.95 ± 1.20	----	----
Trolox	----	13.08 ± 0.02	----

Data are reported as the mean ± standard deviation (SD) of triplicate experiments (n = 3). IC₅₀ = concentration giving 50% of the activity; TE = Trolox equivalents.

2.3.4 α -Amylase and α -Glucosidase Inhibitory Activity

The inhibition of α -amylase and α -glucosidase enzymes was assessed. The findings indicated that CLF extract inhibited both the enzymes, even if the effects on α -glucosidase were higher than on α -amylase. Furthermore, the CLF extract was demonstrated to be 100-fold more potent than acarbose as the standard compound (4.56 vs. 436.47 $\mu\text{g/mL}$). The IC_{50} values of CLF extract are shown in Table 3 and compared to those of acarbose.

Table 3. α -amylase and α -glucosidase inhibition of *C. laciniata* flower (CLF) extract.

	α -Amylase (IC_{50} $\mu\text{g/mL}$)	α -Glucosidase (IC_{50} $\mu\text{g/mL}$)
CLF	517.41 $\pm$ 36.86	4.56 $\pm$ 0.13
Ascarbose	29.07 $\pm$ 1.9	436.47 $\pm$ 58.82

2.3.5 Inhibitory Effects on Tyrosinase Enzyme

The inhibition of *C. Lacinata* flower extract was investigated regarding tyrosinase inhibition, and the results presented in Table 4 were compared to kojic acid, employed as a positive control. The inhibitory effects of CLF extract are already evident in the initial step of the reaction, with a lower IC_{50} value for monophenolase than diphenolase activity (67.47 vs. 103.61 $\mu\text{g/mL}$).

Table 4. Tyrosinase inhibition of *C. laciniata* flower (CLF) extract.

	Monophenolase (IC_{50} $\mu\text{g/mL}$)	Diphenolase (IC_{50} $\mu\text{g/mL}$)
CLF	67.47 $\pm$ 3.86	103.61 $\pm$ 5.46
Kojic acid	9.18 $\pm$ 1.24	2.52 $\pm$ 0.81

Data are reported as the mean $\pm$ standard deviation (SD) of triplicate experiments ($n = 3$). IC_{50} = concentration inhibiting 50% of the enzyme activity.

2.3.6 Inhibitory Effects on Melanogenesis in Zebrafish Embryos

In the present research, a toxicological evaluation of Zebrafish's early-stage development was carried out before evaluating the anti-melanogenic effects of CLF. Demonstrating a

good percentage (90%), the absence of morphological abnormality in treated concentrations of CLF (25–100 $\mu\text{g/mL}$) until the end of the testing period (72 h post-fertilization, hpf) was determined (Figure 3, Panel a). The same treatment supported us to assess the anti-melanogenic properties on zebrafish. After a visual inspection of the embryos under a stereomicroscope, images were taken and processed to quantify the whitening effects elicited by CLF treatment with respect to the control (100% pigmentation) (Figure 3, Panels b and c). Overall, pigmentation in zebrafish embryos was significantly decreased in a dose-dependent manner following treatment with CLF at 25 and 50 $\mu\text{g/mL}$, resulting in pigmentation levels of 33.64% and 29.87% relative to the control, respectively. However, increasing the concentration to 100 $\mu\text{g/mL}$ did not lead to a further enhancement of depigmentation, with residual pigmentation remaining at 30.19% compared to the control. In general, treatment with CLF at concentrations of 25–50 $\mu\text{g/mL}$ produced slightly greater depigmenting effects compared to 1-phenyl-2-thiourea (PTU), which was used as the reference standard (30 $\mu\text{g/mL}$, 36.00%).

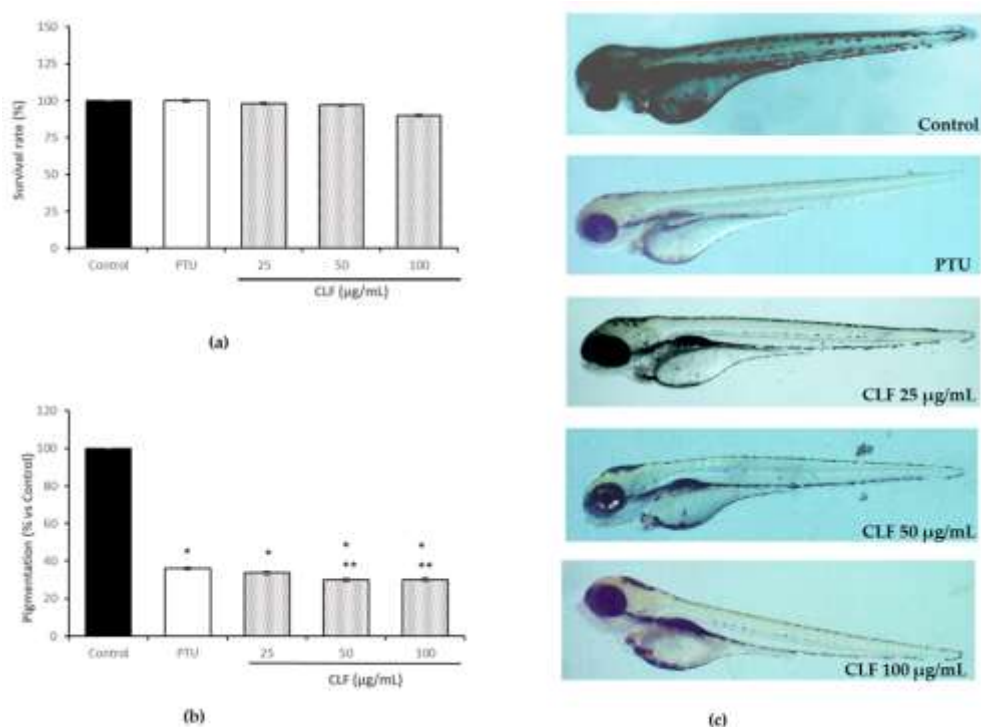


Figure 3. (a) Survival rate of zebrafish embryos following 72 h of exposure to PTU and *C. laciniata* flower extract (25–100 $\mu\text{g/mL}$). (b) Effects of *C. laciniata* flower extract (CLF) on pigmentation in zebrafish embryos after treatment. Pigmentation levels are expressed as percentages relative to the control group. * $p < 0.01$ vs. control; ** $p < 0.05$ vs. PTU. (c) Representative images of zebrafish

embryos at 72 hpf (magnification 20×) were captured using a stereomicroscope (SMZ-171 Series, Motic, San Antonio, TX, USA) equipped with a digital camera (MotiCam® 5 plus, Motic, San Antonio, TX, USA).

2.4 Discussion

Crataegus species (hawthorn) have been used traditionally as edible and medicinal plants since ancient times. Hawthorn includes a variety of flavonoids and anthocyanins that appear to be mainly responsible for its cardioprotective effects (Mustapha et al., 2015). Our research was focused on *C. laciniata*, a species growing in Sicily (Nebrodi and Madonie mountains) described for the first time by Bernardino ab Ucria (Calvo et al., 2015). At first, we quantified the phenolic and flavonoid content of an ethanolic flower extract of *Crataegus laciniata*, which proved to be particularly rich in flavonoids (2531.4 mg/100 g DW). Our findings are similar to those previously reported for samples of *Crataegus oxyacantha* L. flowers from Algeria with a TPC average of 2759.0 mg GAE/100 g DW. Nevertheless, the total flavonoid content reported for *Crataegus oxyacantha* L. flowers (660.7 mg RE/100 g DW) were lower than those for our samples *C. laciniata* (Issaadi, et al., 2021). Among the limited reports available on the flavonoid composition of *Crataegus* flowers of different *Crataegus* species, Edwards et al. (2012) described comparatively lower values, ranging between 317.8 and 1710 mg/100 g DW. Thus, when compared with the literature, variability can be attributed to differences in species and geographic origin. Subsequently, LC-MS/MS analysis was performed to characterize the phytochemical profile of CLF extract, which was found to be particularly enriched in polyphenolic compounds. The most abundant polyphenols detected in CLF consist of flavonoid derivatives (Bardakci et al., 2019). In addition to hyperoside and vitexin, which were identified as the main metabolites, luteolin glycosides were characterized. These findings are in accordance with previous literature on *Crataegus oxyacantha* flowers that were rich in flavones like vitexin, and flavonols like rutin and hyperoside (Issaadi et al., 2021). CLF was also particularly abundant in anthocyanidins, notably containing delphinidin aglycone (Belkhir et al., 2013). In addition, polymers of catechin and epicatechin corresponding to procyanidin derivatives were identified. A comparable procyanidin profile was likewise reported in the flowers of

two *C. azarolus* varieties (*C. azarolus* L. var. *aronia* Batt. and *C. azarolus* L. var. *eu-azarolus* Maire) cultivated in Tunisia (Bahri-Sahloul et al., 2009). Oxidative stress leads to the overproduction of reactive oxygen species, which damage cellular structures, accelerate age-associated dysfunctions, and contribute to the onset of chronic diseases. For the evaluation of the antioxidant potential of our extract, DPPH, TEAC, and FRAP assays were performed. The results demonstrated that CLF exhibited a notable free-radical scavenging activity in the DPPH assay ($IC_{50} = 165.25 \mu\text{g/mL}$). Interestingly, based on the TEAC test, CLF possesses an IC_{50} value of $20.13 \mu\text{g/mL}$, comparable to Trolox ($IC_{50} = 13.08 \mu\text{g/mL}$), because of the synergistic effects of its mixture of polyphenolic compounds. The same extract also displayed high antioxidant activity in the FRAP assay ($75.61 \text{ TE/g extract}$), supporting the reducing ability of its bioactive compounds. The health-promoting potential of CLF extract was also studied *in vitro* for two enzymes (α -amylase and α -glucosidase). Inhibiting α -amylase and α -glucosidase has appeared to be a significant treatment option for diabetes. Postprandial hyperglycemia is controlled by the inhibition of these enzymes, which results in the delay of the carbohydrate digestion and reduced glucose absorption. As shown in Table 3, CLF extract exhibited dual α -amylase ($IC_{50} = 517.41 \mu\text{g/mL}$) and α -glucosidase ($IC_{50} = 4.56 \mu\text{g/mL}$) inhibitory activity. A strong inhibitory effect on α -glucosidase was comparable to that reported for Irish seaweed extracts, which achieved IC_{50} values below $2 \mu\text{g/mL}$ (Lordan et al., 2013). In addition, the considerable antihyperglycemic potential of CLF extract may be attributed to its phytochemical profile, mainly the abundance of glycosides such as hyperoside and vitexin. Furthermore, recent investigations reported that the α -amylase activity has been reduced in the presence of hyperoside in a competitive manner (Shen et al., 2023). Conversely, vitexin showed a strong inhibitory ability on α -glucosidase, with an uncompetitive mechanism of action (Ni, et al., 2020). TYR is widely recognized as a crucial target for screening novel bioactive compounds aimed at treating dermatological disorders associated with excessive melanin accumulation. TYR catalyzes the two rate-limiting steps in melanin biosynthesis: the hydroxylation of L-tyrosine to L-DOPA (monophenolase activity), followed by the oxidation of L-DOPA to dopaquinone (diphenolase activity) (Thawabteh et al., 2023). Well-known TYR inhibitors, such as kojic acid, have been associated with adverse side effects when used over prolonged periods (Cho et al., 2020). Because of these safety issues, many medicinal plants have been

investigated through *in vitro* TYR inhibition assays to identify novel phytocomplexes with potential skin-whitening effects. Among various *Crataegus* species, previous reports indicated that extracts from the aerial parts of *C. azarolus* L. and the seeds of *C. pinnatifida* Bunge reduced melanin content in B16F10 cells through TYR inhibition (Mustapha et al., 2015; Huang et al., 2014). On this basis, we evaluated the inhibitory effects of *C. laciniata* flowers on TYR activity. The results demonstrated that CLF effectively inhibited both monophenolase and diphenolase activities at relatively low concentrations, with IC₅₀ values of 67.47 and 103.61 µg/mL, respectively. Given that hyperoside and vitexin, the principal metabolites identified in CLF, are recognized as effective TYR inhibitors, their contribution to the observed anti-melanogenic activity cannot be ruled out (Ren et al., 2018; Erdogan Orhan et al., 2015). The zebrafish embryo is an accepted model for pharmacological studies because of its high physiological and genetic similarity to mammals. Consequently, zebrafish embryo assays have emerged as alternative approaches for animal testing (Agalou et al., 2018). Zebrafish offer several advantages, including their small size, ease of handling, and efficient skin absorption of test compounds at early developmental stages. In addition, their optically transparent embryogenesis enables direct observation of the pigmentation process (Choi et al., 2007). Accordingly, in the present study, CLF exerted pronounced whitening effects on the pigmentation of early-stage embryos, without impairing their development or survival after 48 h of exposure. Under comparable experimental conditions, dibenzofuran compounds isolated from the aerial parts of *C. pycnoloba* Boiss. and Heldr. were reported to reduce embryonic melanogenesis at a concentration of 10 µg/mL (Agalou et al., 2018). Interestingly, the polyphenol-rich phytocomplex of CLF induced embryo depigmentation at a relatively low concentration (25 µg/mL), proving slightly more effective than the standard PTU.

2.5 Conclusion

In this study, the flower extract of *Crataegus laciniata* was studied in terms of its phytochemical profile and biological properties. The chemical profile revealed a remarkable abundance of phenolic components, such as flavonoid glycosides, procyanidin oligomers, and anthocyanidins, which are widely acknowledged for their health-promoting potential. Such diversity not only supports the traditional medicinal relevance of hawthorn species

but also highlights *C. laciniata* as an underexplored reservoir of bioactive natural products. The CLF extract significantly inhibited α -glucosidase and α -amylase and showed strong antioxidant activity *in vitro*. In addition, the inhibitory activity on tyrosinase, combined with *in vivo* skin-whitening effects in zebrafish embryos, suggests valuable applications in the cosmeceutical sector. Importantly, the absence of developmental toxicity in zebrafish assays reinforces the favorable safety profile of the extract. In conclusion, the findings of this study demonstrate *C. laciniata* flowers as a promising natural source of multifunctional bioactive compounds with potential applications in both pharmaceutical and cosmetic fields. The current findings offer a strong basis for the future evaluation of this species, even though more mechanistic research and clinical confirmations are necessary.

Chapter 3

Polyphenolic characterization, antioxidant and antidiabetic activities of *Crataegus spp.* fruit extracts

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Polyphenolic characterization, antioxidant and antidiabetic activities of *Crataegus* spp. fruit extracts

3.1 Introduction

The genus *Crataegus* constitutes an extremely variable taxon within Rosaceae family, with hundreds of tree and shrub species, along with unknown number of subspecies, varieties, and hybrids some of medicinal interest and in some cases also with edible fruits (Spadaro et al., 2024). In Italy, the island of Sicily represents a biodiversity hotspot for *Crataegus*, due to its unique geological, pedological, and climatic conditions, as well as its central location within the Mediterranean basin. Moreover, hawthorn fruits show significant potential as a medicinal and edible source with different pharmacological activities, including the treatment of heart disease and heart failure (Koçyildiz et al., 2006), atherosclerosis (Lu et al., 2023), urinary retention, hypertension (Walker et al., 2002), and intestinal disorders (Zhang et al., 2019; Liu et al., 2020). Hawthorn is also known for its antioxidant, anti-inflammatory, antimicrobial, hypoglycemic, hypolipidemic and hepatoprotective effects (Kalantari et al., 2016). *Crataegus* fruits are now also known for its nutrients. Recent studies highlight hawthorn fruits as an emerging source of bioactive compounds with antioxidant, anti-inflammatory, and cardiovascular protective properties of bioactive compounds from hawthorn fruits that offer many benefits to human health (Ma et al., 2024). For this reason, in this part of *Crataegus* project a total of 11 *Crataegus* taxa and nothotaxa were collected in the Western part of Sicily with the aim to address a multidisciplinary approach that integrates advanced phytochemical profiling and *in vitro* biological assays targeting key enzymes involved in carbohydrate digestion. To further investigate the relationships between the chemical composition and biological activity of the extracts, multivariate statistical techniques were also performed.

In recent years, the decline in biodiversity has raised concerns about the conservation and sustainable use of plant genetic resources (Salgotra et al., 2023). The present study provides a significant contribution to the understanding of the chemical and biological diversity within the *Crataegus* genus, and could help to promote the value of wild plants in the Mediterranean region, this issue is particularly critical for endemic species. These species are underutilised, but they could be valuable resources for the agri-food and nutraceutical industries (Liu et al., 2020). Their comprehensive characterization could

enhance their use in food, nutritional and medicinal applications and support biodiversity conservation, especially as they can thrive in arid soils, making them a promising asset for climate change adaptation in Sicily. Consequently, the present study aimed to characterize the polyphenolic profile of fruit extracts of different Sicilian *Crataegus* species and to evaluate their antioxidant and antidiabetic potential, establishing correlations between their phytochemical composition and biological activities.

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

UHPLC-grade methanol (MeOH), acetonitrile (ACN), water (H₂O), and formic acid (HCOOH) were obtained from Merck KGaA (Darmstadt, Germany), while analytical-grade solvents were sourced from VWR International S.R.L. (Milan, Italy). Pure standards of Vitexin $\geq$ 98.0% (HPLC), Hyperoside analytical standard ($\geq$ 98% by HPLC) and all reagents for the biological assays were purchased from Sigma-Aldrich Corp. (St. Louis, Mo., USA). Luteolin 7-*O*-glucoside, and quercetin standards were previously isolated and characterized using 1D- and 2D-NMR, as well as HR-MS techniques, in the authors' laboratory from other plant extracts. Hydrogen peroxide (H₂O₂) and 2',7'-dichlorofluorescein diacetate (H₂DCF-DA) were purchased from Sigma-Aldrich Corp. (St. Louis, Mo., USA).

3.2.2 *Crataegus* Fruit Sample Collection

Plant material was collected (October 2022) in the provinces of Palermo and Trapani, where the *Crataegus* populations are very diversified. The specimens were characterized by Professor Francesco Maria Raimondo and preserved as documentation at the herbaria of PLANTA research Center of Palermo (PAL-Gr). The seedless fruits were air-dried at 45 °C for 24 hours and then pulverized. Each sample (5 g) was extracted with ethanol/water (7:3) and the resulting extracts were filtered and concentrated to dryness. The collection and the extraction yields are reported in *Table 1*.

Table 1. Collection site, extraction yields (%), and macroscopic characteristics of *Crataegus* fruits collected from different locations in Sicily.

	Specific and Intraspecific Taxa	Fruit Samples	Macroscopic Characteristics	Collection site	Longitude and Latitude	Extraction Yield (%)
CrF1	<i>Crataegus drepanensis</i> Raimondo, Marino & Scuderi		Yellowish small fruit (2-3 seeds)	San Vito Lo Capo-Tonnara del Secco (Trapani)	38°10'08.40"N - 12°45'55.49"E	50.18
CrF2	<i>Crataegus azarolus</i> var. <i>aronia</i> L.		Yellowish medium fruit (2-3 seeds)	San Vito Lo Capo-Tonnara del Secco (Trapani)	38°10'08.40"N - 12°45'55.49"E	33.84
CrF3	<i>Crataegus azarolus</i> var. <i>aronia</i> L.		Yellowish medium fruit (2-3 seeds)	Macari-San Vito Lo Capo (Trapani)	38°07'48.5"N 12°44'20.8"E	33.88
CrF4	<i>Crataegus zichichii</i> Raimondo, Spadaro & Venturella		Bright red medium fruit (2 seeds)	Fraginesi-Buseto Palizzolo (Trapani)	38°01'32.0"N 12°50'11.0"E	39.96
CrF5	<i>Crataegus zichichii</i> Raimondo, Spadaro & Venturella		Bright red medium fruit (2 seeds)	Paceco-Marsala (Trapani)	37°58'43.0"N 12°33'37.0"E	43.00
CrF6	<i>Crataegus azarolus</i> var. <i>azarolus</i> L.		Reddish large, wrinkled fruit (2-3 seeds)	Custonaci/Purgatorio-Castelluzzo road (Trapani)	38°04'48.7"N 12°44'20.3"E	49.95
CrF7	<i>Crataegus azarolus</i> var. <i>chlorocarpa</i> L. (Moris) K. I. Chr.		Yellowish large, wrinkled fruit (2-3 seeds)	Fraginesi-Buseto Palizzolo (Trapani)	38°01'32.0"N 12°50'11.0"E	49.68
CrF8	<i>Crataegus laciniata</i> Ucria		Wine-red medium fruit (2-3 seeds)	Piano Pomo/Petralla Sottana (Palermo)	37°53'43.9"N 14°04'00.6"E	57.27
CrF9	<i>Crataegus</i> aff. <i>insengae</i> (Tineo ex		Bright red small subglobose	Sempria/Castelbuono (Palermo)	37°54'11.5"N 14°04'06.8"E	48.01

	Guss.) Bertol.		fruit (1-2 seeds)			
CrF10	<i>Crataegus</i> aff. <i>insengae</i> (Tineo ex Guss.) Bertol.		Bright red small subglobose fruit (1-2 seeds)	Piano Pomo/Petrali a Sottana (Palermo)	37°53'43.9"N 14°04'00.6"E	47.89
CrF11	<i>Crataegus</i> <i>monogyna</i> Jacq. subsp. <i>monogyna</i>		Dark red small, elongated fruit (1 seed)	San Guglielmo/Cas telbuono (Palermo)	37°55'31.1"N 14°05'38.4"E	51.2

3.2.3 LC-MS/MS Qualitative Analysis

Based on previous publications (Mirabile et al. 2024), the separation system adopted was an UltiMate 3000 UHPLC system (Thermo Fisher Scientific, Milan, Italy) interfaced through an ESI source to a linear ion trap coupled to a high-resolution mass analyzer (Orbitrap Qexactive PLUS [ThermoFisher Scientific, Milano, Italy]) operating in negative- and positive-ion modes. The MS data were first acquired in the full-mass, within a m/z scan range of 150–1000 and data-dependent-scan modes. A C18 column (Luna C18, 150 × 2.0 mm, 3 μ m) (Phenomenex®, Castel Maggiore, Bologna, Italy) and a binary mobile phase composed of eluent A (ultrapure water–formic acid 0.1% v/v) and eluent B (ultrapure acetonitrile–formic acid 0.1% v/v) were used. The separation conditions ranged from 5% to 60% of B in 35 min and then to 100% in 15 min. The flow rate was set to 0.200 mL/min, and the injection volume was 10.0 μ L.

3.2.4 LC-MS Quantitative Analysis

For quantification analyses, dry extracts were solubilized in a mix of H₂O/MeOH 1:1 to obtain a final concentration of 10 mg/mL. A C18 column (Luna Omega C18, 100 × 2.1 mm, 1.6 μ m) (Phenomenex®, Castel Maggiore, Bologna, Italy) and a binary mobile phase composed of eluent A (ultrapure water–formic acid 0.1% v/v) and eluent B (ultrapure acetonitrile–formic acid 0.1% v/v) were used. The mobile phase composition was linearly changed from 5% to 50% of B in 20 minutes and then to 100% in 30 minutes. The flow rate

was set to 0.300 mL/min, and the injection volume was 10 μ L. The MS analyses were carried out using a Orbitrap Qexactive PLUS (ThermoFisher Scientific, Milano, Italy). The main compounds of *Crataegus* extract, hyperoside, vitexin, luteolin 7-*O*-glucoside and quercetin, were quantified using the external standards method. Calibration curve of reference standards was prepared by dilution with MeOH in the range of 20 ng/mL and 10 μ g/mL. A linear model (R^2 values of 0.9992 and 0.9984 for hyperoside and vitexin respectively) was adopted for quantitative analysis. Quantitative data were expressed in g/Kg $\pm$ standard deviation (SD) of dried weight (DW) of extract (Vitiello et al. 2024).

3.2.5 Total Phenolic Content Assessment

A modified Folin-Ciocalteu method (Singleton et al., 1999) was employed to determine the total phenolic content (TPC) in *Crataegus* extracts. Each extract (100 μ L) was mixed with Folin-Ciocalteu reagent (1000 μ L) and distilled water (900 μ L). Then, Na₂CO₃ (15%, 1000 μ L) was added to the mixture prior to incubation at room temperature in the dark for 1 h. The absorbance was recorded at 765 nm. A calibration curve of gallic acid was utilized to indicate TPC content as mg gallic acid equivalents per gram of dry extract (mg GAE/g).

3.2.6 Total Flavonoid Content Assessment

The total flavonoid content (TFC) was measured following a previously described method (Cioni et al., 2024). Briefly, 100 μ L of the extract (1 mg/mL) was diluted with 400 μ L of distilled water and mixed with 30 μ L of 5% NaNO₂. After 5 minutes, 30 μ L of 10% AlCl₃ was added, followed by the addition of 200 μ L of 1 M NaOH and 240 μ L of distilled water after 6 minutes. The absorbance of the final reaction mixture was determined spectrophotometrically at 510 nm, and the TFC results are expressed as milligrams of catechin equivalents per gram of dry extract (mg CE/g).

3.2.7 In Vitro Antioxidant Capacity Assessment

Antioxidant activity was assessed using three widely used *in vitro* assays, offering a complementary insight into the antioxidant properties of the tested samples. These methods, based on different chemical principles, allow a comprehensive evaluation of

radical scavenging and reducing power. The following sections detail the procedures and standards used for the DPPH radical-scavenging activity, ferric ion reducing antioxidant power (FRAP) assay, and ABTS tests.

DPPH Radical-Scavenging Activity

The scavenging activity was determined using the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical following the method of Yoshida (Yoshida et al., 1989). Then, 0.5 mL of the tested sample was added to 3.0 mL of DPPH methanolic solution (0.1 mM). After shaking, the mixture was placed in the dark for 30 min at room temperature, and finally the absorbance was read at 517 nm. The results are reported as mg Acid Ascorbic Equivalents (mg AAS/g), using ascorbic acid as a reference compound.

Ferric Ion Reducing Antioxidant Power Assay (FRAP)

The FRAP test was assessed following the method reported by (Uysal et al., 2017). The results are reported as mg Trolox Equivalents (mg TE/g), using Trolox as a reference compound.

ABTS Assay

The free radical scavenging activity of *Crataegus* fruit extracts was also determined by the ABTS test (Wang et al., 2013). Results are presented in terms of mg Trolox Equivalents (mg TE/g), used as a reference compound.

3.2.8 Cellular Reactive Oxygen Species (ROS) Assessment

Cell Culture

Embryonic rat heart cardiomyocyte-derived cell line H9c2 (from the American Tissue Culture Collection; Manassas, VA, USA) was grown adherent to Petri dishes with Dulbecco's modified Eagle's medium (DMEM), which was supplemented with 10% fetal bovine serum (FBS), 25 u/mL penicillin, and 25 u/mL streptomycin at a humidified air with 5% CO₂ and 37 °C in an incubator to enable the cells to attach and reach the exponential phase of growth.

Cell ROS Formation Evaluation

H9c2 cells were plated (1×10^4 cells/well) on 96-well plates and allowed to adhere for 24 h at 37 °C in 5% CO₂/95% air atmosphere. Thereafter, the medium was replaced with fresh medium alone or containing graded concentrations (25–3.125 µg/mL) of selected *Crataegus* extracts (C_{R8}, C_{R10}, and C_{R1}) for 1 h. Hydrogen peroxide (H₂O₂, 0.5 mM) was then added as a pro-oxidant stimulus (Sigma-Aldrich Corp., St. Louis, Mo., USA). After 1 h, ROS formation was evaluated using the probe 2',7'-dichlorofluorescein diacetate (H₂DCF-DA; 10 µM) for 30 min in the dark at 37 °C, followed by washing with phosphate buffer saline (PBS). H₂DCF-DA is a non-fluorescent molecule that passively diffuses into cells, where the acetates are cleaved by intracellular esterases to form H₂DCF and is rapidly oxidized to the highly fluorescent DCF in the presence of intracellular ROS. H9c2 cells were washed with PBS and then incubated in PBS containing H₂DCF-DA. After 15 min, fluorescence intensity was recorded at an excitation wavelength of 485 nm and an emission wavelength of 520 nm using a Spectrofluorimeter (PerkinElmer Enspire 2300 Multi-mode Microplate Reader, PerkinElmer Inc., Waltham, Mass., USA).

3.2.9 α -Glucosidase Inhibitory Activity

The α -glucosidase inhibition assay was carried out according to the procedure described by Milella et al., albeit with minor modifications (Milella et al., 2016). The reaction mixture was prepared with 650 µL of phosphate buffer (0.1 M, pH 6.8), 200 µL of the test sample (1–100 µg/mL), and 100 µL of enzyme solution (0.4 U/mL in buffer) and incubated at 37 °C for 15 min. Then, 300 µL of 4-nitrophenyl α -D-glucopyranoside (2.5 mM in buffer) was added to the mixture and re-incubated via the previously described method. Absorbance was recorded at 405 nm. The control with 100% enzyme activity was prepared by replacing the extract with buffer (200 µL). A blank was similarly prepared by replacing the enzyme solution with buffer to determine the absorbance produced by the extract. Acarbose (10–1000 µg/mL) was employed as a positive control. The inhibition percentage was determined via the following equation:

$$\text{Inhibition (\%)} = ((C-S)/C) \times 100$$

where C = absorbance of the control, and S = absorbance of the extract. The results are reported as IC₅₀.

3.2.10 α -Amylase Inhibitory Activity

The α -amylase inhibition assay was carried out using the 3,5-dinitrosalicylic acid (DNSA) method, albeit with minor modifications (Hawash et al., 2020). A mixture of 40 μ L of each extract in DMSO (0.25–1.0 mg/mL), 160 μ L distilled water, and 200 μ L α -amylase enzyme (4 U/mL dissolved in buffer $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 0.02 M containing NaCl 0.006 M, pH 6.9) was incubated at 30 °C for 10 min. Then, 400 μ L of potato starch (0.5%, w/v) were added to the mixture and re-incubated as previously described. At the end, 200 μ L of mixture were removed and, added into a separate tube containing 100 μ L of DNSA reagent solution (96 mM 3,5-dinitrosalicylic acid, 5.31 M sodium potassium tartrate in 2 M NaOH), and placed into a water bath at 90 °C. After 15 min, this mixture was diluted with 900 μ L distilled water, and the absorbance was measured at 540 nm using a UV-Visible spectrophotometer. The control with 100% enzyme activity was prepared by replacing the extract with DMSO (40 μ L). A blank was similarly prepared by replacing the enzyme solution with distilled water to allow absorbance produced by the extract. Acarbose (10–100 μ g/mL) was used as a positive control. The α -amylase inhibitory activity was calculated using the following equation:

Inhibition (%) = $((C-S)/C) \times 100$, where C = control absorbance, and S = extract absorbance. The results are reported as the concentration inhibiting 50% of enzymatic activity (IC₅₀).

3.2.11 Statistical Analysis

All experiments were conducted in triplicate with three independent biological replicates (n=3). Each biological replicate consisted of fruits collected from different individual plants of the same species. For cellular assays, three independent experiments were performed, each conducted in triplicate. Statistical analysis was performed using GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, Calif., USA). Data are presented as mean $\pm$ standard deviation (SD) for plant extracts analysis or mean $\pm$ standard error of the mean (SEM) for cellular studies. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to determine significant differences between groups for plant extract studies. For cellular ROS studies, data was analyzed by variance test analysis, and multiple comparisons were made by Bonferroni's test. A p-value < 0.05 was considered statistically significant. The Relative Antioxidant Capacity Index (RACI) was calculated using

Excel software (Microsoft Corp., Redmond, Wash., USA) by integrating the antioxidant capability values generated from the different methods used (Sun and Tanumihardjo 2007). Principal Component Analysis (PCA) and hierarchical clustering analyses were performed using appropriate statistical software, Past 4.0, to highlight correlations between different fruit extracts (Parisi et al., 2022).

3.3 Results

3.3.1 LC-MS/MS Qualitative Analysis

The detection of individual phenolic compounds of alcoholic extracts of fruits of *Crataegus* spp. was performed using an Orbitrap Q-Exactive ESI-MS instrument. Comparison of retention times, high-resolution full mass spectra, fragmentation patterns, and consideration of a mass tolerance <5 ppm for the experimental molecular formula allowed the tentative identification of a total of 25 compounds. These compounds include mainly hydroxycinnamic acids and their derivatives, flavonoids, anthocyanins and unsaturated fatty acids. All identified compounds were confirmed by previous studies on hawthorn genus.

Table 2. Secondary metabolites tentatively identified by LC-MS/MS analysis in *Crataegus* fruits extracts. Compound numbers correspond to those in Figure 1.

No	Compound	Chemical formula	RT (min)	[M-H] ⁻	[M+H] ⁺	BASE PEAKS	product ions (M/Z)	MSI level ^a	Error (ppm)
1	Chlorogenic acid	C ₁₆ H ₁₈ O ₉	11.36	353.0881		191	161 [M-192] ⁻ 191[M-162] ⁻	1	3.96
2	Caffeoylquinic acid isomer	C ₁₆ H ₁₈ O ₉	12.73	353.0881		191	161 [M-192] ⁻ 191[M-162] ⁻ 431 [M-162] ⁻	2	3.96
3	Luteolin O-Hexoside-Deoxyhexoside	C ₂₇ H ₂₈ O ₁₅	12.75	593.1512		285	285 [M-146-162] ⁺	2	2.02
4	Catechin O-hexoside	C ₂₁ H ₂₄ O ₁₁	12.81		453.1391	291	291 [M-162] ⁺ 245 [M-46] ⁺	1	0.34
5	Catechin	C ₁₅ H ₁₄ O ₆	13.00		291.0864	245	245 [M-46] ⁺	1	0.34
6	Epicatechin O-hexoside	C ₂₁ H ₂₄ O ₁₁	13.05		453.1391	291	291 [M-162] ⁺ 245 [M-46] ⁺	1	0.34
7	Cyanidin O-Glucoside ^b	C ₂₁ H ₂₁ O ₁₁	13.08		449.1092	287	287 [M-162] ⁺	1 ^b	3.11

8	Petunidin <i>O</i> -hexoside	C ₂₂ H ₂₃ O ₁₂	13.61	479.1184	317	317 [M-162] ⁺	2	3.01
9	Petunidin	C ₁₆ H ₁₃ O ₇	13.70	317.0655	302	302 [M-15] ⁺ 287 [M-15-15] ⁺	2 ^b	1.01
10	Peonidin	C ₁₆ H ₁₃ O ₆	13.86	301.0706	286	286 [M-15] ⁺	2 ^b	1.01
11	Quercetin <i>O</i> -Dihexoside	C ₂₇ H ₃₀ O ₁₇	13.96	627.1543	465	465 [M-162] ⁺ 303 [M-162-162] ⁺	2	-1.91
12	Rosinidin	C ₁₇ H ₁₅ O ₆	14.00	315.0863	300	315 [M-15] ⁺	2	-0.50
13	Cyanidin <i>O</i> -Pentoside ^b	C ₂₀ H ₁₉ O ₁₀	14.10	419.0987	287	287 [M-132] ⁺	2	3.57
14	Apigenin <i>O</i> -Hexoside-Deoxyhexoside	C ₂₇ H ₃₀ O ₁₄	15.29	579.1696	433	433 [M-146] ⁺ 271 [M-162-146] ⁺	2	-2.07
15	Quercetin <i>O</i> -Hexoside-Deoxyhexoside	C ₂₇ H ₃₀ O ₁₆	15.30	609.1450	301	433 [M-162] ⁻ 301 [M-162-146] ⁻	2	0.51
16	Quercetin <i>O</i> -Pentoside-Glucuronide	C ₂₇ H ₃₀ O ₁₆	15.64	609.1450	477	477 [M-132] ⁻ 301 [M-132-176] ⁻	2	0.52
17	Vitexin	C ₂₁ H ₂₀ O ₁₀	15.80	433.1120	415	415 [M-18] ⁺ 343 [M-90] ⁺ 313 [M-120] ⁺	1 ^b	-2.07
18	Apigenin <i>O</i> -Hexoside	C ₂₁ H ₂₀ O ₁₀	15.94	431.0975	269	269 [M-162] ⁻	2	0.69
19	Hyperoside	C ₂₁ H ₂₀ O ₁₂	16.19	465.1020	303	303 [M-162] ⁺	1 ^b	-1.50
20	Myricetin <i>O</i> -Deoxyhexoside	C ₂₁ H ₂₀ O ₁₂	16.28	465.1027	319	319 [M-146] ⁺	2	0.23
21	Quercetin <i>O</i> -Glucuronide	C ₂₁ H ₁₈ O ₁₃	16.84	477.0680	301	301 [M-176] ⁻	1	3.56
22	Luteolin <i>O</i> -Glucoside	C ₂₁ H ₂₀ O ₁₁	17.41	447.0921	285	285 [M-162] ⁻	1	0.11
23	Quercetin <i>O</i> -Pentoside	C ₂₀ H ₁₈ O ₁₁	17.46	433.0783	301	301 [M-132] ⁻	2	4.15
24	Quercetin <i>O</i> -Deoxyhexoside	C ₂₁ H ₂₀ O ₁₁	18.01	449.1066	303	303 [M-146] ⁺	2	-2.67
25	Quercetin	C ₁₅ H ₁₀ O ₇	22.98	301.0357	257	257 [M-44] ⁻	1 ^b	4.98
26	Trihydroxy octadecenoic acid	C ₁₈ H ₃₄ O ₅	25.00	329.2331	171	311 [M-18] ⁻ 293 [M-18] ⁻ 309 [M-18] ⁻	2	2.73
27	Trihydroxy-octadecadienoic acid	C ₁₈ H ₃₂ O ₅	25.30	327.2172	211	229 [M-98] ⁻ 211 [M-98-18] ⁻	2	1.83
28	Trihydroxy octadecenoic acid isomer	C ₁₈ H ₃₄ O ₅	25.52	329.2331	171	311 [M-18] ⁻ 293 [M-18] ⁻ 309 [M-18] ⁻	2	2.73
29	Trihydroxy-octadecadienoic acid isomer	C ₁₈ H ₃₂ O ₅	26.88	327.2172	211	229 [M-98] ⁻ 211 [M-98-18] ⁻	2	1.83
30	Hydroxy-octadecatrienoic acid	C ₁₈ H ₃₀ O ₃	39.81	293.2118	275	275 [M-18] ⁻ 231 [M-18-44] ⁻	2	2.38
31	Hydroxy-octadecatrienoic acid isomer	C ₁₈ H ₃₀ O ₃	40.12	293.2118	275	275 [M-18] ⁻ 231 [M-18-44] ⁻	2	2.38

^a MSI level of identification according to Sumner et al., 2007. ^b m/z values are expressed as $[M]^+$. Abbreviations: $[M - H]^-$, negative ion values; $[M+H]^+$ positive ion values; m/z , mass-to-charge ratio; R_t , retention time. ^bCompounds identified by standards.

3.3.2 Phytochemical Characterization

Crataegus fruits phytochemical profiling were acquired in negative and positive ion modes. The most polar compounds are quinic acid derivatives, in particular, compounds **1** and **2** produced the characteristic fragment ion at m/z 161 $[M-192]^-$ corresponding to dehydrated and deprotonated caffeic acid and fragment ion at m/z 191 $[M-162]^-$ corresponding to deprotonated quinic acid. The main compounds in the extract belong to the flavon and flavonol chemical classes, in fact, compounds **17** and **19**, respectively vitexin and hyperoside represent the main polyphenolic compounds, typical of this genus. Moreover, compounds **11**, **15**, **19**, **21**, **23**, **24** and **25** showed the fragments ions at m/z 301 and m/z 303, typical of quercetin aglycone in both polarities. These compounds were glycosylated carrying different sugar moieties, such as hexose, pentose and deoxyhexose. Based on accurate mass value and MS_2 experiments, apigenin (compounds **14** and **18**) and luteolin (compounds **3** and **22**) derivatives were identified, based on the fragment ions at m/z 269 and m/z 285, respectively. Another representative polyphenolic class of fruits of *Crataegus* genus are the anthocyanins. Compounds **7** and **13** showed the same fragment ion at m/z 287 $[M]^+$ corresponding to a cyanidin unit linked respectively to a hexose and to a pentose group. Finally, several hydroxylated polyunsaturated fatty acids were also identified.

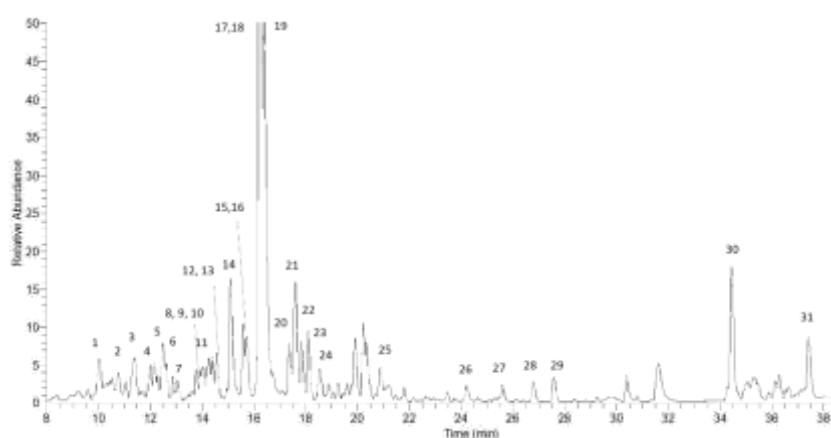


Figure 1. LC-MS chromatogram of specialized metabolites from *Crataegus* fruits

3.3.3 LC-MS Quantitative analysis

The LC-MS quantitative analysis (Table 3) revealed differences in the flavonoid and hydroxycinnamic acid composition among the *Crataegus* species and variety hydroalcoholic extracts (CrF1–CrF11). Overall, the extract showed a relevant accumulation of flavonoids, particularly quercetin derivatives, alongside moderate levels of hydroxycinnamic acids, as shown in Table 3. Sample CrF1 displayed the highest caffeoyl derivatives content, with a value of 2.40 ± 0.07 mg/g DW. Among the quantified compounds, quercetin O-dihexoside emerged as one of the most abundant components, reaching its highest concentration in fraction CrF1 (9.19 ± 0.33 mg/g DW), followed by CrF2 (6.17 ± 0.23 mg/g DW) and CrF3 (3.38 ± 0.13 mg/g DW). CrF1 showed the highest concentration of these metabolites, reaching 16.05 ± 0.58 mg/g DW. These results confirm the predominance of flavone and flavonol glycosides in the extract. Among anthocyanins, cyanidin O-glucoside and cyanidin O-pentoside were detected mainly in CrF6 (1.95 ± 0.13 mg/g DW) and CrF7 (2.13 ± 0.15 mg/g DW) while petunidin, petunidin O-hexoside and rosinidin were present in particular in CrF8 (0.981 ± 0.001 , 0.553 ± 0.005 and 0.121 ± 0.004 mg/g DW, respectively), which is the richest in terms of total anthocyanins. The high abundance of these compounds cause of the intense red colour of the *C. laciniata* fruit. Minor components, such as myricetin O-rhamnoside, were present in lower amounts throughout the samples. Particular attention should be given to the catechin derivatives found exclusively in CrF8, with a content of 5.02 ± 0.20 mg/g DW. These different patterns reflect the chemical heterogeneity of the *Crataegus* species and variety extract and provides a basis for correlating specific fractions with potential biological activities.

Table 3. Amount (mg/g DW $\pm$ SD) of main compounds detected in *Crataegus* ssp. and variety

Compounds	CrF1	CrF2	CrF3	CrF4	CrF5	CrF6	CrF7	CrF8	CrF9	CrF10	CrF11
<i>Caffeoylquinic acid</i> ^a (mg/g DW)	1.17 $\pm$ 0.03	0.76 $\pm$ 0.02	0.28 $\pm$ 0.01	0.13 $\pm$ 0.00	0.40 $\pm$ 0.01	0.82 $\pm$ 0.02	0.39 $\pm$ 0.01	0.26 $\pm$ 0.01	0.33 $\pm$ 0.01	0.82 $\pm$ 0.02	0.05 $\pm$ 0.00
<i>Caffeoylquinic acid isomer</i> ^a (mg/g DW)	1.24 $\pm$ 0.03	0.71 $\pm$ 0.02	0.30 $\pm$ 0.01	0.12 $\pm$ 0.00	0.43 $\pm$ 0.01	0.87 $\pm$ 0.02	0.37 $\pm$ 0.01	0.24 $\pm$ 0.01	0.35 $\pm$ 0.01	0.76 $\pm$ 0.02	0.06 $\pm$ 0.00
<i>Total</i>	2.40 $\pm$ 0.07	1.46 $\pm$ 0.04	0.58 $\pm$ 0.02	0.25 $\pm$ 0.01	0.83 $\pm$ 0.02	1.68 $\pm$ 0.05	0.76 $\pm$ 0.02	0.50 $\pm$ 0.01	0.68 $\pm$ 0.02	0.94 $\pm$ 0.04	0.11 $\pm$ 0.00
<i>Cyanidin O-glucoside</i> ^b (mg/g DW)	0.49 $\pm$ 0.03	1.90 $\pm$ 0.13	0.81 $\pm$ 0.06	0.28 $\pm$ 0.02	0.31 $\pm$ 0.02	1.95 $\pm$ 0.13	2.13 $\pm$ 0.15	0.20 $\pm$ 0.01	0.03 $\pm$ 0.00	0.03 $\pm$ 0.00	0.19 $\pm$ 0.01
<i>Cyanidin O-glucoside isomer</i> ^b (mg/g DW)	N.Q.	N.Q.	0.46 $\pm$ 0.01	N.Q.	0.26 $\pm$ 0.00	N.Q.	N.Q.	0.45 $\pm$ 0.00	N.Q.	N.Q.	N.Q.
<i>Petunidin</i> ^b (mg/g DW)	0.14 $\pm$ 0.00	0.12 $\pm$ 0.00	0.04 $\pm$ 0.00	0.12 $\pm$ 0.00	0.19 $\pm$ 0.00	0.07 $\pm$ 0.00	N.Q.	0.98 $\pm$ 0.00	0.22 $\pm$ 0.00	0.52 $\pm$ 0.00	0.42 $\pm$ 0.00
<i>Petunidin O-hexoside</i> ^b (mg/g DW)	0.18 $\pm$ 0.00	0.17 $\pm$ 0.00	0.03 $\pm$ 0.00	0.11 $\pm$ 0.00	0.17 $\pm$ 0.00	0.09 $\pm$ 0.00	0.03 $\pm$ 0.00	0.55 $\pm$ 0.01	0.28 $\pm$ 0.00	0.30 $\pm$ 0.00	0.26 $\pm$ 0.00
<i>Rosinidin</i> ^b (mg/g DW)	0.06 $\pm$ 0.00	0.03 $\pm$ 0.00	0.05 $\pm$ 0.00	0.05 $\pm$ 0.00	0.04 $\pm$ 0.00	0.01 $\pm$ 0.000	0.27 $\pm$ 0.01	0.12 $\pm$ 0.00	0.18 $\pm$ 0.01	0.06 $\pm$ 0.00	0.03 $\pm$ 0.00
<i>Cyanidin O-pentoside</i> ^b (mg/g DW)	0.086 $\pm$ 0.006	N.Q.	0.04 $\pm$ 0.00	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.
<i>Total</i>	0.95 $\pm$ 0.04	2.22 $\pm$ 0.13	1.42 $\pm$ 0.07	0.55 $\pm$ 0.02	0.98 $\pm$ 0.03	2.11 $\pm$ 0.13	2.29 $\pm$ 0.16	3.30 $\pm$ 0.12	0.71 $\pm$ 0.01	0.90 $\pm$ 0.01	0.90 $\pm$ 0.02
<i>Quercetin O-dihexoside</i> ^b (mg/g DW)	9.19 $\pm$ 0.33	6.18 $\pm$ 0.23	3.38 $\pm$ 0.13	0.40 $\pm$ 0.03	2.15 $\pm$ 0.03	2.52 $\pm$ 0.03	0.64 $\pm$ 0.03	0.27 $\pm$ 0.03	N.Q.	N.Q.	0.66 $\pm$ 0.03
<i>Quercetin O-hexoside-deoxyhexoside</i> ^b (mg/g DW)	1.50 $\pm$ 0.05	1.11 $\pm$ 0.04	0.36 $\pm$ 0.01	1.75 $\pm$ 0.06	3.79 $\pm$ 0.13	0.32 $\pm$ 0.01	0.16 $\pm$ 0.01	1.23 $\pm$ 0.04	1.50 $\pm$ 0.05	2.07 $\pm$ 0.07	0.70 $\pm$ 0.02
<i>Quercetin</i> ^d (mg/g DW)	0.10 $\pm$ 0.00	0.34 $\pm$ 0.016	0.06 $\pm$ 0.01	N.Q.	0.04 $\pm$ 0.01	N.Q.	0.04 $\pm$ 0.00	0.04 $\pm$ 0.00	0.08 $\pm$ 0.02	0.06 $\pm$ 0.00	0.09 $\pm$ 0.00
<i>Quercetin O-pentoside</i> ^b (mg/g DW)	1.46 $\pm$ 0.05	0.52 $\pm$ 0.02	0.54 $\pm$ 0.02	0.16 $\pm$ 0.01	0.33 $\pm$ 0.01	0.44 $\pm$ 0.02	0.18 $\pm$ 0.01	0.28 $\pm$ 0.01	0.25 $\pm$ 0.01	0.26 $\pm$ 0.01	0.30 $\pm$ 0.01
<i>Quercetin O-glucoronide</i> ^b (mg/g DW)	0.60 $\pm$ 0.02	N.Q.	0.81 $\pm$ 0.02	N.Q.	0.32 $\pm$ 0.01	N.Q.	0.60 $\pm$ 0.02	0.81 $\pm$ 0.02	N.Q.	N.Q.	0.32 $\pm$ 0.01

<i>Quercetin O-rhamnoside^b (mg/g DW)</i>	1.42±0.05	5.89±0.20	2.11±0.07	0.80±0.03	0.88±0.03	5.85±0.20	5.44±0.18	1.39±0.05	1.46±0.05	1.53±0.05	0.71±0.02
<i>Myricetin O- deoxyhexoside^b (mg/g DW)</i>	0.55±0.05	0.56±0.04	0.27±0.02	0.03±0.00	0.29±0.02	0.16±0.01	0.05±0.00	0.03±0.00	0.04±0.00	0.03±0.00	0.06±0.00
<i>Luteolin 7 O-glucoside^c (mg/g DW)</i>	0.03±0.00	0.02±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.02±0.00	0.08±0.00	0.001±0.00	0.01±0.00	0.01±0.00
<i>Luteolin O-hexoside^c (mg/g DW)</i>	1.20±0.04	N.Q.	1.03±0.04	0.03±0.00	N.Q.	N.Q.	N.Q.	N.Q.	0.01±0.00	N.Q.	N.Q.
<i>Vitexin^a (mg/g DW)</i>	0.57±0.01	0.92±0.02	0.67±0.01	0.09±0.00	0.17±0.00	0.51±0.01	0.61±0.02	0.06±0.00	0.03±0.00	0.01±0.00	0.69±0.00
<i>Hyperoside^b (mg/g DW)</i>	1.22±0.04	1.14±0.04	0.35±0.01	0.48±0.01	1.22±0.04	0.85±0.03	0.56±0.02	0.98±0.03	1.07±0.03	1.47±0.05	0.76±0.02
<i>Total</i>	17.84±0.63	16.68±0.60	9.57±0.34	3.75±0.14	9.40±0.28	10.66±0.27	8.29±0.29	5.17±0.19	4.44±0.17	5.54±0.19	4.29±0.12
<i>Catechin^d (mg/g DW)</i>	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	1.13±0.07	N.Q.	N.Q.	N.Q.
<i>Epicatechin O-hexoside^d (mg/g DW)</i>	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	1.43±0.01	N.Q.	N.Q.	N.Q.
<i>Catechin O-hexoside^d (mg/g DW)</i>	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	N.Q.	2.47±0.12	N.Q.	N.Q.	N.Q.
<i>Total</i>	5.02±0.20										

^a Compounds quantify using vitexin calibration curve; ^b Compounds quantify using hyperoside calibration curve; ^c Compounds quantify using luteolin O-glucoside calibration curve; ^d Compounds quantify using quercetin calibration curve

To investigate the distribution of specialised metabolites in different *Crataegus* fruits, a quantitative analysis of the main constituents of *Crataegus* fruit extracts was also performed using an external standard UHPLC-MS method. Hyperoside and vitexin, the main polyphenolic compounds of this genus, were chosen as reference compounds for the calibration curves. The quantitative results (Table 4) showed a significant variability in the presence of these components among *Crataegus* fruit extracts. The quantification of these markers in different samples highlighted that their concentrations were consistent with previous studies (Turnalar Ülger et al., 2023). In particular, samples CrF 1, 2 and 3, collected in San Vito Lo Capo (Trapani), showed a remarkable concentration of vitexin compared to other *Crataegus* species.

Table 4. Results are expressed as g of compounds to Kg of dried extract

Sample	Hyperoside (g/Kg)	Vitexin (g/Kg)	Luteolin 7-O-glucoside (g/Kg)	Quercetin (g/Kg)
C _R F1	1.218 ± 0.042	0.574 ± 0.015	0.028 ± 0.006	0.100±0.000
C _R F2	1.140 ± 0.040	0.920 ± 0.024	0.022 ± 0.004	0.344±0.015
C _R F3	0.346 ± 0.012	0.667 ± 0.017	0.007 ± 0.001	0.060±0.010
C _R F4	0.483 ± 0.017	0.094 ± 0.003	0.005 ± 0.001	0.000±0.000
C _R F5	1.224 ± 0.043	0.167 ± 0.004	0.002 ± 0.000	0.042±0.008
C _R F6	0.847 ± 0.030	0.508 ± 0.013	0.012 ± 0.001	0.000±0.000
C _R F7	0.558 ± 0.020	0.607 ± 0.016	0.015 ± 0.001	0.040±0.001
C _R F8	0.981 ± 0.034	0.062 ± 0.001	0.008 ± 0.001	0.044±0.001
C _R F9	1.071 ± 0.038	0.029 ± 0.001	0.005 ± 0.00	0.081±0.023
C _R F10	1.471 ± 0.052	0.010 ± 0.001	0.001 ± 0.000	0.060±0.006
C _R F11	0.755 ± 0.026	0.690± 0.001	0.002± 0.000	0.094±0.001

3.3.4 Total Phenolic (TPC) and Flavonoid Content (TFC)

The total phenolic content of *Crataegus* fruit samples is reported in Table 5. Among the extracts, C_RF8 (*C. laciniata*) shows the highest total phenolic content (25.54 mg GAE/g). Regarding C_RF9 and C_RF10 (*C. aff. insengae*), TPC values notably vary according to the place of collection (7.50 mg GAE/g and 20.81 mg GAE/g, respectively). The total flavonoid content mirrors the previously reported trend for phenols with C_RF8 (*C. laciniata*) showing the highest total flavonoids (12.92 mg CE/g), and a variable content among *C. aff. insengae* samples (C_RF9 and C_RF10) collected from different latitudes.

The total flavonoid content showed a similar trend previously reported for phenols with C_RF8 (*C. laciniata*) presenting the highest total flavonoids (12.92 mg CE/g), and a variable content among *C. aff. insengae* samples (C_RF9 and C_RF10) collected from different latitudes as shown in Table 5.

3.3.5 In Vitro Antioxidant Activity Analysis

Table 5 summarizes the results of all three antioxidant activity assays conducted *in vitro*, such as DPPH, FRAP, and ABTS. These results illustrate the great variation in levels of radical-scavenging activity between species that were collected from the various geographical locations, establishing that species of *Crataegus* can represent outstanding sources of bioactive compounds.

3.3.5.1 DPPH Radical-Scavenging Activity

The DPPH assay highlights *C. laciniata* (C_RF8) as the species with the strongest radical-scavenging activity (27.27 mg AAE/g). Similarly, *C. drepanensis* (C_RF1) demonstrates a notable activity (25.70 mg AAE/g). Among *C. aff. insengae* samples, those collected from Piano Pomo (CRF10) exhibit a stronger activity (25.52 mg AAE/g) compared to samples from Sempria (CRF9, 22.14 mg AAE/g). These results suggest that higher altitude and geographic location may enhance the radical-scavenging potential.

3.3.5.2 Ferric Ion Reducing Antioxidant Power Assay (FRAP)

The FRAP assay confirms *C. laciniata* (CRF8) as the species with the highest reducing power, with 44.29 mg TE/g. *C. aff. insengae* from Piano Pomo (CRF10) also shows remarkable reducing capacity (35.73 mg TE/g), as compared to that from Sempria (CRF9, 20.98 mg TE/g). This trend aligns with the differences observed in their total phenolic and flavonoid contents, further emphasizing the role of environmental factors, such as altitude, in modulating antioxidant properties.

3.3.5.3 ABTS Assay

In the ABTS assay, *C. laciniata* (C_RF8) stands out with the highest activity (34.98 mg TE/g) followed by *C. drepanensis* (C_RF1, 22.16 mg TE/g). Among *C. aff. insengae* samples, those from Piano Pomo (C_RF10) exhibit greater scavenging activity (9.91 mg TE/g) compared to that from Sempria (C_RF9, 5.84 mg TE/g). These results align with the patterns observed in both the DPPH and FRAP assays, highlighting a consistent influence of location on antioxidant potential.

Table 5. Total phenolic content (TPC, mg GAE/g), total flavonoid content (TFC, mg CE/g), and antioxidant activity results: DPPH expressed as Ascorbic Acid Equivalents per gram of extract (mg AAE/g); FRAP and ABTS expressed as Trolox Equivalents per gram of extract (mg TE/g), for 11 *Crataegus* species^a

Sample	TPC (mg GAE/g)	TFC (mg CE/g)	DPPH (mg AAE/g)	FRAP (mg TE/g)	ABTS (mg TE/g)
C _R F1	21.50 ± 0.03 j	6.44 ± 0.08 j	25.70 ± 0.90 j	31.00 ± 4.24 j	22.16 ± 0.01 j
C _R F2	14.02 ± 1.43 b	4.99 ± 0.12 b	22.71 ± 0.21 b	25.11 ± 1.87 b	7.44 ± 0.27 b
C _R F3	22.09 ± 2.40 b	2.87 ± 0.05 b	24.15 ± 0.33 b	25.01 ± 4.62 b	6.66 ± 0.10 b
C _R F4	7.23 ± 0.79 h	5.05 ± 1.00 h	22.70 ± 1.44 h	22.22 ± 0.68 h	4.88 ± 0.01 h
C _R F5	16.72 ± 1.74 i	5.83 ± 0.19 i	23.16 ± 0.23 i	32.76 ± 2.81 i	8.46 ± 0.41 i
C _R F6	5.98 ± 0.70 c	5.26 ± 0.09 c	23.02 ± 0.25 c	32.97 ± 0.22 c	8.40 ± 0.76 c
C _R F7	5.55 ± 0.19 d	4.58 ± 0.26 d	22.17 ± 0.75 d	27.95 ± 0.59 d	5.53 ± 0.30 d
C _R F8	25.54 ± 1.38 e	12.92 ± 0.15 e	27.27 ± 0.78 e	44.29 ± 1.50 e	34.98 ± 0.09 e
C _R F9	7.50 ± 0.85 f	4.04 ± 0.05 f	22.14 ± 2.39 f	20.98 ± 0.89 f	5.84 ± 0.70 f
C _R F10	20.81 ± 1.02 f	9.26 ± 0.63 f	25.52 ± 1.21 f	35.73 ± 0.82 f	9.91 ± 0.55 f
C _R F11	12.67 ± 0.19 g	3.03 ± 0.24 g	22.40 ± 0.74 g	16.36 ± 0.72 g	8.99 ± 0.12 g

^aValues are expressed as mean ± standard deviation. Different letters within each column indicate statistically significant differences at $p < 0.05$ in an ANOVA + Tukey's pairwise t-test analysis.

3.3.5.4 Relative Antioxidant Capacity Index

The Relative Antioxidant Capacity Index (RACI) was calculated to compare the different antioxidant ability among *Crataegus* spp. fruit extracts. RACI is a statistical, adimensional index calculated using Excel software (2010, Microsoft, Redmond, WA, USA) by integrating the antioxidant capability values generated from the different methods used (Sun & Tanumihardjo, 2007). Results are illustrated in Figure 2 where they are expressed as RACI values. In particular, C_RF8 (*C. laciniata*) evidenced the highest value (2.39) followed by C_RF1 (*C. drepanensis*) and C_RF10 (*C. aff. insengae*) (0.76 and 0.42, respectively). A relative lack of

antioxidant activity was attributed to the other extracts for their low RACI values. The high antioxidant activity observed for *C. laciniata*, *C. drepanensis*, and *C. aff. insengae* suggests that these species could be particularly valuable for dietary applications aimed at reducing oxidative stress, which it is known to play a critical role in in the development of chronic diseases.

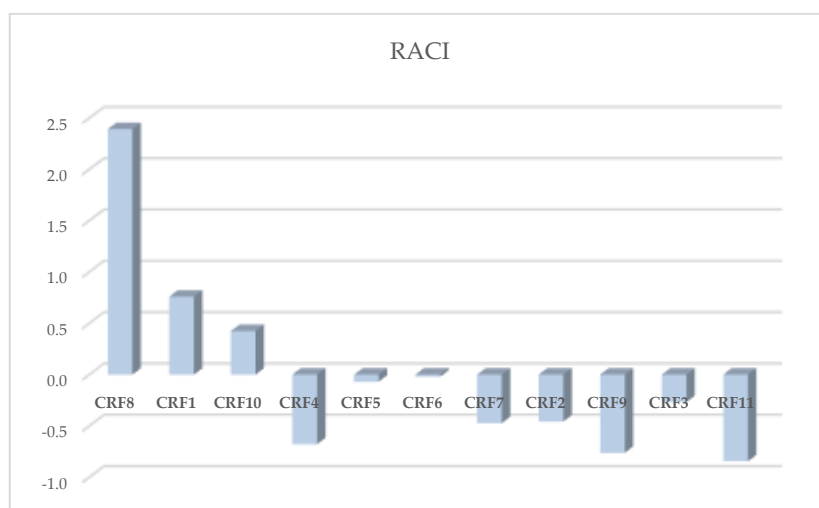


Figure 2. Relative Antioxidant Capacity Index (RACI) of *Crataegus* spp. fruit extracts.

3.3.6 Biostatistic Analysis

Multivariate analyses, principal component analyses, PCAs, were performed to deep inside the phytochemical analysis. In detail, the PCA (Figure 3) highlighted that hyperoside and vitexin are the main compounds responsible for the total variance of the dataset. Also, Hierarchical clustering analyses were employed, and the results showed an interesting distribution of red and yellow *Crataegus* fruits. Furthermore, the species, varieties and collection place were key parameters to discriminate between the different samples. As shown in Figure 4, *Crataegus* fruit clustered together based on the color of their fruits, in fact samples CrF4, CrF5, CrF8, CrF11, CrF9 and CrF10 were characterised by the red color of their fruits, whereas samples CrF3, CrF6, CrF7, CrF2 and CrF1 had yellow fruits. Moreover, samples CrF3, CrF6, CrF7 and CrF2 variety, belong to the same species, *Crataegus azarolus*, were grouped by the minimum distance value. All these meta-analyses highlighted how the genus *Crataegus* was able to change and adapt to different conditions, such as climate and how hyperoside and vitexin could play a fundamental role in this adaptation. These analyses underscore how environmental factors, including climate and

altitude, influence secondary metabolite accumulation. This aligns with previous findings (Cheng et al., 2023; Xipan et al., 2023) and highlights the importance of phytogeographical parameters in shaping phytochemical diversity.

Figure 3. Principal component analysis (PCA) of specialized metabolites of *Crataegus* extracts. Component 1 and 2 elucidated, in that order, 80.4% and 12.4% of the total variance.

Figure 4. Hierarchical clustering analysis of 11 *Crataegus* fruit extracts based on their LC-MS metabolite profiles.

metabolic profile of CrF8 and its antioxidant activity. These findings are consistent with the known antioxidant properties of flavonoids and anthocyanins. The presence of these compounds in CrF8 could suggest a synergistic contribution to the antioxidant capacity of its extract, confirming the role of the qualitative and quantitative composition of secondary metabolites in determining antioxidant activity.

Table 6. Pearson correlation coefficients (ρ s) between metabolites and antioxidants activities of *Crataegus* spp. Only positive statistically significant correlations ($p \leq 0.260$; *: $p \leq 0.05$; **: $0.01 \leq p \leq 0.05$; ***: $p \leq 0.001$) were considered. Blue color intensity is directly proportional to Pearson coefficient significance.

Compounds	RACI
	ρ
Luteolin 7 <i>O</i> -glucoside	0.87643***
Catechin	0.84834***
Epicatechin <i>O</i> -hexoside	0.84834***
Catechin <i>O</i> -hexoside	0.84834***
Petunidin	0.73559***
Petunidin <i>O</i> -hexoside	0.68987**
Quercetin <i>O</i> -glucuronide	0.57098**
Caffeoylquinic acid	0.25055*
Quercetin <i>O</i> -pentoside	0.24648
Caffeoylquinic acid isomer	0.24442
Luteolin <i>O</i> -hexoside	0.12245
Quercetin <i>O</i> -hexoside-deoxyhexoside	0.11219
Quercetin <i>O</i> -dihexoside	0.065443
Myricetin <i>O</i> -deoxyhexoside	0.014562
Rosinidin	0.003821

3.3.7 Cellular ROS Inhibition Activity

To further validate the antioxidant potential observed in chemical assays, the ability of selected *Crataegus* extracts to reduce intracellular reactive oxygen species (ROS) was assessed in H9c2 rat cardio myoblasts. Based on previous results, the three most promising extracts, CRF8 (*C. laciniata*), CRF10 (*C. aff. insengae*), and CRF1 (*C. drepanensis*) were tested at concentrations ranging from 3.125 to 25 $\mu\text{g/mL}$. The results indicate that the tested extracts are able to protect H9c2 cells against H_2O_2 -induced oxidative stress. At the same

time, a moderate increase in H₂DCF-DA fluorescence was observed in cells treated with the extracts alone compared to untreated controls. This result may suggest the presence of a mild intrinsic pro-oxidant effect, potentially related to the redox properties of its constituents. This effect did not adversely affect cell viability nor did it appear to reduce the antioxidant protection observed under conditions of induced oxidative stress, indicating that the overall impact of the extracts remains predominantly protective (Figure 5).

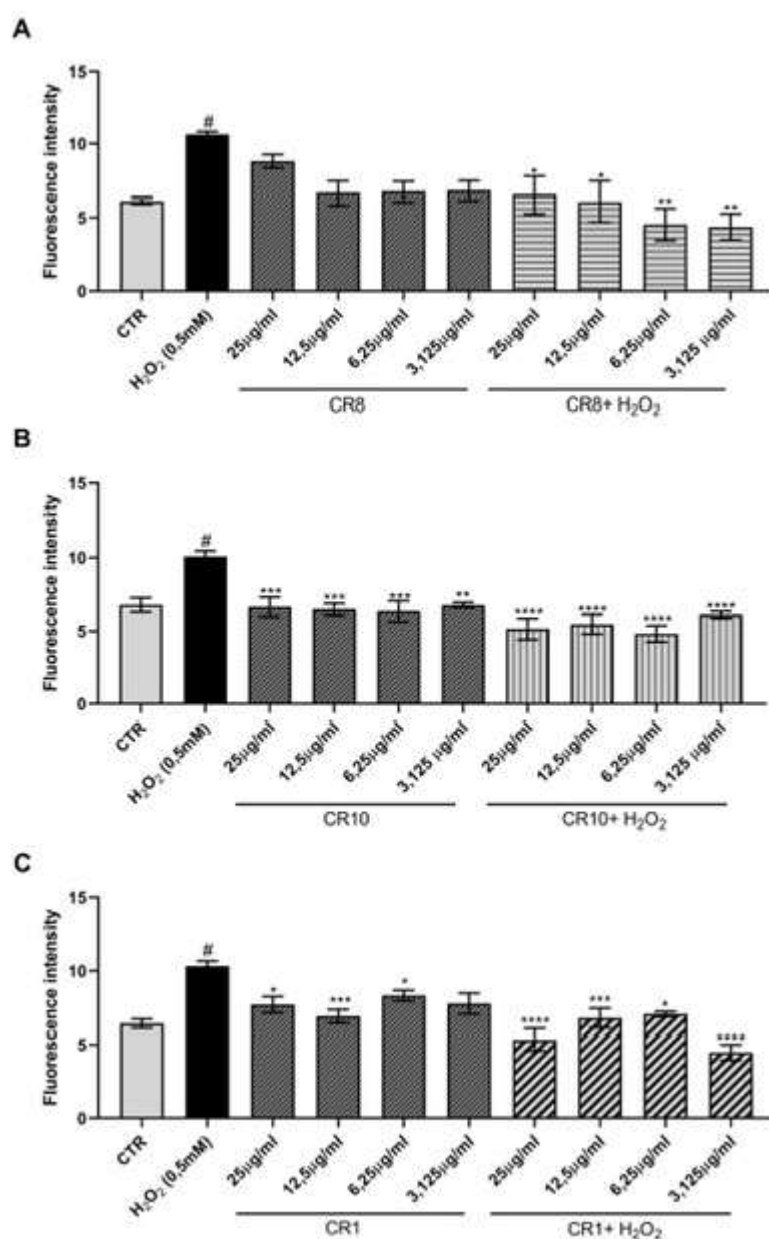


Figure 5. The fluorescent probe 2'-7'-dichlorofluorescein diacetate (H₂DCF-DA) was used to evaluate cytosolic ROS generation, by spectrofluorimetric analysis. Results are reported as mean $\pm$ SEM of the

intensity of fluorescence from at least three independent experiments, each performed in triplicate. Data were analysed by variance test analysis, and multiple comparisons were made by Bonferroni's test. # $p < 0.05$ vs untreated cells; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, and **** $p < 0.001$ vs H_2O_2 treated cells.

Similar observations have been reported in the literature. Maliar et al. (2023) suggest that certain plant secondary metabolites, particularly polyphenolic compounds containing multiple oxygen heteroatoms or carbonyl groups, such as flavonoids may exhibit dual redox behavior. This means they can act as both antioxidants and pro-oxidants, depending on the chemical and cellular context. This behavior, characterized by ambivalence, may result from polyphenols' ability to interact with metal ions or participate in electron transfer reactions. Such reactions can transiently generate reactive oxygen species, which, at controlled levels, may contribute to activating the antioxidant defenses. This mechanism could explain the moderate increase in H_2DCF -DA fluorescence observed in H9c2 cells treated with *Crataegus* extracts without compromising cell viability or overall antioxidant protection in the presence of induced oxidative stress (Figure 5). The extracts' efficacy in mitigating induced oxidative stress is confirmed by the significant reduction in ROS levels observed in the presence of H_2O_2 , while maintaining an active redox potential. This dual redox behavior supports the notion that plant polyphenols can act as antioxidants under oxidative stress conditions while displaying moderate pro-oxidant activity in the absence of external stressors. In conclusion, CRF8 demonstrates superior activity in the assay, consistent with the findings of previous tests.

3.3.8 α -Glucosidase and α -Amylase Inhibitory Activity

The results on α -glucosidase inhibitory activity showed that *C. laciniata* (CRF8) and *C. azarolus* var. *azarolus* (CRF6) exhibit demonstrate the strongest effects with IC_{50} values of 0.074 mg/mL and 0.076 mg/mL, respectively. Among *C. aff. insengae* samples, those from Piano Pomo show a better activity (CRF10, IC_{50} 0.121 mg/mL) compared to that from Sempria (CRF9, IC_{50} 0.306 mg/mL). These differences suggest that altitude and environmental conditions may also influence the enzymatic inhibition (Table 7). In general, it is of particular relevance that all the fruit extracts exhibited a stronger α -glucosidase activity as compared to acarbose. The results on α -amylase inhibitory activity evidenced

that *C. drepanensis* (C_RF1) exhibits the strongest α -amylase inhibitory activity with an IC₅₀ value of 2.892 mg/mL, followed by *C. laciniata* (C_RF8, 4.207 mg/mL). These findings highlight *C. laciniata* and *C. drepanensis* as the most promising species for α -amylase inhibition (Table 7). The inhibitory effects on α -glucosidase and α -amylase highlight the potential antidiabetic activity of *Crataegus* fruits, reinforcing their traditional use in managing blood glucose levels (Cheng et al., 2023). Specifically, in the assessment of potential pharmaceutical applications of *Crataegus* species extracts, targeting α -glucosidase is a more advantageous approach than to α -amylase inhibition. The enzymatic mechanism of α -glucosidase is directly implicated in the final stage of carbohydrate digestion in the small intestine, where it disintegrates disaccharides and oligosaccharides into absorbable monosaccharides. Through the inhibition of α -glucosidase, the degradation and absorption of glucose are effectively showed, resulting in a controlled and gradual elevation in postprandial blood glucose levels (Kashtoh & Baek 2022). Conversely, α -amylase operates earlier in the digestive process, predominantly in the oral cavity and in the stomach, commencing the breakdown of complex starches into smaller oligosaccharides. This is due to the disintegration of oligosaccharides and disaccharides still occurring in the small intestine, where α -glucosidase is operative (Yee & Fong 1996). In light of these insights, the significant inhibitory activity of *C. laciniata*, *C. azarolus* var. *azarolus*, and *C. drepanensis* on α -glucosidase suggests that these species may provide functional benefits in dietary strategies for glycemic control. Therefore, *Crataegus* species with pronounced α -glucosidase inhibitory effects are better suited for pharmaceutical applications aimed at improving blood glucose management and enhancing therapeutic outcomes for diabetic patients compared to acarbose, the known α -glucosidase inhibitor used in clinical practice which is accompanied by several gastrointestinal side effects.

Table 7. α -Glucosidase and α -Amylase inhibitory activity of 11 *Crataegus* species. Results are expressed as IC₅₀ value $\pm$ standard deviation (SD).

Sample	α -Glucosidase (IC ₅₀ mg/mL)	α -Amylase (IC ₅₀ mg/mL)
C _R F1	0.124 $\pm$ 0.015**	2.892 $\pm$ 0.523
C _R F2	0.265 $\pm$ 0.008*	>5 mg
C _R F3	0.256 $\pm$ 0.008*	>5 mg

C _R F4	0.146 ± 0.006**	>5 mg
C _R F5	>1 mg	>5 mg
C _R F6	0.076 ± 0.005**	3.761 ± 0.065
C _R F7	0.169 ± 0.018**	>5 mg
C _R F8	0.074 ± 0.001**	4.207 ± 0.222
C _R F9	0.306 ± 0.000*	>5 mg
C _R F10	0.121 ± 0.005**	>5 mg
C _R F11	0.455 ± 0.016	>5 mg
Acarbose	0.436 ± 0.058 ++	0.029 ± 0.001 ++

* $p < 0.05$ and ** $p < 0.01$ vs control: Student's *t*-test.

3.4 Discussion

The present research demonstrates the significant phytochemical diversity and bioactive potential of *Crataegus spp.* fruits collected from wild plants in Western Sicily. The chemical profiling showed that these fruits are a rich source of polyphenolic compounds. The current study demonstrates the extensive phytochemical diversity and bioactivity present in *Crataegus spp.* fruits collected and harvested from wild plants in Western Sicily. The phytochemical study has proved that these fruits are a rich source of polyphenols and hyperoside particularly; this has already been previously characterized for its antioxidant and metabolism regulatory activity (Ma et al., 2024). The high antioxidant activity exhibited by *C. laciniata*, *C. drepanensis*, and *C. aff. insengae* shows the outstanding value of these species as candidates for dietary applications with the purpose of reducing oxidative stress. This is of relevance, considering that oxidative stress has been identified as one of the main elements responsible for the initiation and progression of chronic diseases, including cardiovascular disorders, diabetes, and neurodegenerative disorders. The inhibitory activity against α -glucosidase and α -amylase observed in *Crataegus* fruit extracts underscores their antidiabetic potential, aligning with and providing biochemical support for their traditional use in regulating blood glucose levels (Cheng et al., 2021). The enzymatic mechanism of α -glucosidase is critically involved in the terminal step of carbohydrate digestion within the small intestine, catalyzing the breakdown of disaccharides and oligosaccharides into monosaccharides readily available for absorption.

By mitigating the hydrolysis and absorption of glucose, inhibition of α -glucosidase causes a steady rise in postprandial blood glucose levels (Kashtoh et al., 2022). On the other hand, α -amylase acts earlier in the digestion process, mainly in the oral cavity and the stomach, initiating the breakdown of complex starches into smaller oligosaccharides. This is because of the breakdown of oligosaccharides and disaccharides continues in the small intestine, where α -glucosidase remains functionally active (Yee & Fong, 1996). In the light of these insights, the significant inhibitory activity of *C. laciniata*, *C. azarolus* var. *azarolus*, and *C. drepanensis* on α -glucosidase suggests that these species may provide functional benefits in dietary strategies for glycemic control. Based on this, *Crataegus* species with pronounced α -glucosidase inhibitory effects may be suited for nutraceutical applications aimed at improving blood glucose control. In fact, their use can provide diabetic patients with results comparable to those of the well-known α -glucosidase inhibitor acarbose, but without the gastrointestinal side effects associated with the latter. Furthermore, their phytochemical profile may contribute to unique flavors and aromas, potentially enriching the sensory appeal of artisanal jams, syrups, or functional confectionery items. Their antioxidant content also justifies their potential use in functional beverages, such as antioxidant juices and herbal infusions, as candidates for metabolic health and overall well-being enhancement (Xipan et al., 2023). The variability we observed in polyphenolic composition and bioactivity among *Crataegus* species and varieties point toward the role of environmental conditions, especially those of climate and altitude, in governing the accumulation of secondary metabolites. As reported in previous studies, the phytochemical profile of various *Crataegus* fruits largely depends on their geographical origin and growth environment (Xipan et al., 2023). Given the growing consumer demand for natural sources of bioactive compounds, the detailed phytochemical characterization of these local varieties provides a scientific basis for their sustainable application. These local *Crataegus* varieties provide a scientific basis in the agri-food and nutraceutical industries while also providing environmental and economic benefits through land rehabilitation initiatives.

3.5 Conclusion

Our findings demonstrate that *Crataegus* fruits from Western Sicily represent a valuable source of bioactive compounds with strong antioxidant and potential antidiabetic

properties. The high polyphenolic content, particularly in *C. laciniata* and *C. azarolus* var. *azarolus*, suggests their potential application in functional foods and dietary supplements. Furthermore, the observed enzymatic inhibition activity across species and varieties reinforces their possible role in glycemic control, supporting their traditional medicinal use. The validation of antioxidant activity through cellular ROS inhibition in H9c2 cardiomyoblasts confirms the biological relevance of these compounds beyond chemical assays. The present study highlights the significant phytochemical diversity and bioactive potential of *Crataegus* spp. fruits collected from wild plants in Western Sicily. The chemical profiling revealed that these fruits are a rich source of polyphenolic compounds, particularly hyperoside, which has been previously recognized for its antioxidant and metabolic regulatory properties.

From a nutritional and functional food perspective, *Crataegus* spp. fruits could serve as valuable ingredients in the development of health-promoting food products, due to the presence of bioactive compounds and their bioactivity. Additionally, their phytochemical composition may contribute to unique flavors and aromas, potentially enriching the sensory appeal of artisanal jams, syrups, or functional confectionery items. Moreover, their antioxidant content suggests potential applications in functional beverages, such as antioxidant-rich juices or herbal infusions, which could support metabolic health and enhance overall well-being. The remarkable biodiversity of *Crataegus* in this Mediterranean hotspot not only offers an opportunity for nutritional exploitation but also raises the need for conservation strategies to preserve these valuable genetic resources. Furthermore, the differences in polyphenolic content and bioactivity observed between different *Crataegus* species and varieties highlight the influence of environmental factors, including climate and altitude, on the accumulation of secondary metabolites. This is in accordance with previous studies demonstrating that the phytochemical profile of *Crataegus* fruits is significantly modulated by their geographical origin and growth conditions. Additionally, efforts should be directed towards developing sustainable supply chains to integrate these fruits into the modern food industry while ensuring biodiversity conservation. The present study provides a strong foundation for future research and commercialization of the investigated *Crataegus* fruits as functional food ingredients, in agreement with the global trend of utilizing natural bioactive compounds for health

promotion. Given the promising results, further studies on bioavailability enhancement and formulation strategies will be essential to optimize the commercial viability and efficacy of *Crataegus*-based nutraceuticals. Furthermore, the potential adaptability of *Crataegus* species to challenging environmental conditions, including poor soils, suggests their potential role in sustainable land management strategies. Growing these species on marginal or uncultivated land could not only enhance their commercial value but also contribute to soil restoration and desertification control. By promoting their growth in semi-arid regions, *Crataegus* could serve as a model for sustainable agriculture, promoting biodiversity, improving soil quality and creating economic opportunities in otherwise unproductive areas.

Chapter 4

8',9'-Bis-*nor*-neolignans from *Cedrela odorata* and their antiangiogenic activity

Based on the paper

Iobbi, V., Arabi, S. P., Bisio, A., D'Angelo, V., Germano, M. P., Braca, A., & De Tommasi, N. (2024). 8', 9'-Bis-*nor*-neolignans from *Cedrela odorata* and their antiangiogenic activity. *Phytochemistry Letters*, 64, 13-18.

8',9'-Bis-nor-neolignans from *Cedrela odorata* and their antiangiogenic activity

4.1 Introduction

Pathological angiogenesis, or the abnormal production of blood vessels from pre-existing ones, is an intrinsic mechanism in the development of various diseases. Among these diseases, the most significant is undoubtedly cancer, where angiogenesis plays a key role in tumor invasiveness. Aberrant vascularization is also implicated in autoimmune disorders, ocular pathologies such as age-related macular degeneration, and cardiovascular conditions, including atherosclerosis (Folkman, 2007). Hence, modulation of angiogenesis has emerged as an attractive therapeutic strategy across diverse clinical contexts. In particular, natural products represent a prolific source of structurally diverse bioactive molecules, many of which have shown promising antiangiogenic activity in preclinical studies. Their chemical diversity and evolutionary optimization provide unique scaffolds for the development of new agents capable of targeting vascular growth, either alone or in synergy with existing chemotherapeutics. In recent decades, lignans and neolignans have emerged as a prominent class of plant-derived secondary metabolites attracting the attention of natural product chemists and pharmacologists alike. Their biological activities, most of which reported in cell-based assays, are considerable (Zálešák et al., 2019), including antioxidant properties, cytotoxic and antitumor effects that highlight them as potential anticancer candidates, as well as substantial anti-inflammatory activity relevant to chronic degenerative diseases (Zálešák et al., 2019). In addition, several lignans and neolignans have shown neuroprotective properties, antimicrobial and antiviral activity against a broad range of pathogens, and the ability to inhibit platelet aggregation, which emphasizes their multifunctional pharmacological potential (Cui et al., 2020). Lignans are present in plant-based foods including grains, legumes, fruits, and vegetables. Their occurrence in the human diet has drawn particular interest in nutritional and pharmacological research. Actually, several epidemiologic studies showed that diets rich in lignan compounds alleviate various health conditions, including hormone-related cancers and cardiovascular diseases (Rodríguez-García et al., 2019). However, the broad-spectrum of bioactivities of lignans and neolignans emphasizes their importance not only as dietary phytochemicals but also as promising scaffolds for the discovery of therapeutic agents

targeting different pathological processes. The anti-cancer potential of these compounds can be attributed mainly to the induction of apoptosis, inhibition of cell growth, and prevention of metastasis and angiogenesis (Zálešák et al., 2019). Several neolignans have been characterized with antiangiogenic properties in previous studies. For instance, honokiol, was shown to inhibit endothelial proliferation and tumor-induced angiogenesis both *in vitro* and *in vivo*. A ferulate-derived dihydrobenzofuran neolignan was found to modulate angiogenic and steroidogenic pathways in swine granulosa cells (Basini et al., 2014). Additional studies further confirmed that neolignans from diverse plant sources, including *Vitex* and *Lumnitzera* species, exhibit the ability to suppress vascular development (Yu et al., 2018). These findings reinforce the ongoing significance of neolignans as a structurally diverse class of natural products with consistent antiangiogenic potential. Lignans are secondary metabolites of plants, biosynthetically derived from the shikimic acid pathway, and are characterized as dimers of two phenylpropanoid units linked through a β - β' (8-8') bond. Classical lignans follow this structural arrangement, whereas neolignans are characterized by coupling patterns distinct from the β - β' linkage, giving rise to a wide structural diversity within this class (Teponno et al., 2016). Fifteen subtypes of neolignan scaffolds have been recognized, although no trivial or semi-trivial names have yet been assigned to them (Teponno et al., 2016). *Nor*-lignans and *bis-nor*-lignans possess one and two fewer carbon atoms, respectively, and demonstrate structural arrangements that differ from those of lignans (Frezza et al., 2020). Lignans are mainly found in both woody and non-woody dicotyledons, whereas only a limited number have been reported in monocotyledons. However, their distribution across various plant families has not yet been adequately clarified (Peterson et al., 2010). Neolignans illustrate remarkable structural diversity within the Magnoliidae clade. They are distributed across numerous plant families, among which the Meliaceae Juss., a family of woody plants belonging to the order Sapindales, represents one of the most important (Yadav et al., 2015). *Cedrela odorata* L., commonly known as Spanish cedar, is a semi-deciduous tree native to the tropical regions of Central and South America, and later naturalized in West Africa (POWO, 2024). For centuries, the stem bark of *C. odorata* has been widely used in traditional African and American folk medicine to treat a broad range of ailments, including wounds, skin disorders, diarrhoea, fever, inflammatory conditions, vomiting, haemorrhage,

haemorrhoids, leukorrhea, malaria, diabetes, cancer, and gastrointestinal disturbances (Barajas-Ramírez et al., 2023).



Figure 1. *Cedrela odorata* L. Credits by <https://www.flickr.com>

The chloroform and *n*-hexane extracts of the bark have shown promising anti-parasitic activity, particularly against *Leishmania infantum* and *Trypanosoma cruzi* (González-Coloma et al., 2012). The aqueous and ethanolic extracts of *Cedrela odorata* stem bark exhibited moderate antimicrobial activity *in vitro*, specifically against *Streptococcus mutans*. Besides, the hydroethanolic extract of the inner stem bark has also been shown to possess beneficial effects in alleviating postprandial hyperglycaemia and mitigating oxidative stress-related damage. Moreover, the ethanolic extract of the stem bark was found to decrease cholesterol levels and prevent hepatic lipid peroxidation in diabetic and hypercholesterolemic animal models, effects likely attributable to the presence of bioactive constituents with antioxidant properties (Almonte-Flores et al., 2015). Phytochemical investigations of *Cedrela odorata* have led to the isolation of various secondary metabolites, including terpenoids, tocopherols, and flavonoids from its leaves and bark, alongside some limonoids (Nogueira et al., 2020). Analysis of the essential oil obtained from the bark of *C. odorata* resulted in the identification of thirty-seven sesquiterpenoids. From *C. odorata* grafted onto *Toona ciliata* var. *australis*, several classes of metabolites, including sesquiterpenes, triterpenoids, limonoids, and flavonoids, have been isolated (De Paula et al., 1997). Several compounds isolated from *C. odorata* have been reported to exhibit

diverse biological activities. These include strong binding affinity for Hsp90 (Chini et al., 2016), inhibition of protein tyrosine phosphatase 1B (PTP1B) (Wu et al., 2014), as well as hypoglycaemic (Almonte-Flores et al., 2015), anticancer (Wan et al., 2018), antimalarial (Omar et al., 2003), and antifeedant effects. In the search for natural lignan and neolignan derivatives with potential antiangiogenic activity, the present study aimed to extend previous phytochemical investigations on the apolar and medium polarity extracts of *C. odorata* (Bellone et al., 2021). The objectives were to isolate lignan derivatives and to evaluate their effects on angiogenesis using the zebrafish embryo model. The study was further designed to include additional lignans and neolignans from our chemical library, which were selected due to their structural similarities with the newly isolated compounds.

4.2 Material and Methods

4.2.1 Plant material

The stem bark of *C. odorata* L. was collected in April 2010 in Mérida, Venezuela, and authenticated by Dr. Juan Carmona. A voucher specimen (No. 06) is preserved in the MERF Herbarium, Universidad de Los Andes, Mérida, Venezuela.

4.2.2 Extraction and isolation

The dried and powdered stem bark of the plant (300 g) was extracted for 72 h with solvents of increasing polarity: *n*-hexane, CHCl₃, CHCl₃-MeOH (9:1), and MeOH by exhaustive maceration as previously reported (Faiella et al., 2012). The CHCl₃ extract (5.0 g) was submitted to flash silica gel column chromatography (CC) by Biotage (SNAP 340 g column, flow rate 90 mL/min, collection volume 30 mL), eluting with CHCl₃ followed by increasing concentrations of MeOH in CHCl₃ (between 1 % and 30 %) and collecting twelve major fractions (A-L). Fraction B (103.0 mg) was subjected to RP-HPLC with MeOH-H₂O (13:7) as eluent to give compound **1** (1.3 mg, R_t 38 min). Part of the CHCl₃-MeOH extract (4.4 g) was submitted to flash silica gel CC by Biotage (SNAP 340 g column, flow rate 90 mL/min, collection volume 30 mL), eluting with a *n*-hexane-CHCl₃ (8:2) mixture followed by increasing concentrations of CHCl₃ (from 80 % to 100 %), continuing with CHCl₃ followed by increasing concentrations of MeOH in CHCl₃ (between 1 % and 100 %) and collecting thirteen major fractions (A-M). Fraction B (382.0 mg) was tested to RP-HPLC with MeOH-H₂O (7:13) as eluent

to give homovanillic alcohol (1.6 mg, R_t 9 min) and scopoletin (2.8 mg, R_t 10 min). Fraction H (170.0 mg) was subjected to RP-HPLC with MeOH-H₂O (2:3) as eluent to give 4,5-dihydroblumenol A (1.1 mg, R_t 11 min) and compound **2** (4.5 mg, R_t 13 min). Fractions J (187.6 mg) and K (222.0 mg) were subjected to RP-HPLC with MeOH-H₂O (3:7) as eluent to yield 7-megastigmene-3,6,9-triol (1.1 mg, R_t 11 min) from fraction J and 2-(3,4-dimethoxyphenyl) ethyl- β -D-glucopyranoside (1.1 mg, R_t 39 min) from fraction K.

Compound (**1**):

Yellow residue; $[\alpha]_D - 54$ (c 0.1, MeOH); ¹H and ¹³C NMR data, see Table 1; HRESIMS m/z 423.1048 [M + Na]⁺ (calcd. for C₂₁H₂₀O₈Na 423.1056), 363.0838 [M + Na – 60]⁺.

Compound (**2**):

Yellow residue; $[\alpha]_D - 9$ (c 0.1, MeOH); ¹H and ¹³C NMR data, see Table 1; HRESIMS m/z 401.1204 [M + Na]⁺ (calcd. for C₁₉H₂₂O₈Na 401.1212), 205.0475 [M + Na – 196]⁺.

4.2.3 Zebrafish embryo culture and treatment protocol

Adult male and female zebrafish were kept in flow-through aquaria at 28.5 °C on a 14/10 h (light/dark) photoperiod and they were provided with live brine shrimp (*Artemia salina*) according to the standard protocol (Westerfield, 2009). The eggs generated by natural mating were collected and investigated under a stereomicroscope (SMZ-171 Series, Motic, San Antonio, TX, USA) equipped with a digital camera (Moticam® 5 plus, Motic, San Antonio, TX, USA). The fertilized eggs containing embryos with normal morphology were selected, manually dechorionated with forceps and distributed in 96 single well microplates (one embryo per well). The embryos were treated with the obtained compounds (5, 10, and 20 μ M/embryo) and incubated from 24 hpf (hours post-fertilization) to 72 hpf at 28.5 °C. Test samples were dissolved in DMSO and then diluted at the final concentrations with the embryo medium. The final concentration of DMSO was maintained at 0.2% (v/v) in all samples. The control group encountered only DMSO (0.2% v/v). All tests were carried out in compliance with the European Directive 2010/63/EU and the ethical guidelines described in the “National Institutes of Health Guide for Care and Use of Laboratory Animals.

4.2.4 Quantitative determination of endogenous alkaline phosphatase (EAP) activity

Quantitative assessment of EAP activity was conducted following the method suggested by De Leo et al. (De Leo et al., 2021), using p-nitrophenyl phosphate disodium salt as a substrate of EAP. The optical density of end products was determined at 405 nm via a microplate reader (Mutiskan GO, Thermo Scientific). Vessel growth was determined as a percentage in optical density compared with the control and finally expressed as inhibition %. Each assay was repeated at least three times.

4.3 Results and discussion

The dried and powdered stem bark of *Cedrela odorata* L. was subjected to successive extraction following the procedure previously described by Bellone and co-workers (2021). Fractionation of the chloroform and chloroform–methanol (9:1) extracts through chromatographic separation led to the isolation of a set of secondary metabolites. Among these, two previously undescribed 8',9'-bis-nor-neolignans (compounds **1** and **2**; *Figure 2*) were obtained as the major new constituents. In addition, the extracts yielded two known megastigmanes, one coumarin, and two phenolic derivatives, which were unambiguously identified as 4,5-dihydroblumenol A, 2-(3,4-dimethoxyphenyl) ethyl- β -D-glucopyranoside, scopoletin, homovanillic alcohol (Cardinal et al., 2016), and 7-megastigmene-3,6,9-triol (Kawakami et al., 2011).

The co-occurrence of structurally diverse metabolites corresponding to distinct chemical classes illustrates the complex phytochemical profile of *C. odorata*, confirming previous reports that members of the Meliaceae family represent a rich source of bioactive natural products with potential pharmacological significance. Compound **1** was obtained as a yellow powder. Its molecular formula ($C_{21}H_{20}O_8$) was established from the sodiated molecular ion peak in the HRESIMS at m/z 423.1048 $[M + Na]^+$, which indicates that **1** exerted 12 hydrogen deficiency index. Another fragmentation peak was detected at m/z 363.0838 $[M + Na - 60]^+$, because of the loss of acetic acid. The NMR spectroscopic data in *Table 1* showed that eight of the 12 elements of unsaturation came from six double bonds into aromatic rings and two ester carbonyls; consequently, the molecule was tetracyclic. The analysis of the 1D and 2D NMR spectra revealed the presence of one phenylpropanoid unit in **1**, consisting of a 1-piperonylpropane moiety [δ_H 3.77 (H-8), 4.38 (H-9b), 4.43 (H-9a), 5.61 (H-7), 5.97 (OCH₂O), 6.83 (H-5), 6.86 (H-2), 6.96 (H-6)]. This assumption was indicated

by the singlet resonance at δ_{H} 5.97 (OCH₂O), which correlates with the resonance of the quaternary carbon at δ_{C} 149.2 (C-4) in the HMBC spectrum, thereby supporting the presence of a dioxymethylene group in a phenyl ring system. COSY correlations detected between H-7–H-8 and H-8–H-9, along with the HMBC cross peak observed between H-2–C-7 verified the structural features of the phenylpropanoid unit. The remaining NMR spectral signals were allocated to a 1,3,4,5-tetrasubstituted aromatic ring consisting of a benzofuran moiety, which was substituted with one methoxy and one carboxymethyl group, resonating at δ_{H} 3.93 (s, OMe-3') and 3.90 (OMe-7') in the ¹H NMR, respectively. A distinct singlet resonance at δ_{H} 2.04 (9-OCOCH₃), showing a correlation with the carbonyl signal at 172.4 (9-OCOCH₃) ppm in the HMBC spectrum, was assigned to an acetyl substituent. The above data suggested that **1** was a dihydrobenzofuran-type 8',9'-bis-*nor*-neolignan and its general structure was substantially similar to that of toonin C (Dong et al., 2013). The key structural variation between the two molecules is the represented by the presence of an acetyl group at C-9 in compound **1**. The HMBC spectrum displayed a clear long-range correlation between H-9 and the carbonyl resonance of the 9-OCOCH₃ group, which allowed the precise localization of the acetyl substituent at the C-9 position. This key evidence strongly established the structure of compound **1** as 9-acetyltoonin C.

Compound **2** was isolated as a yellow residue. The HRESIMS spectrum showed a sodiated molecular ion peak at m/z 401.1204 [M + Na]⁺, from which the molecular formula C₁₉H₂₂O₈ was deduced. The calculated hydrogen deficiency index of nine indicated the presence of a moderately unsaturated carbon skeleton within the molecule. The fragmentation peak observed at m/z 205.0475 [M + Na - 196]⁺ pointed to the loss of one phenylpropanoid unit. Comprehensive analysis of the NMR spectroscopic data (Table 1) identified compound **2** as a guaiacylglycerol derivative. The structure closely paralleled that of *threo*-guaiacylglycerol- β -vanillic acid ether (Katayama et al., 1981; Sakushima et al., 2003), with the defining distinction being the substitution of a carboxymethyl group at C-1' in compound **2**, instead of the free carboxylic acid group. Accordingly, the ¹H NMR signals detected at δ_{H} 7.08 (H-2), 6.88 (H-6), 6.73 (H-5), 4.82 (H-7), 4.61 (H-8), 3.88 (H-9), and 3.81 (3-OMe) were assigned to the *threo*-guaiacylglycerol moiety, which constituted the phenylpropanoid subunit of compound **2**. This structural interpretation was confirmed by long-range HMBC correlations, specifically between H-7 and C-1, and between 3-OMe and C-3. Additional

evidence was provided by 1D-TOCSY and COSY spectra, which unambiguously demonstrated the spin coupling system involving H-7 and H₂-9, thereby substantiating the proposed connectivity of the guaiacylglycerol moiety. The remaining ¹H NMR signals, observed at δ_{H} 7.56 (H-6'), 7.52 (H-2'), 7.04 (H-5'), 3.87 (7'-OMe), and 3.83 (3'-OMe), were attributed to a vanillic acid methyl ester moiety. This assignment not only confirmed the presence of the substituted aromatic domain in compound **2** but also complemented the connectivity deduced from the HMBC and COSY data. All the assignments were confirmed by the analysis of the 2D NMR spectra. The HMBC correlations detected between H-8 and C-1, as well as between H-8 and C-4', unequivocally established that the C-8 position of the *threo*-guaiacylglycerol fragment is linked through an ether bond to C-4' site of the vanillic acid methyl ester moiety, thereby confirming the connectivity within compound **2**. Accordingly, compound **2** was characterized as *threo*-guaiacylglycerol- β -methyl vanillate ether, with its complete structure illustrated in Figure 2.

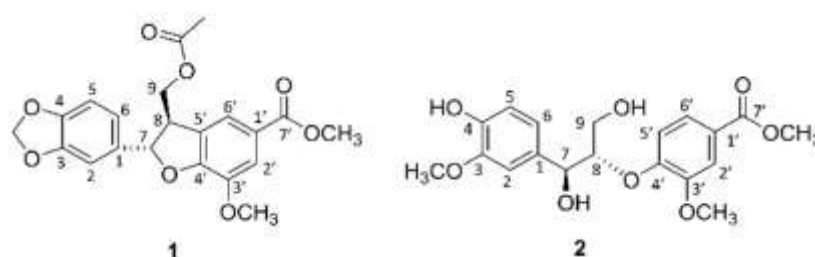


Figure 2. New compounds from *C. odorata*

Bis-*nor*-neolignans have been recognized in various Magnolidae, particularly among families such as Rosaceae, Asteraceae, Fabaceae, and Araceae. Moreover, their presence in another *Cedrele* species has been highlighted (Lee et al., 2010), emphasizing the relevance of this genus as a reservoir of structurally diverse neolignan derivatives. Lignans and neolignans are known to exhibit cytotoxic and anticancer properties, and little investigation on evaluating anticancer activity in relation to the various lignan subclasses has been carried out to date (Maia et al., 2023). Dihydrobenzofuran neolignans have been reported to display anti-inflammatory, antioxidant, neuroprotective, antimicrobial, cytotoxic, and antiangiogenic activities (Basini et al., 2014). In addition, secoisolariciresinol has also been described as cytotoxic.

Table 1 ^1H NMR (CD_3OD , 600 MHz, J in Hz) and ^{13}C NMR (CD_3OD , 150 MHz) data of **1-2**.

position	1		2	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1		135.5		133.7
2	6.86 d (1.5)	106.7	7.08 d (1.5)	111.0
3		148.0		148.5
4		149.2		146.0
5	6.83 d (8.0)	108.0	6.73 d (8.0)	114.0
6	6.96 dd (8.0, 1.5)	120.3	6.88 dd (8.0, 1.5)	120.4
7	5.61 d (6.7)	89.7	4.82 ^a	73.9
8	3.77 br dd (7.8, 5.3)	50.0	4.61 br dd (5.0, 10.6)	84.8
9a	4.43 dd (10.5, 5.3)	65.9	3.88 br d (7.7)	61.8
9b	4.38 dd (10.5, 7.8)			
3-OMe			3.81 s	55.7
9-COCH ₃	2.04 s	20.0		
9-COCH ₃		172.4		
-OCH ₂ O-	5.97 s	101.4		
1'		120.2		123.4
2'	7.63 br s	113.0	7.52 d (2.0)	113.0
3'		145.2		150.7
4'		153.7		153.0
5'		128.9	7.04 d (8.3)	115.0
6'	7.62 br s	120.0	7.56 dd (8.3, 2.0)	124.0
7'		168.0		167.8
3'-OMe	3.93 s	56.7	3.83 s	55.6
7'-OMe	3.90 s	52.6	3.87 s	51.8

^a Overlapped signal. J values are in parentheses and reported in Hz; chemical shifts are given in ppm; assignments were confirmed by COSY, 1D-TOCSY, HSQC, and HMBC experiments.

Accordingly, the present research evaluated the antiangiogenic potential of compounds **1** and **2** and other lignans that previously purified and isolated from our chemical library, namely erythro-4,7,9,9'-tetrahydroxy-3,3'-dimethoxy-8-O-4'-oxyneolignan (**3**) (Faiella et al., 2012a), urolignoside (**4**) (De Leo et al., 2021), secoisolariciresinol 9-O- β -D-glucopyranoside (**5**) (Vitiello et al., 2024), icariside E3 (Faiella et al., 2012b), erythro-4,7,9,9'-tetrahydroxy-3,3'-dimethoxy-8-O-4'-oxyneolignan-9'-O- β -D-glucopyranoside (Faiella et al., 2012a), and dihydrodehydroconiferyl alcohol (unpublished results). Their antiangiogenic effects were systematically assessed using the zebrafish (*Danio rerio*) *in vivo* model, a well-established system for evaluating vascular development and inhibition.

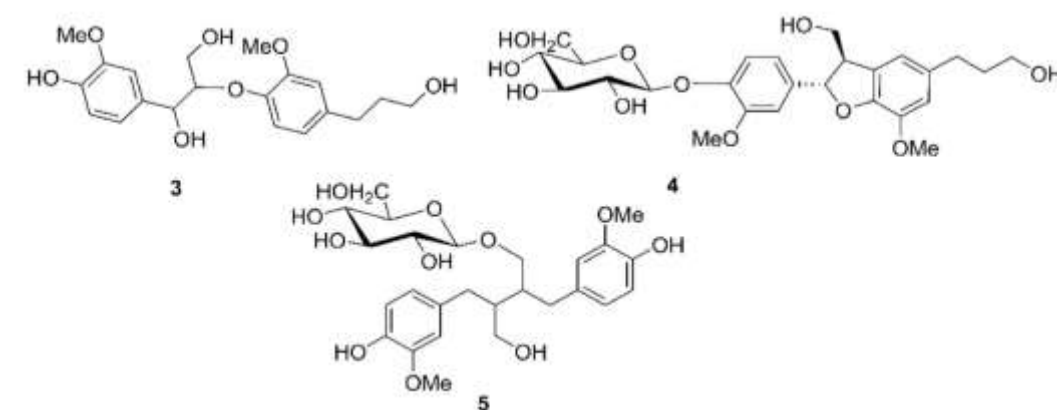


Figure 3. Chemical structures of compounds **3-5**

The results of the biological evaluation of compounds **1**, **2**, and **3** demonstrated the most significant antiangiogenic activity, highlighting their potential as lead candidates for further pharmacological investigation. Remarkably, compound **2** (the new 8',9'-bis-*nor*-neolignans 9-acetyl toonin C) exerted significant inhibitory effects in the zebrafish embryo EAP assay, achieving 42.56% inhibition at 10 μ M ($P < 0.01$), and this level of activity was comparable to that of 2-methoxyestradiol, a well-established antiangiogenic standard, which induced 49.50% inhibition at 2 μ M (Figure 4).

Mild inhibitory effects on EAP release were observed in zebrafish embryos treated with compounds **4** and **5**. On the other hand, no measurable antiangiogenic activity was observed for icariside E3, *erythro*-4,7,9,9'-tetrahydroxy-3,3'-dimethoxy-8-O-4'-oxyneolignan-9'-O- β -D-glucopyranoside, and dihydrodehydroconiferyl alcohol under the same tested conditions (data not shown). The findings could be an important starting basis for the discovery of new therapeutic agents capable of regulating angiogenesis in tumor progression (Kerbel, 2000).

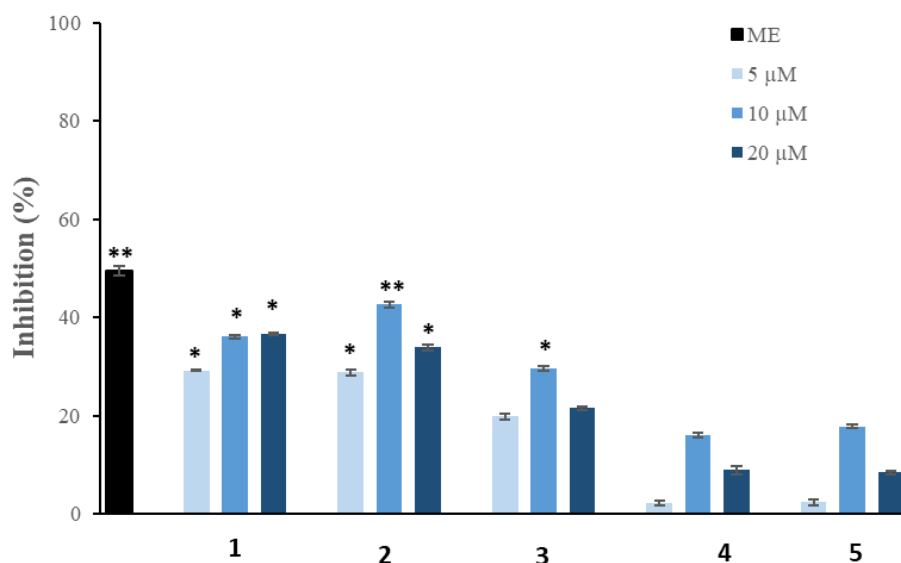


Figure 4. Antiangiogenic evaluation of the active isolated compounds, expressed as percentage inhibition relative to control, determined through the zebrafish embryo endogenous alkaline phosphatase (EAP) assay. Control group received only DMSO (0.2 % v/v). Data were compared with the reference standard, ME = 2-methoxyestradiol, 2 μ M. All treated embryos (10 for each group) were incubated from 24 hpf to 72 hpf (total 48 hours of exposure). * $P < 0.05$ and ** $P < 0.01$ vs control: Student's t-test

4.4. Conclusion

Lignans have long been well noted for their antitumor, antimitotic, and antiviral properties, as well as for their ability to inhibit distinct enzymatic targets; however, information concerning their effect on angiogenesis remains limited (Zálešák et al., 2019). In this research, *C. odorata* was examined with particular focus on lignan derivatives, highlighting their potential as new therapeutic candidates capable of modulating angiogenesis and laying the groundwork for future pharmacological applications. Beyond its potential anticancer effects, *C. odorata* exhibits a broad range of noteworthy biological activities, making it a plant species of significant phytochemical and extractive interest. Nevertheless, it is important to emphasize that this species is considered as endangered. Indeed, *C. odorata* is included in Appendix III of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Finch et al., 2019), which allows unrestricted trade in some countries, while in other regions its commercialization is subject to stricter protection and regulation (Galván-Hernández et al., 2018). According to the IUCN Red List,

C. odorata is classified as Vulnerable under the A1cd+2cd criteria (Galván-Hernández et al., 2018). This species ranks as the second most highly demanded tropical timber worldwide (Cotrina Sánchez et al., 2021) and is also recognized for its significant potential in agroforestry systems (Tenorio and Moya, 2019). *Cedrela odorata* is ranked as the second most commercially demanded tropical hardwood and is also considered a species of high relevance for integration into agroforestry systems (Tenorio & Moya, 2019). Due to its sensitivity to climate variations, *C. odorata* is increasingly regarded as a notable species for elucidating tree responses to climate change. *C. odorata* is recognized for its high economic impact in the forestry sector and the pressing need for conservation within the Amazon Biome; the implementation of environmental sustainability principles is of paramount importance. It is therefore vital to expand the research on its bioactive compounds, also considering their application as fundamental structures for the synthesis of analogous compounds with the same or increased biological activity.

Chapter 5

Phytochemical Characterization and Bioactivity Assessment of *Lavandula pubescens* Decne

5.1 Introduction

The genus *Lavandula* (Lamiaceae) grows mainly around the Mediterranean basin, including Southern Europe, North and East Africa, the Middle East, Southwest Asia, and Southeastern India. These perennial evergreen shrubs, generally 40–60 cm in height, thrive on calcareous, well-drained soils under full sunlight exposure. The plants are easily recognized by their narrow lanceolate or linear leaves covered by a silvery tomentum that protects them from direct sunlight and water loss, and by their inflorescences, which display characteristic violet or lilac flowers, although pink or white varieties also exist (Batiha, G. E. S. et al., 2023; Ghushash, 2006). The genus *Lavandula* includes more than 176 species and numerous hybrids and subspecies, among which *L. angustifolia*, *L. latifolia*, *L. stoechas*, and *L. x intermedia* are the most studied for their economic and pharmacological value.

Plants belonging to this genus are recognized for their significantly high content of monoterpenes and sesquiterpene components in their essential oils. They are employed in perfumery, cosmetics, aromatherapy, as well as the food industry. Beyond their aromatic appeal, *Lavandula* species have been widely used in traditional medicine for centuries, largely because of their well-recognized antimicrobial, anti-inflammatory, and antioxidant activities (Eissa et al., 2014). In addition to their cultural and aromatic value, *Lavandula* species also exhibit great phytochemical diversity. They produce compounds such as essential oils, polyphenols, flavonoids, tannins, and triterpenes molecules associated with a wide range of biological activities, such as antioxidant, antimicrobial, and anti-inflammatory activities. For instance, *L. angustifolia* oil contains linalool and linalyl acetate, responsible for its sedative and anxiolytic properties, while *L. latifolia* is rich in 1,8-cineole and borneol, compounds with respiratory and antimicrobial benefits. This chemical variability among species contributes to the extensive range of pharmacological and industrial applications attributed to the genus. Within this diverse genus, *Lavandula pubescens* Decne remains comparatively underexplored despite its ecological and medicinal potential. The species is native to arid and semi-arid regions of the Middle East particularly Saudi Arabia, Yemen, Jordan, and Iran, where it has adapted to harsh environments such as rocky slopes and palm groves, characterized by strong sunlight and limited water availability (Bordbar & Upson, 2021). Morphologically, *L. pubescens* is

characterized by deeply pinnate, pubescent leaves and abundant glandular trichomes, which secrete both volatile and non-volatile metabolites responsible for its strong and acrid odor. Botanical studies report that *Lavandula pubescens* is a resilient shrub adapted to harsh environments. These distinctive morphological and ecological properties not only allow this species to thrive in including arid and rocky habitats but also make it a notable part of local biodiversity, contributing to soil stabilization and providing nectar sources that sustains a variety of pollinators.

Traditionally, *Lavandula pubescens* has played an important role in folk medicine and rural economies across the Arabian Peninsula. In Yemen and surrounding countries, decoctions of its leaves are used to treat headaches, colds, and respiratory discomforts, while the essential oil serves as an antiseptic, carminative, and insect repellent (Bordbar & Upson, 2021). The antibacterial and antifungal activity of its main volatile constituents such as carvacrol, β -caryophyllene, and p-cymen-8-ol supports these traditional uses and has prompted further pharmacological investigations (Rahman et al., 2004). Beyond medicinal applications, *L. pubescens* demonstrates significant ecological and economic versatility and adaptability. It is cultivated as an ornamental plant in urban parks in Saudi Arabia for its aesthetic value and drought tolerance, reducing the prevalence of invasive non-native plants and enhancing the resilience of urban ecosystems (Banan et al., 2023). Moreover, *Lavandula pubescens* specifically supports apiculture through the production of high-value mono floral honey well known as “Seyfi honey,” characterized by its delicate lavender aroma and commercial value ranging from 50 to 120 USD per kilogram (Nuru et al., 2015). Moreover, the extracts have been studied for their application in the food industry as natural antioxidants. They can be considered alternatives to chemicals such as BHT and TBHQ because of their phenolics' strong scavenging ability. Interestingly, recent works have also investigated the use of *L. pubescens* for other environmental purposes. In fact, the powdered aerial parts of the plant were found to have a remarkable capacity to bio-sorb lead ions (Pb^{2+}) in wastewater. These findings underline the multi-purpose potential of this species, extending from pharmacognosy to environmental biotechnology (Alorabi et al., 2020). The pharmacological interest in *Lavandula pubescens* Decne is increasing, but the available information on the phytochemicals of this species remains limited, especially about its non-volatile components. However, the composition of essential oils has been the

target of some studies. These volatile constituents are responsible not only for the characteristics of the aroma of the plant but also for its biological properties. As pointed out in previous studies, the essential oils from the species belonging to the Lamiaceae family have commercial and therapeutic importance. The essential oil of *L. pubescens* is a complex mixture of terpenoids—mainly oxygenated monoterpenes (such as carvacrol and methyl carvacrol) which together constitute approximately 70% of the oil. Beyond volatile constituents, *Lavandula pubescens* is also a source of non-volatile secondary metabolites, especially flavonoids (hypolaetin 8-O-glucuronide, hypolaetin 4'-methyl ether 8-O-glucuronide, isoscutellarein 8-O-glucuronide, xanthomicrol, hypolaetin 7-O-glucoside, and luteolin 7-O-glucoside) and triterpenes (such as oleanolic acid and ursolic acid) (Shen et al., 2022). The ethyl acetate extracts of various parts of *Lavandula pubescens* also examined for their antimicrobial activity against various microorganisms, including gram-positive, gram-negative bacteria, and yeast. The antimicrobial activity was determined using standardized methods, such as Minimum Inhibitory Concentration, which determines the minimum amount for each extract to inhibit bacterial growth. The ethyl acetate extracts from leaves had the highest antimicrobial activity against *Escherichia coli*, *Staphylococcus haemolyticus*, *Aeromonas hydrophila*, *Cronobacter sakazakii*, and *Aeromonas salmonicida* bacteria, followed by extracts from flowers and then stems. On the other hand, the extract of roots exhibited no significant antimicrobial properties (Park et al., 2019). In addition to its antimicrobial activity, the ethyl acetate leaf extract of *L. pubescens* exhibited a remarkable antioxidant capacity. Such strong effects are largely attributed to its high triterpene content, notably oleanolic and ursolic acids, as well as its elevated levels of total phenolics and flavonoids (Park et al., 2019). The data reveal that there is no synergistic antioxidant interaction, hence supporting the usage of extracts of *L. pubescens* as natural alternatives to BHT and TBHQ antioxidants. Apart from antimicrobial and antioxidant activity, in recent years, emphasis has also shifted to the immune-modulatory activity of natural compounds derived from plants, which act on immune and inflammation processes with reduced side effects in contrast to synthetic drugs. Extracts of *L. pubescens* have demonstrated the ability to regulate prominent inflammatory factors, including nitric oxide (NO), tumor necrosis factor-alpha (TNF- α), and cyclooxygenase-2 (COX-2) in LPS-activated macrophages. By suppressing the excessive production of those inflammatory factors, *L.*

pubescens extracts appear to play a part in reducing tissue damage and preventing chronic inflammation. Furthermore, the methanolic extract showed an IC₅₀ value of approximately 100 µg/mL in macrophage proliferation assays, one of the lowest among several medicinal plants tested, indicating a strong bioactivity. Overall, the extract showed the ability to enhance the activity of phagocytosis, indicating the role of the plant in the stimulation of the immune response and protection against infections in the body. In conclusion, these findings substantiate the ethnopharmacological use of *L. pubescens* for treatment of inflammatory and respiratory diseases and demonstrate its potential as a natural source of chemo-preventive agents (Ghonime et al., 2015). Taken together, these findings underline the high pharmacological versatility of *Lavandula pubescens* and justify additional investigation into its bioactive properties of this plant. Therefore, the present study aimed to explore both the phytochemical composition and biological properties of this species.

Aim of the present study

This project focuses on the chemical-biological study of *Lavandula pubescens* Decne, collected in Saudi Arabia, with the objective of valorizing pruning residues from ornamental shrubs cultivated in urban gardens and parks. To this end, a phytochemical investigation of the leaf and leaf surface extracts of *Lavandula pubescens* was carried out, aimed at characterizing the phytocomplex and isolating pure bioactive compounds. Moreover, considering the traditional use of *Lavandula pubescens* in folk medicine and its chemical profile, the anti-bacterial and anti-inflammatory activities of the parts extract and aerial exudate and of the isolated molecules were evaluated. The project was organized according to the following experimental steps:

(i) Separation of the aerial parts and leaf surface extract by chromatographic techniques in order to obtain compounds in pure form; (ii) Structural characterization of the isolated compounds by spectroscopic and mass-spectrometric techniques (UV, 1D and 2D NMR, and HRMS); (iii) Evaluation of antibacterial and anti-inflammatory activity of the extracts and the pure compounds.

5.2 Materials and methods

5.2.1 General experimental procedures

Optical rotations were measured on an Atago AP-300 digital polarimeter with 1 dm microcell and a sodium lamp (589 nm). NMR data were acquired on a Bruker DRX-600 NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with a Bruker 5 mm TCI CryoProbe at 300 K. Data processing was carried out with Topspin 3.2 software. All 2D NMR spectra were acquired in methanol-*d*₄ (99.95%, Sigma-Aldrich, Milano, Italy), and standard pulse sequences and phase cycling were used for COSY, HSQC, HMBC, 1D-TOCSY, and ROESY spectra. HRESIMS data were obtained in the positive ion mode on a Q Exactive Plus mass spectrometer, Orbitrap-based FT-MS system, equipped by an ESI source (Thermo Fischer Scientific Inc., Bremen, Germany). Column chromatography was performed over silica gel (70-220 mesh, Merck). RP-HPLC separations were carried out using a Shimadzu LC-8A series pumping system equipped with a Shimadzu RID-10A refractive index detector and Shimadzu injector (Shimadzu Corporation, Kyoto, Japan) on Waters XTerra Semiprep MS C₁₈ column (300 mm × 7.8 mm i.d.) and a mobile phase consisting of MeOH-H₂O mixture at a flow rate of 2.0 mL/min. TLC separations were conducted using silica gel 60 F254 (0.20 mm thickness) plates (Merck, Darmstadt, Germany) and Ce(SO₄)₂/H₂SO₄ as spray reagent (Sigma-Aldrich, Milano, Italy).

5.2.2 Plant materials

The aerial parts of *Lavandula pubescens* Decne (Lamiaceae) have been collected from Wadi Thee Gazal, near Taif, Makkah Al-Mukarramah Region, Saudi Arabia, during the summer of 2023. A voucher specimen (number SA/IT-2023/1) was deposited in the Laboratory of Pharmacognosy at Umm Al-Qura University, Saudi Arabia.

5.2.3 Extraction

Both the surface exudate and the aerial-part extracts were obtained using different extraction procedures, as described below, respectively. Fresh material (1000 g) was dipped in dichloromethane (5 L) for 20 s, and the filtrate was concentrated under reduced

pressure using a rotary evaporator to afford the exudate (yield 1% of the fresh weight). The washed plant material was then left to dry at room temperature until constant weight for further phytochemical studies. The dried material was powdered and subjected to exhaustive extraction at room temperature with solvents of increasing polarity (*n*-hexane, chloroform, and finally methanol).

5.2.4 UHPLC-HRESI-Orbitrap/MS/MS analysis

LC-HRESIMS analysis on *L. pubescens* *n*-hexane, chloroform and leaf surface extract was performed in positive ion mode using a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Scientific®) coupled with Thermo Scientific® UltiMate 3000 UHPLC system. Capillary temperature was set at 320 °C, flow rate of sheath gas and auxiliary gas were set at 35.0 and 15 arbitrary units. A Luna® C18 150 × 2 mm, 3 μm (100 Å) column (Phenomenex®, Castel Maggiore, Bologna, Italy) and a binary mobile phase composed of eluent A (H₂O–formic acid 0.1% v/v) and eluent B (acetonitrile) were used. The separation conditions are from 5% to 100% of B in 45 minutes. Flow rate and injection volume were 0.2 mL/min and 10.0 μL, respectively.

5.2.5 Isolation of pure compounds from *n*-hexane extract of *Lavandula pubescens* Decne

Part of the *n*-hexane extract (4.5 g) was subjected to flash column chromatography (Figure 1) by Biotage Selekt automated flash chromatography system (Biotage Sweden AB, Uppsala, Sweden). The extract was loaded onto a 10 g silica gel samplet and then separated using a Biotage® Sfär HC Duo 50 g eluting with *n*-hexane, followed by increasing concentrations of CHCl₃ in *n*-hexane (between 10% and 100%), and MeOH in CHCl₃ (between 1% and 100%) at a flow rate of 100 mL/min. A total of 280 fractions of 22 mL were collected and pooled together after screening by TLC into ten fractions (A₁–J₁). Fraction C₁ (90 mg) was submitted to RP HPLC with MeOH–H₂O (18:7) as eluent to yield compounds **9** (1.1 mg, R_t 44 min), **4** (0.9 mg, R_t 72 min), **20** (1.2 mg, R_t 88 min), **18** (1.0 mg, R_t 98 min). An aliquot (105 mg) of fraction E₁ was separated by RP HPLC eluting with MeOH–H₂O (18:7) to give compounds **17** (1.0 mg, R_t 47 min), **3** (1.5 mg, R_t 50 min), **16** (0.8 mg, t_R 56 min), **15** (1.3 mg, R_t 68 min) and **11** (2.8 mg, R_t 114 min). Fraction F₁ (110 mg) was separated by RP HPLC eluting with MeOH–H₂O (18:7) to give compounds **19** (1.6. mg, R_t 44

min), **16** (0.6 mg, R_t 50 min), **5** (1.2 mg, R_t 64 min), **10** (1.8 mg, R_t 172 min), and **13** (3.2 mg, R_t 190 min). Compound **13** (22 mg) was directly obtained as pure molecule from fraction G₁. Fraction H₁ (190 mg) was separated by RP HPLC eluting with MeOH–H₂O (18:7) to give compounds **5** (0.9 mg, t_R 66 min), **12** (2.2 mg, R_t 180 min), and **13** (4.3 mg, R_t 190 min).

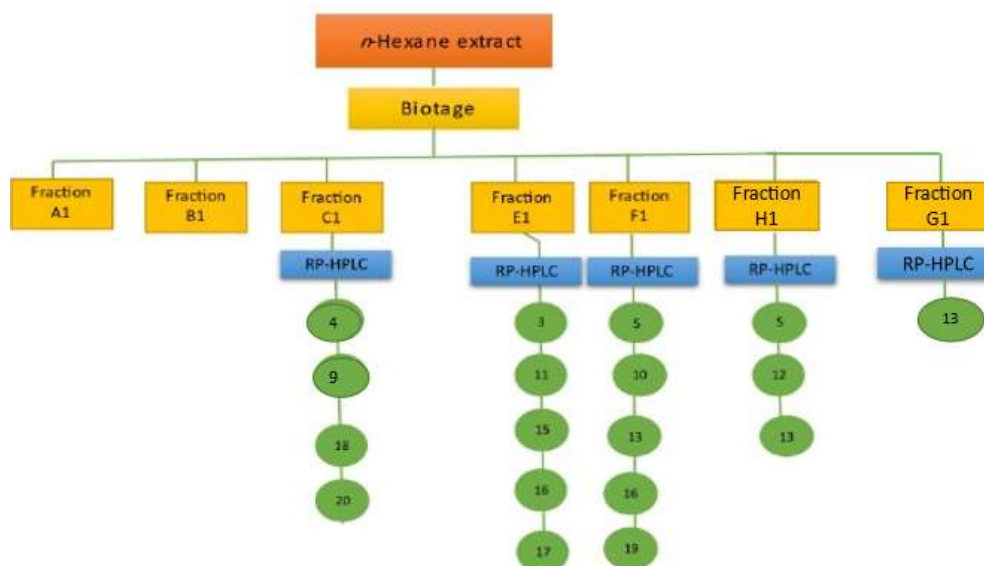


Figure 1. Isolation of pure compounds from *n*-hexane extract of *Lavandula pubescens* Decne

Compound (3): white amorphous powder; $[\alpha]^{25}_D$ -30.0 (c 0.09, MeOH); ^1H and ^{13}C NMR (MeOD- d_4), see Table 2; HRESIMS m/z 323.1981 $[M + Na]^+$ (calcd for $C_{20}H_{28}NaO_2$, 323.1993).

Compound (4): white amorphous powder; $[\alpha]^{25}_D$ -24.9 (c 0.06, MeOH); ^1H and ^{13}C NMR (MeOD- d_4), see Table 2; HRESIMS m/z 325.2138 $[M + Na]^+$ (calcd for $C_{20}H_{30}NaO_3$, 325.2149).

Compound (5): white amorphous powder; $[\alpha]^{25}_D$ -13.5 (c 0.02, MeOH); ^1H and ^{13}C NMR (MeOD- d_4), see Table 3; HRESIMS m/z 345.2403 $[M + Na]^+$ (calcd for $C_{20}H_{34}NaO_3$, 345.2400).

Compound (9): white amorphous powder; $[\alpha]^{25}_D$ -12.7 (c 0.07, MeOH); ^1H and ^{13}C NMR, see Table 4; HRESIMS m/z 343.2253 $[M + Na]^+$ (calcd for $C_{20}H_{32}NaO_3$, 343.2243).

Compound (15): white amorphous powder; $[\alpha]^{25}_D$ -30 (c 0.08, MeOH); ^1H and ^{13}C NMR (MeOD- d_4), see Table 5; HRESIMS m/z 341.2096 $[M + H]^+$ (calcd for $C_{20}H_{30}NaO_3$, 341.2087).

Compound (16): white amorphous powder; $[\alpha]^{25}_D$ -85.0 (c 0.007, MeOH); ^1H and ^{13}C NMR (MeOD- d_4), see Table 5; HRESIMS m/z 341.2096 $[M + Na]^+$ (calcd for $C_{20}H_{30}NaO_3$, 341.2087), 319.2268 $[M + H]^+$, 301.21 $[M + H - 18]^+$.

Compound (17): white amorphous powder; $[\alpha]^{25}_D$ -25.5 (c 0.04, MeOH); ^1H and ^{13}C NMR (MeOD- d_4), see Table 6; HRESIMS m/z 341.2097 $[M+Na]^+$ (calcd for $C_{20}H_{30}O_3$, 341.2087), 301.21 $[M+H-18]^+$, 283.17 $[M+H-2H_2O]^+$.

Compound (18): White amorphous powder; $[\alpha]^{25}_D$ -32.8 (c 0.02, MeOH); ^1H and ^{13}C NMR (MeOD- d_4): see Table 6; HRESIMS m/z 323.1981 $[M+Na]^+$ (calcd for $C_{20}H_{28}NaO_2$, 323.1981), 301.2162 $[M+H]^+$, 283.2064 $[M+H-18]^+$.

Compound (19): white amorphous powder; $[\alpha]^{25}_D$ -23.8 (c 0.05, MeOH); ^1H and ^{13}C NMR (MeOD- d_4), see Table 7; HRESIMS m/z 341.2089 $[M+Na]^+$ (calcd for $C_{20}H_{30}NaO_3$, 341.2087).

Compound (20): White amorphous powder; $[\alpha]^{25}_D$ -32.6 (c 0.04, MeOH); ^1H and ^{13}C NMR (MeOD- d_4): see Table 7; HRESIMS m/z 323.1995 $[M+Na]^+$ (calcd for $C_{20}H_{28}NaO_2$, 323.1981), 283.20 $[M+H-18]^+$, 151.11 $[M+H-C_6H_{12}]^+$, 133.10 $[M+H-C_6H_{12}-18]^+$.

5.2.6 Isolation of pure compounds from chloroform extract of *Lavandula pubescens* Decne

Part (3 g) of the chloroform extract was subjected to CC by Biotage Selekt automated flash chromatography system (Biotage Sweden AB, Uppsala, Sweden). The extract was loaded in a 10 g silica gel samplet and then separated using a Biotage SNAP KP-Sil 100g eluting with *n*-hexane, followed by increasing concentrations of $CHCl_3$ in *n*-hexane (between 10% and 100%), and MeOH in $CHCl_3$ (between 1% and 100%) at a flow rate of 60 mL/min. A total of 196 fractions of 22 mL were collected and pooled together after screening by TLC into nine fractions (A_2 - I_2). Compound **21** identified as Carvacrol (5.6 mg) was directly obtained as pure molecule from fraction A_2 . Fraction B_2 was separated by RP HPLC with MeOH- H_2O (65:35) as eluent to give compounds **25** (15.8 mg, t_R 22 min) and **28** (8.8 mg, t_R 86 min). Fraction C_2 (130 mg) was submitted to RP HPLC with MeOH- H_2O (7:3) as eluent to yield compounds **8** (0.8 mg, t_R 24 min), **5** (1.3 mg, t_R 62 min), **7** (0.9 mg, t_R 78 min), **24** (3.4 mg, t_R 98 min), **13** (1.8 mg, t_R 190 min), **23** (2.5 mg, t_R 192 min), **22** (1.3 mg, t_R 193 min). Furthermore, **13** (6.5 mg) was also obtained in pure form from fraction D_2 . An aliquot (160 mg) of fraction E_2 was separated by RP HPLC eluting with MeOH- H_2O (65:35) to give compound **26** (5.6 mg, t_R 14 min) and **27** (0.9 mg, t_R 62 min). Fraction F_2 (106 mg) was separated by RP HPLC eluting with MeOH- H_2O (65:35) to give compounds **1** (1.2 mg, t_R 48 min), **14** (1.6 mg, t_R 56 min), **6** (1.1 mg, t_R 82 min), **4** (1.4 mg, t_R 130 min). An aliquot (160

mg) of fraction H₂ was separated by RP HPLC eluting with MeOH–H₂O (7:3) to give compound **2** (0.7 mg, t_R 36 min).

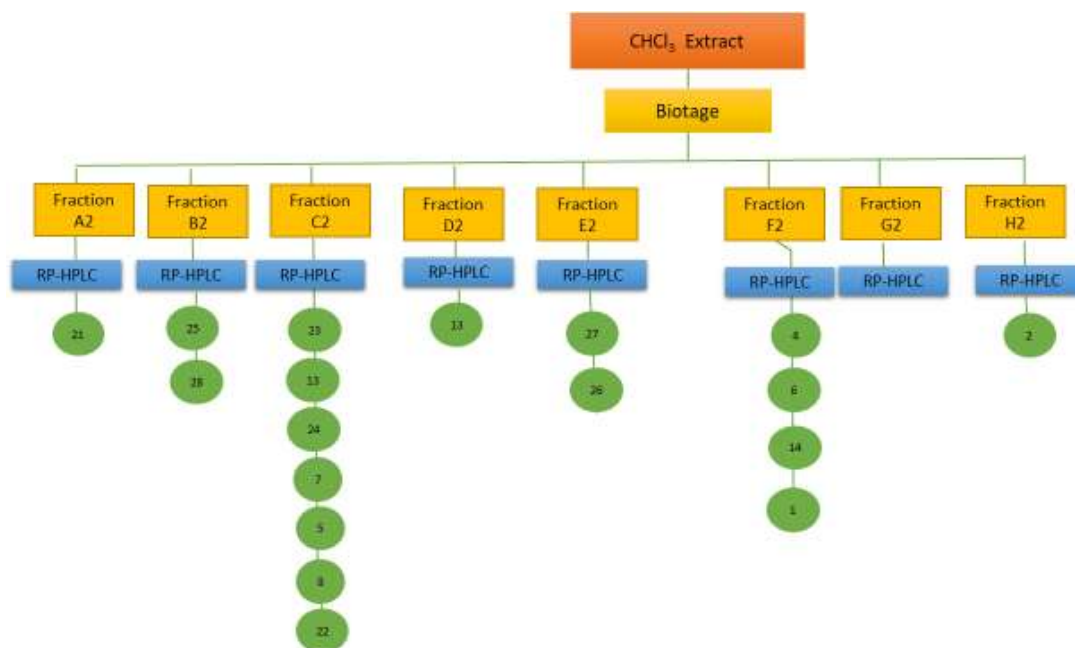


Figure 2. Isolation of pure compounds from chloroform extract of *Lavandula pubescens* Decne

Compound (1): white amorphous powder; $[\alpha]^{25}_{\text{D}}$ - 42.8 (c 0.007, MeOH); ¹H and ¹³C NMR (MeOD-d₄), see Table 1; HRESIMS m/z 343.2255 [M + Na]⁺ (calcd for C₂₀H₃₂O₃, 343.2243).

Compound (2): white amorphous powder; $[\alpha]^{25}_{\text{D}}$ -7.4 (c 0.03, MeOH); ¹H and ¹³C NMR (MeOD-d₄), see Table 1; HRESIMS m/z 345.2394 [M + Na]⁺ (calcd for C₂₀H₃₄NaO₃, 345.2400).

Compound (4): white amorphous powder; $[\alpha]^{25}_{\text{D}}$ -24.9 (c 0.06, MeOH); ¹H and ¹³C NMR (MeOD-d₄), see Table 1; HRESIMS m/z 325.2149 [M + Na]⁺ (calcd for C₂₀H₃₀NaO₂, 325.2138).

Compound (5): white amorphous powder; $[\alpha]^{25}_{\text{D}}$ -13.6 (c 0.05, MeOH); ¹H and ¹³C NMR (MeOD-d₄), see Table 1; HRESIMS m/z 345.2403 [M + Na]⁺ (calcd for C₂₀H₃₄NaO₃, 345.2400).

Compound (6): white amorphous powder; $[\alpha]^{25}_{\text{D}}$ + 9.3 (c 0.07, MeOH); ¹H and ¹³C NMR (MeOD-d₄), see Table 3; HRESIMS m/z 361.2341 [M + Na]⁺ (calcd for C₂₀H₃₄NaO₄, 361.2349).

Compound (7): white amorphous powder; $[\alpha]^{25}_{\text{D}}$ - 7.5 (c 0.04, MeOH); ¹H and ¹³C NMR (MeOD-d₄), see Table 4; HRESIMS m/z 361.2342 [M + Na]⁺ (calcd for C₂₀H₃₄NaO₄, 361.2349).

Compound (8): white amorphous powder; $[\alpha]^{25}_{\text{D}}$ -21.2 (c 0.073, MeOH); ¹H and ¹³C NMR, see Table 4; HRESIMS m/z 331.2257 [M + Na]⁺ (calcd for C₁₉H₃₂NaO₃, 331.2243).

5.2.7 Antimicrobial activity

Preliminary Screening of the Antimicrobial Activity of *L. pubescens* Extracts

A preliminary assessment of the antimicrobial activity of the *L. pubescens* chloroform and hexane extracts was conducted by monitoring bacterial growth curves using a Tecan microplate reader. The bacterial strains tested included *Escherichia coli*, *Staphylococcus aureus*, *Citrobacter* spp., *Salmonella* spp., *Enterococcus faecalis*, *Acinetobacter baumannii*, *Streptococcus epidermidis*, and *Listeria monocytogenes*. For each strain, liquid cultures were prepared and adjusted to an initial density of $OD_{600} = 0.05$. The *L. pubescens* leaves extracts were added directly to the wells of the microplate at final concentrations of 500, 250, 125, 60, and 0 $\mu\text{g/mL}$ (untreated control). Starting from a stock concentration of 10 mg/mL , the extracts were diluted with BHI (Brain Heart Infusion). The plates were incubated under controlled temperature conditions and bacterial growth was monitored for 940 minutes by measuring optical density at 600 nm. *L. pubescens* chloroform extract exhibited antimicrobial activity against *L. monocytogenes* and *S. epidermidis*, no activity was observed for *n*-hexane residue.

Analysis of Fractions from the Chloroform Extract

Based on the screening results, which indicated higher susceptibility of *L. monocytogenes* and *S. epidermidis* to the chloroform extract, subsequent analyses were focused on these two microorganisms. Nine fractions derived from the chloroform extract (A_2 – I_2) were tested. Each fraction was added to standardized bacterial suspensions (initial $OD_{600} = 0.05$) at different concentrations 100 $\mu\text{g/mL}$, 50 $\mu\text{g/mL}$, 25 $\mu\text{g/mL}$, 12 $\mu\text{g/mL}$, and 0 $\mu\text{g/mL}$. Starting from a stock concentration of 10 mg/mL , the fractions were diluted with BHI (Brain Heart Infusion). Bacterial growth was monitored under the same microplate conditions described above, and growth profiles were compared with the untreated control.

Testing of Individual Molecules Isolated from Fraction C_2

The minimum inhibitory concentration (MIC), defined as the lowest concentration of the molecule capable of inhibiting bacterial growth, was assessed using the serial dilution method, in accordance with the CLSI protocol (2009). Several molecules (**13**, **22**, **23**, isolated from fraction C_2 , the most active from fractions screening, were evaluated against *S.*

epidermidis. Serial dilutions were performed in a 96-well plate to achieve the desired antimicrobial concentrations (50 µg/mL, 25 µg/mL, 12 µg/mL, 6 µg/mL and 0 µg/mL) with a volume of 100 µL per well. All tests were performed in triplicate. At this point, 100 µL of bacteria, previously diluted according to the initial OD values obtained from spectrophotometric measurements, was added to each well. *S. mutans* was inoculated into the plate at a density of 1×10^6 CFU/ml. The plate was then incubated at 37 °C. After 24 hours, the bacterial growth inhibition values were evaluated using a spectrophotometer at a wavelength of 600 nm. Growth was monitored using a Tecan Infinite 200 Pro microplate reader with measurements at 600 nm, and growth curves were used to compare the effects of the different compounds.

5.2.8 Isolation of pure compounds from leaf surface extract of *Lavandula pubescens*

5.2.8.1 Silica Gel Column Chromatography

The extract was first dissolved in approximately 20 ml CHCl₃: *n*-hexane 1:1 and centrifuged for 10 min at 3000 rpm. The supernatant was removed and dried, obtaining 3.6 g of residue soluble in the mixture used. The residue thus obtained was subsequently separated by adsorption chromatography on silica gel (approximately 200 g), using as mobile phase: CHCl₃: *n*-hexane 1:1, CHCl₃: *n*-hexane 7:3, CHCl₃: *n*-hexane 9:1, CHCl₃, CHCl₃: MeOH 99:1, CHCl₃: MeOH 98:2, CHCl₃: MeOH 95:5, CHCl₃: MeOH 9:1, CHCl₃: MeOH 1:1, MeOH. The eluate was collected in 22 mL tubes, for a total of 300 fractions, concentrated using a rotary evaporator and subjected to spot test analysis on TLC, using mixtures of *n*-hexane: CHCl₃ (7:3, 1:1), CHCl₃, CHCl₃: MeOH (99:1, 98:2, 95:5, 9:1) as mobile phase. Detection was performed by UV analysis at wavelengths of 254 and 366 nm and subsequently using a spray reagent consisting of a saturated solution of cerium sulfate in 65% sulfuric acid, diluted to 5% with ethanol, followed by heating at 120 °C for 15 minutes. Based on the similarities revealed by TLC, the fractions were grouped into 26 main fractions (A₃-Z₃).

Fraction A₃ (800 mg) was submitted to RP HPLC with MeOH–H₂O (70:30) as eluent to yield compounds (**29**) (1.1 mg, R_t 30 min). An aliquot (117 mg) of fractions of B₃, C₃, and D₃ was separated by RP HPLC eluting with MeOH–H₂O (60%) to give compounds (**41**) (0.9 mg, R_t 30 min). Fraction E₃ (141.5 mg) was separated by RP HPLC eluting with MeOH–H₂O (60:40) to give compounds (**39**) (1 mg, R_t 27 min) and (**40**) (0.5 mg, R_t 42 min). Fraction F₃ (175 mg)

was submitted to RP HPLC with MeOH–H₂O (65:25) as eluent to yield compounds (**38**) (0.8 mg, R_t 44 min). The pooling of fractions G₃ and H₃ afforded a subfraction that, after separation by RP HPLC with MeOH–H₂O (65:25), lead to purification of compound (**30**) (0.8 mg, R_t 28 min) and (**37**) (0.8 mg, R_t 164 min).

Compound 29: white amorphous powder; ¹H and ¹³C NMR (MeOD-d₄), see Table 8; HRESIMS *m/z* 303.2316 [M + H]⁺ (calcd for C₂₀H₃₁O₂, 303.2318).

Compound 30: White amorphous powder; ¹H and ¹³C NMR (MeOD-d₄): see Table 8; HRESIMS *m/z* 359.2205 [M + Na]⁺ (calcd for C₃₀H₃₂NaO₄, 359.2192).

5.2.8.2 Flash Chromatography (Biotage® Selekt)

Part of surface extract (**7.0 g**) was subjected to flash column chromatography by the Biotage Selekt automated flash chromatography system (Biotage Sweden AB, Uppsala, Sweden). The extract was loaded onto a 10 g silica gel samplet and then separated using a Biotage® Sfär HC Duo 50 g eluting with *n*-hexane, followed by increasing concentrations of CHCl₃ in *n*-hexane (between 10% and 100%) and MeOH in CHCl₃ (between 1% and 100%) at a flow rate of 100 mL/min. A total of 185 fractions of 22 mL were collected and pooled together after screening by TLC into 18 fractions (A₄–R₄).

Fraction A₄ (767mg) was submitted to RP HPLC with MeOH–H₂O (7:3) as eluent to yield compounds (**34**) (1.1 mg, R_t 114 min), (**35**) (1.1mg, R_t 68 min), (**14**) (1.2 mg, R_t 26 min), (**31**) (0.9 mg, R_t 80 min). Then fraction B₄ was separated by RP HPLC with MeOH–H₂O (65:25), lead to purification of compound (**32**) (0.4 mg, R_t 124 min), (**33**) (0.5 mg, t_R 75min), and (**31**) (0.8 mg, R_t 206 min). An aliquot (326 mg) of fraction C₄ was separated by RP HPLC eluting with MeOH–H₂O (65:25) to give compounds (**33**) (1.0 mg, R_t 75 min). Fraction D₄ (326 mg) was separated by RP HPLC eluting with MeOH–H₂O (65:25) to give compounds (**14**) (1.6. mg, R_t 60 min), (**37**) (0.6 mg, R_t 185 min), (**36**) (1 mg, R_t 228min). Fraction E₄ (223 mg) was separated by RP HPLC eluting with MeOH–H₂O (65:25) to give compounds (**14**) (0.9 mg, R_t 57 min), (**36**) (1.0 mg, R_t 248 min). Fraction F₄ (71.2 mg) was submitted to RP HPLC with MeOH–H₂O (65:25) as eluent to yield compounds (**33**) (0.8 mg, R_t 70 min).

Compound 31: White amorphous powder; ¹H and ¹³C NMR (MeOD-d₄): see Table 9; HRESIMS *m/z* 343.2245 [M + Na]⁺ (calcd for C₂₀H₃₂NaO₃, 325.2143).

Compound 32: White amorphous powder; ^1H and ^{13}C NMR (MeOD- d_4): see Table 9; HRESIMS m/z 359.2181 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{20}\text{H}_{32}\text{NaO}_4$, 359.2192).

Compound 33: White amorphous powder; ^1H and ^{13}C NMR (MeOD- d_4): see Table 9; HRESIMS m/z 359.2197 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{20}\text{H}_{32}\text{NaO}_4$, 359.2192).

Compound 34: White amorphous powder; ^1H and ^{13}C NMR (MeOD- d_4): see Table 10; HRESIMS m/z 359.2583 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{21}\text{H}_{36}\text{O}_3$, 359.2556).

Compound 35: White amorphous powder; ^1H and ^{13}C NMR (MeOD- d_4): see Table 10; HRESIMS m/z 361.2376 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{20}\text{H}_{34}\text{O}_4$, 361.2349).

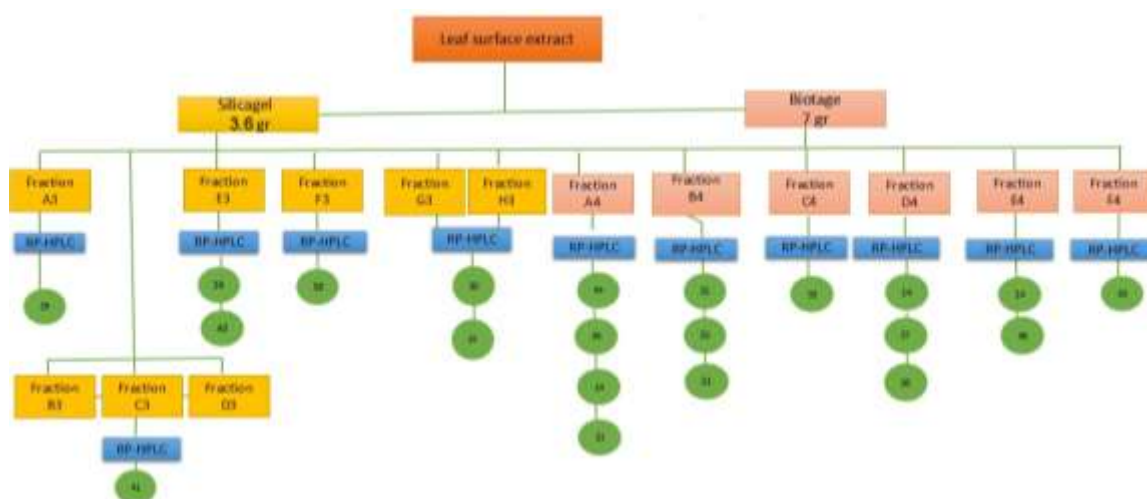


Figure 3. Isolation of pure compounds from leaf surface extract of *Lavandula pubescens* Decne

5.2.9 Cell Culture.

THP-1 (human acute monocytic leukemia cells) were cultured in RPMI 1640 medium supplemented with 10% FBS, 50 U/mL penicillin, 50 $\mu\text{g}/\text{mL}$ streptomycin, 2 mM-glutamine, and 1mM sodium pyruvate, at 37°C in a humidified atmosphere of 5% CO_2 . Before each experiment, THP-1 cells were differentiated in macrophage-like cells by treatment with 100 nM phorbol-12 myristate-13-acetate (PMA) for 24h. After, the medium with PMA was removed and fresh medium was added for another 24h. Stock solutions of extracts, fractions and compounds in DMSO were stored in the dark at 4°C . Appropriate dilutions

were prepared in the culture medium immediately before use. In all experiments, the final concentration of DMSO did not exceed 0.1% (v/v).

5.2.10 Cytotoxicity effects

THP-1 (human acute monocytic leukemia) were plated in 96-well plates at a cell density of 5×10^5 cells/well and differentiated in macrophage-like cells as above with 100 nM PMA for 24 h. The differentiated THP-1 cells, after 24 h, were incubated for 24 h in the presence of *L. pubescens* extracts and Leaf surface extract (6.25 to 0 $\mu\text{g/mL}$), fractions from Leaf surface extract (25 $\mu\text{g/mL}$), compounds **14** and **36** from fractions D₄ and E₄ (3.25 to 50 $\mu\text{g/mL}$), or DMSO alone. The number of viable cells was quantified by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. Absorption at 550 nm for each well was assessed using Multiskan GO (Thermo Scientific, Waltham, MS, USA). The viability (% of control) was obtained using the following formula:

$$\% \text{Viability} = (\text{mean OD treated well}) / (\text{mean OD control well}) \times 100$$

The experiments were performed in technical triplicates.

5.2.11 Enzyme-Linked Immuno-Sorbent Assay (ELISA).

ELISA kits for IL-6 detection were from Diaclone Company (France). THP-1 cells were plated in 12-well plates at a cell density of 6×10^5 cells/well and differentiated in macrophage-like cells as above with 100 nM PMA for 24 h. The THP-1-activated macrophages were incubated for 3 h with LPS (100 ng/mL) and then incubated for 6 h with *L. pubescens* extracts and Leaf surface extract (25, 50, and 100 $\mu\text{g/mL}$), fractions D₄ and E₄ from Leaf surface extract, pure compounds **14** and **36** from fractions D₄ and E₄ (3.125 to 50 $\mu\text{g/mL}$) or DMSO alone. The supernatants were centrifuged and stored at -20°C until testing by ELISA. The procedures for measuring cytokine IL-6 levels followed the manufacturer's instructions. The values were normalized to the LPS sample and reported as percentages in three technical triplicates.

5.3a Results and discussion

5.3.1 LC-MS profiling of *L. pubescens* *n*-hexane, chloroform and leaf surface extracts

A preliminary UHPLC-HRESI-MS analysis in positive ion mode was carried out on the *n*-hexane (A) and chloroform (B) residues, as well as on the leaf-surface extract (C), with the aim of obtaining an initial overview of the chemical constituents present. Examination of the mass spectra (Image 1) revealed a cluster of ions with m/z values ranging from 301.21 to 361.22. These ions could be assigned to molecular formulas of the general type $C_{20+n}H_{28+n}O_{2+n}$, a pattern typically associated with polyoxygenated diterpenes. The presence of these signals in all extracts, non-polar (*n*-hexane), medium polar (chloroform) and epicuticular wash, suggests that this class of molecules is widely distributed in *L. pubescens* tissues and is present both in the inner tissues and on the leaf surface. Furthermore, the detection of multiple congeners that differ by small mass increments indicates the presence of a diverse range of structurally similar compounds, which may include those with varying degrees of oxidation or functional group substitution. LC-MS annotation of diterpenes is poorly represented in the literature, and the apparent chemical complexity and potential bioactivity of these components highlighted the need for a comprehensive phytochemical investigation. A detailed isolation and structural characterisation study was therefore undertaken to obtain pure diterpenes from *L. pubescens*.

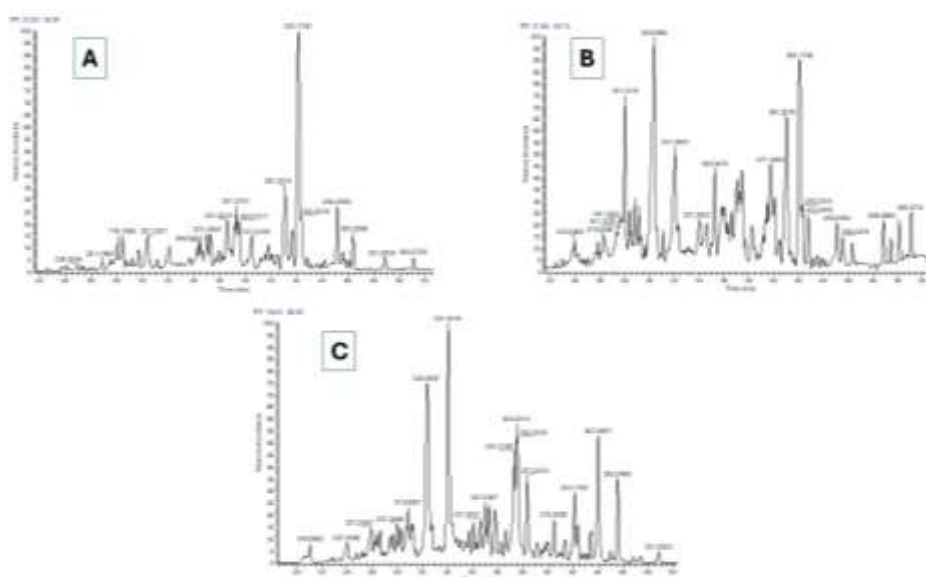


Image 1. UHPLC-HRESI-MS profiles of *n*-hexane (A) and chloroform (B) and leaf surface (C) extracts from *L. pubescens* aerial parts. Spectra were acquired in positive ion mode, main *m/z* values are reported on the top of the peaks.

5.3.2a Isolation and characterization of pure compounds from *n*-hexane and chloroform extract of *Lavandula pubescens* Decne

The phytochemical study of *L. pubescens* aerial parts led to the isolation of nine new diterpenes (**1-9**), and six new dimeric monoterpenes (**15-20**), together with five known diterpenes: ent-8(14)-pimarene-15R,16-diol (**10**) (Han et al., 2005); 15,16dihydro, 15-hydroxy, 14 α , 16epoxyisopimar8(9)ene (**11**) (Seca et al., 2005); 15(S)-isopimar-7-en-15,16-diol (**12**) (Subrahmanyam et al., 1999); ent-8(9)-pimaren-15,16-diol (**13**) (Zhao et al., 1988); 15S,16-dihydroxy-7-oxopimar-8(9)-ene (**14**) (Politi et al., 2002). Furthermore, one known monoterpene, carvacrol (**21**) (Shafiee et al., 1997), and seven known triterpenes, ursolic acid (**22**) (Leal et al., 2012); oleanolic acid (**23**) (Melo et al., 2016); hyptadienic acid (**24**) (Rao et al., 1990); rotundic acid (**25**) (Le et al., 1990); 1 β ,2 α ,3 β ,19 α ,23-pentahydroxy-urs-12-en-28-oic acid (**26**) (Abdel et al., 2020; Guang-Yi, Gray & Waterman, 1989); arjunolic acid (**27**) (Le et al., 2025); tormentic acid (**28**) (Olech, 2021) were also isolated and identified.

Compound **1** was isolated as an amorphous powder and yielded a molecular ion peak in the positive HRESIMS spectrum at *m/z* 343.2255 [M+H]⁺, consistent with a molecular formula of C₂₀H₃₂O₃, and equating to five double bond equivalents (Fig. 4). Immediately identifiable from NMR spectroscopic data for **1** (Table 1) were resonances consistent with one tetrasubstituted double bond (δ_C 142.8 and 155.0), as well as one α,β unsaturated carbonyl carbon (δ_C 200.6). In the absence of any other sp or sp² carbon, the structure of **1** must be tricyclic. The ¹H NMR spectrum (Table 1) of **1** exhibited four methyl singlets at δ 0.91, 0.93, 0.95, 1.21, a carbinol methine signal at δ_H 3.36 (1H, *d*, *J* = 9.5 Hz, H-15) coupled with a carbinol methylene at δ_H 3.67 (1H, *dd*, *J* = 11.0 and 2.5 Hz) and 3.45 (1H, *dd*, *J* = 11.0 and 9.0 Hz), H-16. The carbonyl resonance at C-11 was assigned one the base of HMBC correlations between H-12 (δ_H 2.00) and C-11. The HMBC correlations between H-12 (δ_H 2.00) and C-9, C-8, C-14 and between H-14 (δ_H 1.99) and C-7, C-9, C-13, C-17, C-15 supported the presence of an α,β -unsaturated carbonyl moiety at C-11 (Politi et al., 2002). Results obtained from 1D-TOCSY and COSY experiments established the correlations of protons H-1–H-3, H-5–H-7, H-15–H-16. HSQC and HMBC experiments provided unambiguous assignments of all the proton and carbon resonances. Long-range

correlations were observed between Me-18 and Me-19, C-3, C-5; between Me-20 and C-1, C-9, C-5; between Me-17 and C-12, C-14 and C-15. The relative stereochemistry of **1** was determined through analysis of proton coupling constants and correlations observed in the ROESY spectrum. The coupling constant observed for H-5, $J = 13.0$ and 2.5 Hz suggested that H-5 was axial, which was further confirmed by ROE correlations between H-5 and Me-19. Thus, compound **1** was defined as 15,16-dihydroxy-*ent*-pimar-8(9)en-11 one.

The molecular formula of **2** was determined as $C_{20}H_{34}O_3$ by HRMS at m/z 345.2394 $[M+Na]^+$ as well as from its ^{13}C NMR data (Fig. 4). The ^1H NMR, 1D TOCSY and COSY spectra of **2** revealed the presence of four methyl singlets (δ 0.84, 0.90, 0.94, 0.99), an exocyclic diol group (δ 3.50, 3.49, 3.72), and a carbinol proton at δ 3.82 (1H, br d $J = 3.5$ Hz). This signal indicated the presence of a C-7 hydroxyl group, a conclusion that was further supported by ^{13}C NMR data (Table 1) (Seca et al., 2005). The 7 β - OH substitution was evident from the chemical shifts and J value of the proton assigned to C-7 (Table1). All the observed data can be accommodated only on an *ent*-pimarene diterpenoid structure for compound **2** with two secondary hydroxyl groups at C-7, and C-15 and one hydroxymethylene at C-16 position (Politi et al., 2002). The HSQC and HMBC spectra of **2** confirmed the above deductions. Consequently, the new compound **2** can be formulated as 7 β ,15,16-trihydroxy-8,9-*ent*-pimarene.

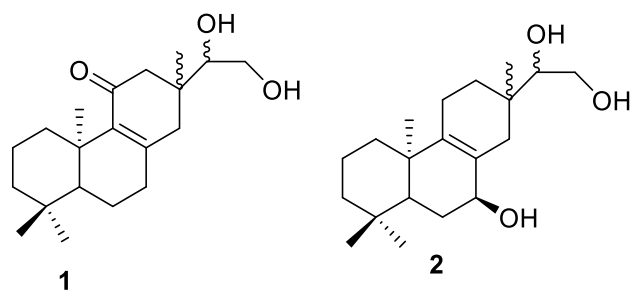


Figure 4. Chemical structure of compounds **1-2**

Table 1. ^1H and ^{13}C NMR spectroscopic data of compounds **1** and **2**

position	1		2	
	δ_C , type	δ_H	δ_C , type	δ_H
1	37.2, CH_2	2.74 br dd (11.5, 5.0) m 0.95 m	37.9, CH_2	1.81 ^b 1.14 ddd (15.0, 13.0, 3.0)
2	20.5, CH_2	1.70 ^b 1.47 ^b	20.0, CH_2	1.69 m 1.53 ^b
3	42.9, CH_2	1.45 ^b 1.21 ^b	42.9, CH_2	1.51 ^b 1.24 ddd (16.0, 14.0, 4.0) ^a

4	30.8, C	-	33.9, C	-
5	53.5, CH	1.14 dd (13.0, 2.5)	47.0, CH	1.54 ^b
6	19.8, CH ₂	1.79 ^b	30.0, CH ₂	1.81 ^b
		1.50 ^b		1.75 dd (13.5, 5.0)
7	35.4, CH ₂	2.29 ^b	70.4, CH	3.82 br d (3.5)
		2.29 ^b		
8	155.0, C	-	127.3, C	-
9	142.8, C	-	143.0, C	-
10	37.1, C	-	35.9 C	-
11	200.6,	-	21.2, CH ₂	2.09 m
				2.02 m
12	50.3, CH ₂	2.65 ^b	33.0, CH ₂	1.59 m
		2.00 ^b		1.35 m
13	38.7, C	-	30.8, C	-
14	41.4, CH ₂	2.65 ^b	39.1, CH ₂	2.53 d (16.0)
		1.99 ^b		1.53 ^b
15	79.5, CH	3.37 ^b	77.1, CH	3.50 ^b
16	63.5, CH ₂	3.67 dd (11.0, 2.5)	63.9, CH ₂	3.72 d (9.0)
		3.45 dd (11.0, 9.0)		3.49 ^b
17	20.0, CH ₃	0.95 s	20.9, CH ₃	0.84 s
18	34.0, CH ₃	0.93 s	33.6, CH ₃	0.94 s
19	22.2, CH ₃	0.91 s	22.1, CH ₃	0.90 s
20	20.0, CH ₃	1.21 s	18.7, CH ₃	0.99 s

^a Spectra were recorded in methanol-d₄, at 600 MHz (¹H) and 150 MHz (¹³C); chemical shifts are given in ppm; J values are in parentheses and reported in Hz; assignments were confirmed by COSY, 1D-TOCSY and HSQC experiments. ^b Overlapped signal.

Compound **3** had molecular weight 323.1993 [M+Na⁺] and molecular formula C₂₀H₂₈O₂, derived from HRMS and ¹³C NMR analyses. The ¹H NMR data displayed signals attributable to three tertiary methyl groups (δ_H 1.29, 1.07, 0.99), one secondary methyl group (δ_H 1.30), and three protons (δ_H 7.43, 7.51, 7.84) (Table 2) corresponding to a 1,3,4-trisubstituted aromatic ring. The ¹³C NMR spectrum suggested that **3** is a diterpene derivative, consisting of four methyls (δ_C 18.2, 21.7, 23.7, 33.0), five methylenes (δ_C 19.9, 30.8, 39.1, 42.5, 68.8), five methines (δ_C 43.2, 50.8, 125.3, 126.7, 135.1), two sp³ quaternary carbons (δ_C 34.2, 37.3), three sp² quaternary carbons (δ_C 139.1, 156.1, 143.9), and one carbonyl carbon (δ_C 200.8) (Table 2). Analysis of the COSY, 1D-TOCSY and HSQC spectra revealed the presence of four molecular fragments, H₂-1-H₂-3, H-5-H₂-6; H-11-H-14, H-15-H-17, three-carbon chain fragment, C-16- C-15-C-17, in which C-16 is a hydroxy methylene carbon and C- 17 is a secondary methyl group suggesting the presence of a hydroxy-iso-propyl moiety. The connections among the carbon-chain fragments, isolated methyl, methine groups, and the quaternary carbons, including a carbonyl carbon, were established based on HMBC correlations. The observed cross-peaks from H-1 to C-5, C-10, and C-20, and from H-6 to C-

7, and H-14 to C-7, indicated that the carbonyl group was located at C-7. Furthermore, the correlations from H-11 to C-8 and C-14, from H-12 to C-9 and C-13, and from H-14 to C-7, C-9, and C-12 suggested that C-8, C-9, and C-11–C-14 form an aromatic ring. In addition, the correlations observed from H-16 and Me-17 to C-13 supported the attachment of C-15 to the aromatic ring. Taken together, these findings indicate that compound **3** possesses an abietan- type skeleton characterized by a 1,3,4-trisubstituted aromatic ring. The NOESY spectrum provided information about the stereochemistry of this diterpenoid: H-5 signals show NOE correlations with Me-19 (Xiong, 2016). Thus, the complete assignment of the NMR spectra and ECD data, reported in Appendix 1, allowed the elucidation of the absolute stereochemistry of compound **3**. Accordingly, the structure of **3** was defined as (5*S*, 10*S*, 15*S*) 16-hydroxy-8,11,13 -abietatrien-7-one (Fig. 5).

Compound **4** showed a molecular formula $C_{20}H_{30}O_2$, derived from HRMS and ^{13}C NMR analyses. Comparison of the NMR data of **4** with those of **3** indicated that **4** is abietane derivative (Yahara et al., 1990). In particular, protons and carbons due to rings A, and C resonated at almost the same frequencies as the corresponding signals in **3**, while ring B NMR signals were observed at different positions. The major difference in the ^1H NMR spectra of **4** was the presence of the signals for the hydroxymethine group and the absence of the signals for the carbonyl group (Table 2). Furthermore, NOE correlations between Me-19 and H-5 showed an abietane skeleton. The analysis of chemical shifts showed close similarity with those of compound **3**, suggesting the same stereochemistry. These data, together with MS data were consistent with **4** being 7*S*,16-dihydroxy-8,11,13-abietatriene (Fig. 5).

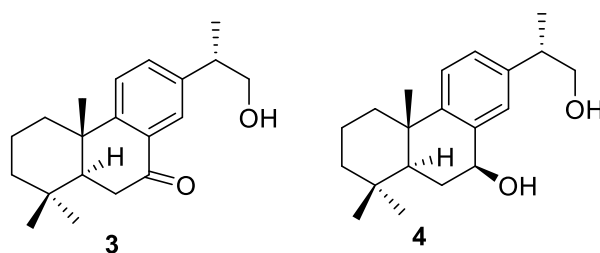


Figure 5. Chemical structure of compounds **3-4**

Table 2. ^1H and ^{13}C NMR spectroscopic data of compounds **3** and **4**

position	3		4	
	δ_C , type	δ_H	δ_C , type	δ_H

1	39.1, CH ₂	2.45 m	39.8, CH ₂	2.32 m
		1.54 ^b		1.38 ^b
2	19.9, CH ₂	1.88 ^b	20.4, CH ₂	1.85 ^b
		1.73 m		1.63 m
3	42.5, CH ₂	1.58 ^b	42.8, CH ₂	1.52 m
		1.34 m		1.31 ^b
4	34.2, C	-	33.6, C	-
5	50.8, CH	1.88 ^b	45.6, CH	1.78 dd (11.0, 4.0)
6	30.8, CH ₂	2.71 dd (12.0, 8.0)	29.9, CH ₂	1.99 ^b
		2.73 dd (14.0, 12.0)		1.99 ^b
7	200.8, C	-	68.8, CH	4.76 br t (2.5)
8	139.1, C	-	135.2, C	-
9	156.10, C	-	148.9, C	-
10	37.3, C	-	39.1 C	-
11	125.3, CH	7.43 d (8.3)	125.4, CH	7.23 d (8.0)
12	135.1, CH	7.51 dd (8.3, 2.0)	128.1, CH	7.10 dd (8.0, 2.0)
13	143.9, C	-	144.4, C	-
14	126.7, C	7.85 d(2.0)	130.5, C	7.19 d (2.0)
15	43.2, CH	2.94 m	43.2, C	2.86 m
16	68.8, CH ₂	3.67 dd (11.0, 7.0)	69.1, CH ₂	3.68 dd (11.0, 7.0)
		3.62 dd (11.0, 7.0)		3.67 dd (11.0, 7.5)
17	23.7, CH ₃	1.30 d (6.5)	18.3, CH ₃	
18	33.0, CH ₃	0.99 s	33.9, CH ₃	1.00 s
19	21.7, CH ₃	1.07 s	22.1, CH ₃	0.98 s
20	18.2, CH ₃	1.29 s	24.4, CH ₃	1.15 s

^a Spectra were recorded in methanol-*d*₄, at 600 MHz (¹H) and 150 MHz (¹³C); chemical shifts are given in ppm; J values are in parentheses and reported in Hz; assignments were confirmed by COSY, 1D-TOCSY and HSQC experiments. ^b Overlapped signal.

Compound **5** exhibited a molecular ion peak at *m/z* 345.2403 [M+Na]⁺ (calcd. 345.2400), consistent with the molecular formula C₂₀H₃₄O₃ as determined by HRMS and supported by ¹³C NMR data. The NMR characteristics were similar to those of darutigenol (Wang et al. 2009) suggesting that **5** was a trihydroxy-*ent*-pimarane diterpene. Owing to the presence of an oxygenated quaternary carbon at δ_C 85.9 (s), this hydroxy group should be located at C-5 or C-9 in **5**. In the HMBC spectrum, the correlations from the Me-20 (3H, δ_H 0.98, s) protons to the carbons at δ_C 46.4 (C-5), 33.2 (C-1), 85.9 (C-9), and from H-14 and 34.5 (C-7), 81.5 (C-15), 29.7 (C-12), and 85.9 (C-9) were observed, which allowed us to locate the hydroxy group at C-9 (Table. 3). In the ¹H NMR spectrum, the H-5 signal at δ_H 1.96 showed a remarkable downfield chemical shift (Δ ≈ 0.8 ppm) in comparison with that of pimar-8(14)-15,16 diol (**10**), indicative of the β-orientation of the C-9 hydroxy group (Han et al., 2005). NOE correlations between H-5 and Me-18; Me-20 and Me-19 confirmed the *ent*-pimarane skeleton. Accordingly, the structure of **5** was established as *ent*-8(14)-pimarane-9,15,16-triol (Fig. 6).

The molecular formula $C_{20}H_{34}O_4$ of compound **6** was determined from the sodiated molecular ion at m/z 361.2341 $[M + Na]^+$ in its HRESIMS (Fig. 6). In the HRESIMS/MS fragment at m/z 343.22 $[M + Na - 18]^+$, due to the loss of one water molecule was observed. The ^1H NMR spectrum (Table 3) exhibited the presence of four tertiary methyl groups at δ_H 0.91, 0.96, 0.97, and 1.04, three hydroxymethines at δ 3.09 (br s), 3.89 (br d, $J = 4.5$ Hz), and 4.35 (br s), and one hydroxymethylene at δ_H 3.45 (br d, $J = 13.0$ Hz) and 3.85 (dd, $J = 13.0$ and 1.5 Hz). The ^{13}C NMR spectrum (Table 3), showing signals attributable to four methyls, seven methylenes (one oxygenated), four methines (three oxygenated), two olefinic quaternary carbons, three quaternary carbons, provided the evidence of a diterpene skeleton (Politi et al., 2002). The connectivity between each proton signal and its corresponding carbon was determined through an HSQC experiment, while 1D TOCSY and COSY experiments were used to establish the correlations among all protons in the molecule. The HMBC cross peaks showing correlations between, Me-20/C-9, Me-20/C-5, Me-20/C-1, H-14/C-7, H-14/C-9, H-14/C-15, H-14/C-17, H-5/Me-20, H-11/Me-20, H-11/C-12, H-15/C-14, H-15/C-17 helped in the location of the three hydroxy groups at C-7, C-11 and C-15, respectively. The presence of a C-16-hydroxyl was determined by HMBC cross peaks between H₂-16-C-13, while the double bond was positioned at C-8, C-9 by correlations between H₂-6-C-8, H₂-14-C-9, Me-20-C-9. Extensive analysis of NMR data and ROESY correlations were used to establish the relative configuration of **6**. The coupling constant of H-7 (δ_H 3.89, br d, $J = 4.5$ Hz) indicated that the OH-7 group was β -oriented, and the β -orientation of OH-11 was established basing on the multiplicities of the H-11 proton signal (δ_H 4.35, br s) (González, M. A., 2016; Yahara et al., 1990). Moreover compound **6** had ROE correlations between H-5 with Me-18 and H-11 with Me-17. Thus, the structure of 7 β ,11 β ,15,16-tetrahydroxy-*ent*-pimar-8(9)-ene was assigned to compound **6** (Fig. 6).

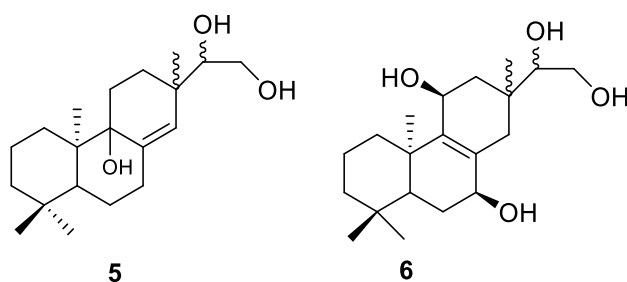


Figure 6. Chemical structure of compounds **5-6**

Table 3. ^1H and ^{13}C NMR spectroscopic data of compounds **5** and **6**

position	5		6	
	δ_{C} , type	δ_{H}	δ_{C} , type	δ_{H}
1	33.2, CH ₂	1.72 ddd (16.0, 11.5, 3.0) 1.45 ^b	36.5, CH ₂	1.81 ^b 1.29 ^b
2	19.8, CH ₂	1.56 ^b 1.46 ^b	19.7, CH ₂	1.68 m 1.52 m
3	42.9, CH ₂	1.39 ^b 1.19 ^b	42.9, CH ₂	1.47 m 1.26m
4	34.3, C	-	33.7, C	-
5	46.4, CH	1.96 ^b	46.9, CH	1.54 dd (13.0, 2.0)
6	23.9, CH ₂	1.62 ^b 1.35 ^b	29.7, CH ₂	1.83 ^b 1.77 ddd (17.0, 13.0, 4.0)
7	34.5, CH ₂	2.51 dddd (15.0, 7.0, 5.0, 2.0) 2.11m	69.3, CH	3.89 br d (4.5)
8	136.6, C	-	135.4, C	-
9	85.9, C	-	138.7, C	-
10	44.03, C	-	39.2, C	-
11	26.0, CH ₂	1.91 ^b 1.89 ^b	67.7, CH	4.35 br s
12	29.7, CH ₂	1.96 ^b 1.37 ^b	34.7, CH ₂	2.16 dd (12.5, 4.5) 1.02 ^b
13	38.6, C	-	32.5, C	-
14	134.3, CH	5.64 s	41.3, CH ₂	2.32 d (18.0) 1.85 ^b
15	81.5, CH	3.37 ^b	74.7, CH	3.09 br s
16	64.5, CH ₂	3.71 dd (11.5, 2.5) 3.46 dd (11.5, 9.0)	65.3, CH ₂	3.85 dd (13.0, 1.5) 3.45 br d (13.0)
17	22.0, CH ₃	1.01 s	27.4, CH ₃	1.04 s
18	33.7, CH ₃	0.92 s	33.4, CH ₃	0.97 s
19	22.7 CH ₃	0.89 s	22.0 CH ₃	0.91 s
20	19.2 CH ₃	0.98 s	18.8 CH ₃	0.96 s

^a Spectra were recorded in methanol-*d*₄, at 600 MHz (^1H) and 150 MHz (^{13}C); chemical shifts are given in ppm; *J* values are in parentheses and in Hz; assignments were confirmed by COSY, 1D-TOCSY and HSQC experiments. ^b Overlapped signal.

The NMR data of compound **7** were quite similar to those of **6** (Table 3), the main differences being in the resonances of H₂-12, H₂-14, H-15, Me-17, and C-12, C-14, C-15 and Me-17. This suggested the presence of a C-13 epimer. Thus, the structure of 7 β ,11 β ,15,16-tetrahydroxy-*ent*-pimar-8(9)-en was assigned to compound **7** (Fig.7).

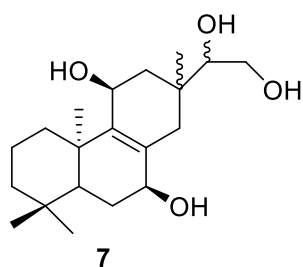


Figure 7. Chemical structure of compounds 7

The molecular formula of compound **8** was established to be $C_{19}H_{32}O_3$ (m/z 331.2257 $[M+Na]^+$ (calcd. 331.2243) in the HRESIMS). The ^1H NMR spectrum of **8** displayed signals for four methyl groups δ_H 0.76 (Me-19), 0.91 (Me-18), 1.01 (Me-17), and 1.03 (Me-16) (Table. 4). (Habtemariam et al., 1990). COSY correlations were obtained between the signal at δ_H 2.25 (H-9) and H₂-11, and H₂-12, and between the signals at δ_H 3.57 and 3.70 (H₂-15), and H-14 (δ_H 2.76). The other connectivities of the diterpenoid skeleton and the other functional groups was established from its COSY and HSQC spectra. HMBC correlations between the signals at δ_H 2.37, 2.44 (H₆-7) and δ_H 2.25 (H-9) with carbon resonance at δ_C 213.5 (C-8) confirmed the presence of a keto group at C-8, while cross-peaks between Me-16 (δ_H 1.04) and C-13 (δ_C 214.6) located the second carbonyl at C-13 (Table 4). The relative configuration of **8** was determined by studying ^1H NMR coupling constants and NOESY correlations. The NOESY correlations between H-5 Me-18 made it possible to hypothesize the relative stereochemistry of bicyclic structure of **8** as an *ent*-labdane. Thus, the structure of **8** could be proposed as 15-hydroxy-17-nor-labdan-8,13-dione (Fig. 8).

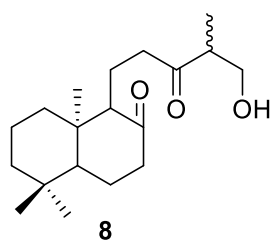


Figure 8. Chemical structure of compounds 8

The molecular formula of **9** was established as $C_{20}H_{32}O_3$ based on the HREIMS molecular peak at m/z 343.2253 (calculated value: 343.2243), which indicates five degrees of unsaturation (Fig.9).

The ^1H NMR spectrum of **9** (Table 4) showed one olefinic methine at δ_{H} 6.70 (s), two terminal vinyl protons at δ_{H} 4.63 (br s), 4.82 (br s), one oxygenated methine at δ_{H} 3.54 (dd, $J=9.0, 3.8$ Hz), one hydroxymethylene δ_{H} 3.55 (dd, $J=8.0, 11.0$ Hz), and 3.70 (dd, $J=11.0, 3.0$ Hz), one methine, seven methylene groups, three methyl signals at δ_{H} 0.84, 0.95, 1.21.

The ^{13}C NMR spectrum (Table 4) displayed 20 carbon resonances, including one carbonyl carbon (δ_{C} 199.9), four olefinic carbons (δ_{C} 109.7, 141.7, 150.4, and 153.7), one oxygenated methine (δ_{C} 79.5), one hydroxymethylene (δ_{C} 64.2), three methyl groups (δ_{C} 21.1, 24.8, and 33.8), along with two quaternary carbons, seven methylenes, and one additional methine carbon. Spectroscopic evidence indicated that compound **9** features a B-seco-ring-pimarane skeleton (Tanaka et al., 2004). Interpretation of the HSQC and COSY spectra revealed that compound **9** was composed of four distinct partial structures: a C-1–C-3 fragment-A, a C-5–C-7 fragment-B, a C-11–C-12 fragment-C, and a C-15–C-17 fragment-D. The HMBC spectrum showed correlations from H-1 to C-10, from H₂-20 to C-1, H₂-20 to C-5, from H₃-18 to C-3, C-4, C-5 and C-19, and from H₃-19 to C-3, C-4, C-5 and C-18. These mentioned HMBC correlations established connectivity of partial structures A and B. Furthermore, the HMBC spectrum showed correlations from H-14 to C-9, C-7 and C-8, from Me-17 to C-12, C-13 C-14 and C-15 establishing the connectivity from the partial structure C to the other partially structure. NOE correlations were observed between Me-18 and H-5 suggesting that are located on the same face of the molecules. These data accounted that **9** had unusual structure of *seco*-pimarane type diterpenoid. Thus **9** was established as 15,16-dihydroxy-9,10- *seco*-pimara-8(14),10(20)-dien, 9-one (Fig. 9).

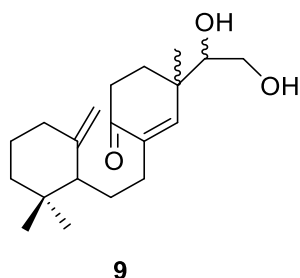


Figure 9. Chemical structure of compounds **9**

Table 4. ^1H and ^{13}C NMR spectroscopic data of compounds **7**, **8** and **9**

position	7		8		9	
	δ_{C} , type	δ_{H}	δ_{C} , type	δ_{H}	δ_{C} , type	δ_{H}
1	37.2, CH ₂	1.90 m 1.53 ^b	39.8, CH ₂	1.84 ^m 1.85 m	33.8, CH ₂	2.18 ^b 2.05 ^b
2	19.9, CH ₂	1.72 m 1.56 ^b	19.7, CH ₂	1.65 ^b 1.56 ^b	28.9, CH ₂	1.56 ^b 1.56 ^b
3	43.0, CH ₂	1.51 ^b 1.29 ^b	43.1, CH ₂	1.49 ^b 1.33 ^b	37.1, CH ₂	1.51 ^b 1.23 ^b
4	33.8, C	-	33.9, C	-	34.0, C	-
5	46.7, CH	1.62 dd (14.0, 2.0)	55.3, CH	1.61 ^b	54.8, CH	1.76 dd (11.5, 3.5)
6	29.9, CH ₂	1.83 ddd (15.0, 4.0, 2.0) 1.77 ddd (15.0, 12.5, 4.0)	24.0, CH ₂	2.11 m 1.69 ^b	26.7, CH ₂	1.50 ^b 1.50 ^b
7	70.9, CH	3.81 ^b	43.1, CH ₂	2.44 m 2.36 ^b	29.8, CH ₂	2.21 ^b 1.89 m
8	132.5, C	-	213.5, C	-	141.7, C	-
9	145.2, C	-	64.1, CH	2.25 br t (9.5)	199.9, C	-
10	39.5, C	-	40.6, C	-	150.4, C	-
11	64.0, CH	4.33 br t (3.5)	16.8, CH ₂	1.76 ^b 1.65 ^b	35.6, CH ₂	2.53 dd (12.0, 6.0) 2.47 br t (5.0)
12	45.2, CH ₂	1.99 dd (14.0, 2.5) 1.40 dd (14.0, 5.0)	41.5, CH ₂	2.64 m 2.37 m	31.6, CH ₂	2.066 ^b 1.82 m
13	30.8, C	-	214.6, C	-	40.7, C	-
14	37.1, CH ₂	2.65 d (15.0) 1.67 d (15.0)	49.6, CH	2.76 m	153.7, CH	6.70 s
15	79.2, CH	3.60 dd (8.5, 3.0)	64.7, CH ₂	3.57 m 3.70 m	79.5, CH	3.54 dd (8.0, 3.0)
16	64.0, CH ₂	3.84 ^b 3.52 dd (11.0, 8.5)	13.8, CH ₃	1.04 d (6.5)	64.1, CH ₂	3.70 dd (11.0, 3.0) 3.55 dd (11.0, 8.0)
17	26.4, CH ₃	0.90 s	33.6, CH ₃	1.02 s	21.1, CH ₃	1.21 s
18	33.5, CH ₃	0.95 s	22, CH ₃	0.91 s	33.8, CH ₃	0.95 s
19	22.1, CH ₃	0.90 s	14.9, CH ₃	0.76 s	24.8, CH ₃	0.84 s
20	19.2, CH ₃	0.95 s			109.7, CH ₂	4.82 br s 4.63 br s

^a Spectra were recorded in methanol-*d*₄, at 600 MHz (^1H) and 150 MHz (^{13}C); chemical shifts are given in ppm; *J* values are in parentheses and in Hz; assignments were confirmed by COSY, 1D-TOCSY and HSQC experiments. ^b Overlapped signal

Compound **15** was obtained as colorless crystals. Its molecular formula was determined as C₂₀H₃₀O₃ by positive ion HRESI-MS (*m/z* 341.2096 [*M* + Na]⁺ analysis, with six degrees of unsaturation. The [*M*+H]⁺ ion at *m/z* 319.1893 undergoes the sequential loss of two water molecules (*m/z* 283 [*M*+H-18-18]⁺), which is consistent with the presence of multiple hydroxyl groups in the structure. The diagnostic fragment at *m/z* 151.11 corresponds to the carvacrol unit (C₂₀H₁₄O), while the ion at *m/z* 169.11 could correspond to the second

monoterpene moiety after dehydration. Additionally, the fragment at m/z 109 originates from the carvacrol unit following the loss of an isopropyl group (C_3H_6), a characteristic feature of phenolic monoterpenes. Its ^1H NMR spectrum showed a set of distinguishable signals for a carvacrol moiety: three methyl groups at δ_H 1.22 (6H, d, $J=6.5$ Hz), 2.15 (3H, s), a 1,2,5-trisubstituted aromatic ring at δ_H 6.69 (1H, dd, $J = 8.0, 1.5$ Hz), 6.85 (1H, d, $J = 1.5$ Hz), and 7.02 (1H, d, $J= 8.0$ Hz) (Numpaque et al., 2011). In addition to this moiety, three methyl groups, one hydroxymethyne, two methylenes, and a tri-substituted double bond were also observed in the ^1H NMR spectrum of **15** (Table 5). Its ^{13}C NMR spectrum displayed 20 carbon signals (Table 5), indicating that compound **15** could be a carvacrol-based dimer. In fact, the number of resonances exceeds that expected for a single monoterpenoid unit, suggesting the presence of an additional structural fragment. To satisfy the degree of unsaturation required by the molecular formula, this second moiety have adopted a cyclized structure. The planar structure of compound **15** was assembled by analysis of its 2D NMR data. Key HMBC correlations of H-3'/C-1', C-5'; H-4'/C-6', C-8', C-2'; H-6'/C-2', C-4', C-8'; Me-7'/C-2', C-3', C-1', proved the presence of a carvacrol moiety. Similarly, another monoterpene moiety (from C-1 to C-10) was determined by HMBC, HSQC and COSY spectra, C-1, C-2 and C-8 were classified as oxygenated carbons. Moreover, HMBC correlations of Me-7/C-1, C-2, C-6, H-2/C-4, H-3/C-1, C-5, C-8, and Me-9/C-8, C-9 demonstrated the structure of the second monoterpene unit as *p*-menth-3-en-1,2,8 triol. The connectivity between the two monoterpene units was determined by the analysis of ^{13}C NMR data and HMBC correlations (Table 5). The downfield chemical shift of the C-8' signal (δ_C 81.3) and the upfield chemical shift of the C-4 (δ_C 146.1) compared with that of the monoterpene moiety (Brewer, 2011; Becker & Martini, 1999) and the HMBC correlations between Me-9 and C-1' clearly indicated the C-8-O-C-1' linkage of the two units. Therefore, a planar structure of the compound **15** was established (Fig. 10). In the NOESY spectrum of **15**, the cross-peaks of H-2—Me-7 implied that these protons were on the same side of molecules. Therefore compound **15** was established as 8-(carvacryl)-*p*-menth-3-en-1,2,8-triol (Fig. 10).

HRESIMS of compound **16** showed a molecular ion peak at 341.2096 m/z [$M + Na$]⁺ (calcd. 341.2087), implying a molecular formula of $C_{20}H_{30}O_3$ which corresponds to five indices of

hydrogen deficiency (Fig. 10). The fragmentation pattern observed in the mass spectrum of compound **16** showed similarities to those of compound **15**. A sequential loss of two water molecules was observed from the molecular ion at m/z 319 $[M+H]^+$. Moreover, two additional fragment ions were detected at m/z 170.12 $[M+H-C_{10}H_{14}O_2]^+$, and 151.11 $[M+H-C_{10}H_{16}O_2]^+$, both consistent with monoterpenoid units, supporting the hypothesis that compound **16** is a monoterpenoid dimer. Its ^1H NMR (Table 5) showed the presence of four tertiary methyl signals at δ_H 2.21 (3H, s) and 1.37 (3H, s), 1.20 (3H, s) and 1.14 (3H, s); one pair of secondary methyls at δ_H 1.23 (3H, d, $J = 7.0$ Hz), and one methine at δ_H 2.80 (1H, m) indicating the existence of one isopropyl group.

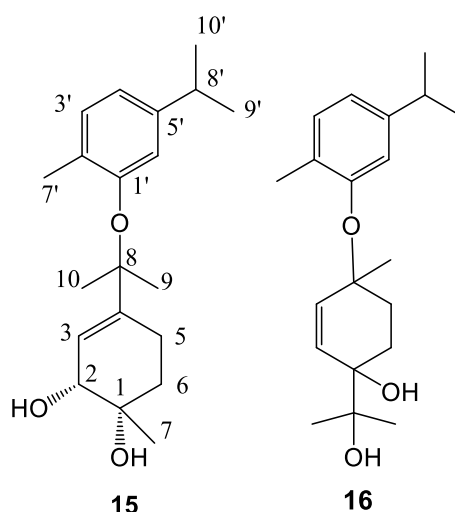


Figure 10. Chemical structure of compounds **15** and **16**

The signals at δ_H 6.03 (1H, dd, $J=11.0, 2.0$) and 5.95 (1H, dd, $J = 11.0, 1.0$ Hz) revealed the presence of one disubstituted olefinic bond. As illustrated in Fig. 11, the *p*-menthene skeletons were concluded based on 1D-TOCSY, COSY as well as HMBC spectra. Considering the molecular weight and the low field of carbon signals at C-1 (δ_C 74.5) and C-1' (δ_C 155.0), we deduced that the two monoterpenoid units were connected by ether linkage (Table 5). The connection between C-1 and C-1' was further supported by HMBC correlations between Me-7 and H-6'. Thus, compound **16** was established as 1-(carvacryl) *p*-menth-2(3)-en-1,4,8-triol (Fig. 10).

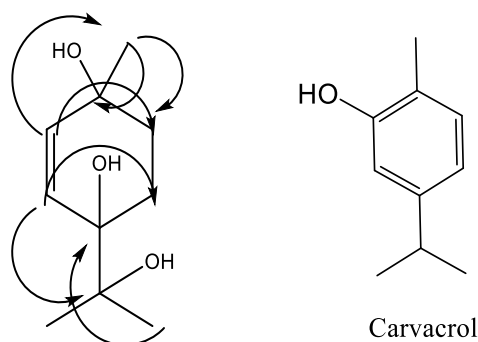


Figure 11. Partial structures of compound **16** and Carvacrol

Table 5. ^1H and ^{13}C NMR spectroscopic data of compounds **15** and **16**

position	15		16	
	δ_{C} , type	δ_{H}	δ_{C} , type	δ_{H}
1	72.5, C	-	74.5, C	-
2	75.1, CH	4.10 dd (4.0, 2.5)	135.0, CH	5.95 dd (11.0, 1.0)
3	125.1, CH	5.64 br s	131.0, CH	6.03 dd (11.0, 2.0)
4	146.1, C	-	74.6, C	-
5	24.1, CH_2	2.34 m	28.2, CH_2	2.13 ^b
6	35.1, CH_2	2.25 m	34.0, CH_2	1.63 m
		1.76 m		2.13 ^b
7	21.5, CH_3	1.71 m	25.0, CH_3	1.98 ^b
		1.23 s		1.37 s
8	81.3, C	-	74.5, C	-
9	27.1, CH_3	1.50 s	22.4, CH_3	1.14 s
10	28.0, CH_3	1.53 s	27.4, CH_3	1.20 s
1'	155.8, C		155.0, C	
2'	127.1, C		122.1, C	
3'	131.3, CH	7.02 d (8.0)	132.0, CH	7.06 d (8.0)
4'	119.8, CH	6.69 dd (8.0, 1.5)	121.8, CH	6.80 dd (8.0, 1.5)
5'	148.5, C	-	143.0, C	-
6'	116.0, CH	6.85 d (1.5)	121.6, CH	6.89 d (1.5)
7'	16.6, CH_3	2.15 s	17.1, CH_3	2.21 s
8'	35.0, CH	2.78 m	37.1, CH	2.80 m
9'	24.6, CH_3	1.22 d (6.5)	24.6, CH_3	1.23 d (7.0)
10'	24.5, CH_3	1.22 d (6.5)	24.6, CH_3	1.23 d (7.0)

^a Spectra were recorded in methanol- d_4 , at 600 MHz (^1H) and 150 MHz (^{13}C); chemical shifts are given in ppm; J values are in parentheses and reported in Hz; assignments were confirmed by COSY, 1D-TOCSY and HSQC experiments. ^b Overlapped signal.

HRESIMS analysis of compound **17** indicated a molecular formula of $\text{C}_{20}\text{H}_{30}\text{O}_3$ (calcd. for $[\text{M} + \text{Na}] + 341.2087$, found 341.2097). NMR revealed a monoterpene dimer. Comparison of the NMR data (Table 5) revealed that the carbon and proton resonances of the carvacrol unit closely matched those of compound **16**, with the primary difference arising from the second monoterpenoid unit. The difference between them was indicated by their ^1H NMR

spectra that **17** has one trisubstituted olefinic bonds (δ_{H} 5.73 (1H, br d, $J = 10.0$ Hz), while **16** contained one disubstituted olefinic bond, moreover the spectra showed also the presence in **17** of an additional hydroxy methine. Analysis of the COSY and 1D-TOCSY spectra led to the determination of the spin systems H-2–H-3 and H-5–H-6 (Table 6). This was confirmed from HSQC and HMBC data. NMR analysis of the second monoterpene unit of **17** supported its identification as *p*-meth-3(4)-en-1 α ,2 α ,8-triol (Yahara *et al.*, 1990). The *cis* orientation of the hydroxyl groups at C-1 and C-2, inferred from the NOESY correlation between Me-7 and H-2, is consistent with the previously reported structure (Yahara *et al.*, 1990). Furthermore, HMBC correlations between H-2 and C-1, C-7, C-4, and C-1', as well as between Me-17 and C-1 enabled the assignment of the linkage position between the two monoterpene units. From all these data was concluded that **17** is 1-(carvacryl)- *p*-menth-3(4)-1,2,8 triol (Fig. 12).

Compound **18** ($\text{C}_{20}\text{H}_{28}\text{O}_2$) showed spectral features characteristic of a dimeric monoterpenoid. Its ^{13}C -NMR spectrum (Table 6) showed 20 signals ascribable to a carvacrol unit (carvacrol unit of compound **16**) linked via an ether linkage to a 4,8-epoxy-3-hydroxy-*p*-menth-1-ene unit (Fig. 12). The second monoterpene unit was identified as a *p*-menth-1-ene derivative bearing a hydroxyl group and a 4,8-epoxy bridge, based on NMR analysis. In particular, the HMBC spectrum showed clear long-range correlations supporting this structural assignment: H-3 exhibited cross-peaks with C-5, C-2, and C-8, while H-2 correlated with C-4 and C-6. In addition, Me-9 and Me-10 displayed a key HMBC correlation with C-4. These combined data strongly indicated the presence of an oxygenated functionality involving C-3 and the C-4/C-8 carbon. The location of the hydroxyl group at C-3 was further confirmed by the COSY spectrum, which revealed a proton coupling sequence from the proton at δ_{H} 4.43 to the proton at δ_{H} 5.76 (Table 6). The ^{13}C NMR spectrum showed characteristic resonances at δ_{C} 62.8 and 66.8, consistent with carbons involved in an epoxy ring. These chemical shifts and the observed HMBC correlations are in excellent agreement with the values reported in the literature for monoterpenes with a 4,8-epoxy functionality. This reinforced the proposed structural assignment (Becker and Martini, 1999). The linkage was proposed to be between C-3 of the 4,8-epoxy-3-hydroxy-*p*-menth-1-ene and C-1' of the *carvacrol* moiety, on the basis of the resonances of C-3 and of C-1' (Table 6). The HMBC experiment confirmed this bond through the observed correlations between H-3 and C-1',

C-1, C-4. Thus, the structure of compound **18** was determined to be 1-(carvacryl)-*p*-menth-1-en-4,8-epoxy (Fig. 12).

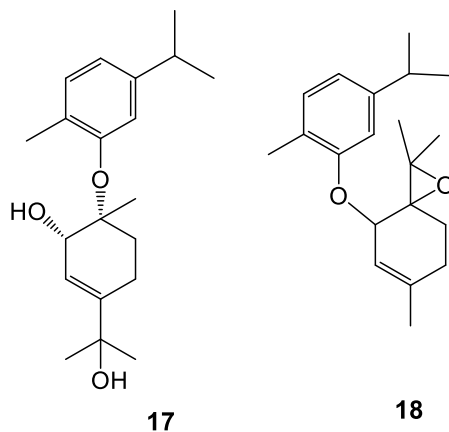


Figure 12. Chemical structure of compounds **17** and **18**

Table 6. ^1H and ^{13}C NMR spectroscopic data of compounds **17** and **18**

position	17		18	
	δ_{C} , type	δ_{H}	δ_{C} , type	δ_{H}
1	81.6		141.3, C	-
2	72.0	4.34	121.5, CH	5.76 d (5.0)
3	120.4	5.73	75.5, CH	4.43 d (5.5)
4	144.9		66.8, C	-
5	35.6	2.20 ^b	24.5, CH ₂	2.68 m 1.62 m
6	25.1	1.65 ^b	31.6, CH ₂	2.31 ^b 2.31 ^b
7	17.8	1.30 s	22.2, CH ₃	1.80 s
8	72.0		62.8, C	-
9	27.4	1.32 ^b	20.8, CH ₃	1.44 s
10	27.4	1.32 ^b	21.2, C	1.43 s
1'	154.5		156.0, C	
2'	128.1		124.6, C	
3'	130.2	7.05 d (8.0)	131.4, CH	7.02 d (8.0)
4'	120.1	6.73 dd (8.0, 1.5)	114.5, CH	6.73 dd (8.0, 1.5)
5'	146.3		147.5, C	-
6'	119.5	6.91 d (1.5)	119.7, CH	6.91 d (1.5)
7'	15.6	2.19 s	16.4, CH ₃	2.20 s
8'	33.6	2.83 m	35.3, CH	2.83 m
9'	23.1	1.23 d (6.7)	23.4, CH ₃	1.24 d (6.7)
10'	22.9	1.25 d (6.7)	23.5, CH ₃	1.24 d (6.7)

^a Spectra were recorded in methanol-*d*₄, at 600 MHz (^1H) and 150 MHz (^{13}C); chemical shifts are given in ppm; *J* values are in parentheses and reported in Hz; assignments were confirmed by COSY, 1D-TOCSY and HSQC experiments. ^b Overlapped signal.

Compound **19** was obtained as an amorphous solid with a molecular formula of $C_{20}H_{30}O_3$, as determined by ^{13}C NMR and HRESIMS analyses. The positive ESIMS spectrum showed a sodiated molecular ion peak at m/z 341.2089 $[M + Na]^+$, while its fragmentation pattern was characterized by a peak at m/z 249.15 $[M + Na - 18 - 18 - C_3H_6 - 15]$. The ^{13}C NMR spectrum (Table 7) displayed signals for twenty carbons: six methyl groups (δ_c 16.0, 23.0, 24.4, 24.8, 24.9, 25.5); five methine carbons, four of which were olefinic (δ_c 115.0, 125.2, 129.7, and 143.2) and one aliphatic (δ_c 27.9); four quaternary aromatic carbons (δ_c 121.6, 135.5, 147.5 and 157.8), including one oxygenated aromatic carbon, one quaternary aliphatic carbon (δ_c 37.5); and two nonprotonated oxygenated carbons (δ_c 72.8 and 75.0) (Table 7). The ^1H NMR spectra of **19** exhibited in the aromatic region characteristic signals of not coupled type aromatic protons at δ 6.72 (1H, s) and 7.05 (1H, s), two sp^2 protons that appeared as doublet at δ 5.97 (1H, dd, $J=10.0, 1.5$ Hz) and 5.80 (1H, dd, $J=10.0, 1.0$ Hz), two methylenes at δ 2.31 (1H, ddd, $J = 17.0, 13.5, 4.0$ Hz), 1.57 (1H, m), and 2.05 (1H, ddd, $J = 17.0, 14.0, 4.0$ Hz), 1.69 (1H, m) (Table 7). One methine proton appeared as a septet at δ 3.85 (1H, m). Its relatively downfield chemical shift indicated close proximity to the aromatic ring, a hypothesis later confirmed by the HMBC experiment. In the same spectrum, a methyl signal at δ 2.16 (3H, s) was observed, consistent with a methyl group attached to an aromatic quaternary carbon. The overall spectral features showed similarities to carvacrol (Figure 11), indicating that the molecule consists of a carvacrol-based structure comprising a carvacrol moiety linked to a second, non-aromatic monocyclic monoterpene unit, in full agreement with the HRMS data. HMBC correlations enabled the unambiguous assignment of protons and carbons within each monomeric unit. Furthermore, the HMBC cross-peaks involving H-2 and C-6, C-4, together with those between H-3 and C-5, C-1, and Me-7 to C-1, C-6, C-2 allowed the second monoterpene unit to be identified as showed in figure 13.

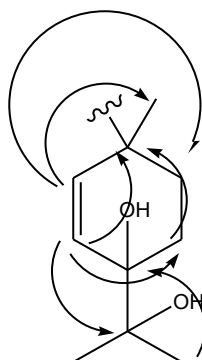


Figure 13. Partial structure of compound **19**

Moreover, cross-peaks were detected between Me-7 and C-4', these correlations established the presence of a C-4'/C-1' linkage connecting the two monomeric units. From all these data, it was concluded that compound **19** is carvacrol-1'-yl-(1)-*p*-menth2(3)-en 4,8 diol (Fig. 15).

The ^1H NMR spectrum of compound **20** showed the presence of a 1,2,4,5-tetrasubstituted aromatic ring at δ_{H} 6.72 (1H, s), and 7.01 (1H, s), three olefinic protons at δ_{H} 5.72 (1H, m), 5.78 (1H, br d, $J=10.0$ Hz) and 6.01 (1H, dd, $J=10.0, 1.5$ Hz), one methylene at δ_{H} 2.72 (1H, dd, $J=19.0, 3.0$ Hz), and 2.22 (1H, dd, $J=19.0, 5.5$ Hz), one isopropyl group at δ_{H} 3.70 (1H, m), 1.13 (6H, d, $J=7.0$ Hz), and four methyl singlets at δ_{H} 2.16, 1.42, 1.37, 1.35. Its HRESIMS (m/z 323.1995 $[\text{M}+\text{Na}]^+$) indicated a molecular formula of $\text{C}_{20}\text{H}_{28}\text{O}_2$ (calcd 323.1981), this was also confirmed by ^{13}C NMR data (Table. 7). The fragmentation pattern showed prominent ions at m/z 151.11 and m/z 133.10 $[\text{M}+\text{H}^+-\text{C}_{10}\text{H}_{14}\text{O}-18]$, once again supporting the presence of a monoterpene dimer. The ^{13}C NMR spectrum of compound **20** displayed twenty carbon resonances, which were assigned to six methyls, one methylene, six methines, six quaternary carbons, and one non-protonated oxygenated carbon, these data are in good agreement with the above analysis. COSY and 1D-TOCSY experiments established the key proton/proton correlations, revealing three main spin systems: H-8'/Me-10', H-2/H-3, and H-5/H-6. These coupling patterns provided the basis for defining the connectivity within each subunit of the molecule. Combined with the observed HMBC correlations from H-6 to C-2, C-3, C-4, C-7; H-10 to C-8, C-4; H-2 to C-7, C-4, C-7, C-6, the presence of a partial structure of unit A (Fig. 14) in the structure of **20** was suggested. In the HMBC spectrum of compound **20**, H-3' showed correlations with C-4', C-7', C-1' and C-

1. Additionally, H-6' correlated with C-2', C-4', and C-8', and H-8' exhibited cross-peaks with C-5' and C-10'. Me-7' displayed correlations with C-2', C-3', and C-1'. Taken together, these data supported the presence of a second structural subunit corresponding to a carvacrol moiety. The connectivity between the two monomeric units was established based on the key correlations observed between H-3' and C-1 and between Me-7' and C-4'. Consistent with these findings, the NOESY spectrum of compound **20** revealed a key spatial correlation between δ_{H} 1.42 (Me-7) and δ_{H} 3.70 (H-8'), thereby defining the positions at which the two units are linked. It follows that **20** can be formulated as 1-(carvacryl)-*p*-menth-2(3), 4(5)-dien-8 olo (Fig. 15).

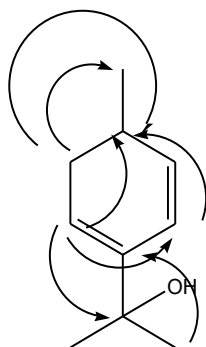


Figure 14. Partial structures of compound **20**

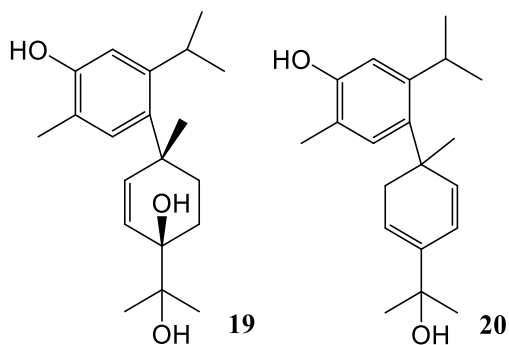


Figure 15. Chemical structure of compounds **19** and **20**

Table 7. ^1H and ^{13}C NMR spectroscopic data of compounds **19** and **20**

position	19		20	
	δ_{C} , type	δ_{H}	δ_{C} , type	δ_{H}
1	37.5, C	-	39.4, C	-
2	143.2, CH	5.97 dd (10.0, 1.5)	120.4, CH	6.01 dd (10.0, 1.5)
3	125.2, CH	5.80 dd (10.0, 1.0)	139.4, CH	5.78 br d (10.0)
4	72.8, C	-	141.6, C	-
5	27.6, CH ₂	2.05 ddd (17.0, 14.0, 4.0) 1.69 m	116.3, CH	5.72 m
6	34.9, CH ₂	2.31 ddd (17.0, 13.5, 4.0) 1.57 m	39.9, CH ₂	2.72 dd (19.0, 3.0) 2.22 dd (19.0, 5.5)
7	25.5, CH ₃	1.43 s	26.7, CH ₃	1.42 s
8	75.0, C	-	82.2, C	-
9	24.9, CH ₃	1.25 s	28.5, CH ₃	1.37 s
10	24.8, CH ₃	1.28 s	28.5, CH ₃	1.35 s
1'	157.8, C		155.4, C	
2'	121.6, C		121.6, C	
3'	130.7, CH	7.05 s	128.9, CH	7.02 s
4	135.5, C	-	138.2, C	-
5'	147.5, C	-	148.2, C	-
6'	115.0, CH	6.72 s	115.0, CH	6.73 s
7'	16.0, CH ₃	2.16 s	16.0, CH ₃	2.16 s
8'	27.9, CH	3.85 m	28.5, CH	3.70 m
9'	23.0, CH ₃	1.14 d (6.8)	24.3, CH ₃	1.13 d (7.0)
10'	24.4, CH ₃	1.14 d (6.8)	24.3, CH ₃	1.13 d (7.0)

^a Spectra were recorded in methanol-*d*₄, at 600 MHz (^1H) and 150 MHz (^{13}C); chemical shifts are given in ppm; *J* values are in parentheses and reported in Hz; assignments were confirmed by COSY, 1D TOCSY and HSQC experiments. ^b Overlapped signal.

5.3.3a Antibacterial activity of extracts and isolates

The preliminary screening of the antimicrobial activity of leaf chloroform and hexane extracts (LPA) was carried out by monitoring bacterial growth curves using a Tecan microplate reader. The bacterial strains tested included *Escherichia coli*, *Staphylococcus aureus*, *Citrobacter spp.*, *Salmonella spp.*, *Enterococcus faecalis*, *Streptococcus epidermidis*, and *Listeria monocytogenes*. Bacterial growth was monitored up to 940 minutes starting from an initial OD of 0.05, in the presence of various concentrations of the LPA chloroform

and hexane extracts (500 µg/mL, 250 µg/mL, 125 µg/mL, 60 µg/mL, and 0 µg/mL) (Figure 16). Optical density was continuously measured at 600 nm (OD₆₀₀) to obtain detailed growth profiles over time. The growth curves were compared to the control (0 µg/mL) to assess potential inhibitory effects. Unlike disk diffusion, this method enables real-time observation of bacterial proliferation dynamics. It provides valuable insight into the stage of bacterial growth at which the extract or compound exerts its effect. This could be an alternative to disk diffusion for identifying the antimicrobial potential in natural extracts.

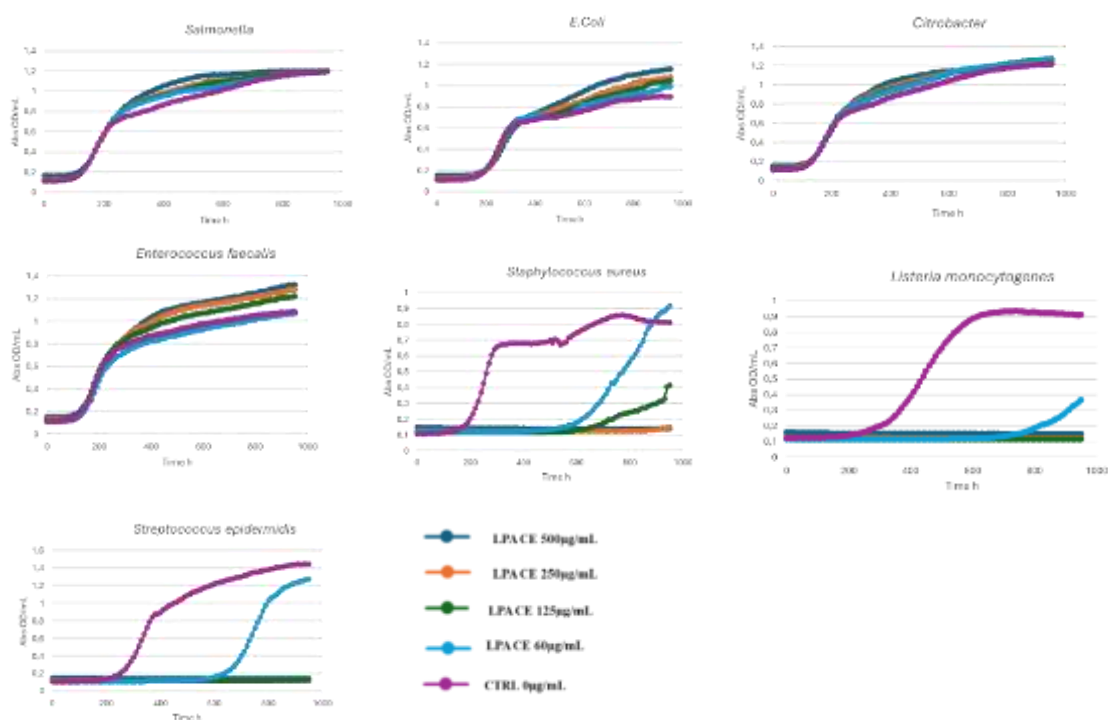


Figure 16. Bacterial growth curves in the presence of *L. pubescens* chloroform extract (LPA CE): Growth of *Escherichia coli*, *Staphylococcus aureus*, *Citrobacter* spp., *Salmonella* spp., *Enterococcus faecalis*, *Streptococcus epidermidis*, and *Listeria monocytogenes* was monitored over 940 minutes using a Tecan microplate reader at 600 nm (OD₆₀₀). Each strain was exposed to different concentrations of the extracts (500, 250, 125, 60, and 0 µg/mL). The untreated control (0 µg/mL) served as a reference for normal growth.

These results demonstrated that the chloroform extract exhibited antimicrobial activity against *L. monocytogenes* and *S. epidermidis*, while a weak activity was observed for *n*-hexane residue. Even at a concentration of 60 µg/mL of chloroform extract, clear variation in bacterial growth was observed, indicating an inhibitory effect on proliferation and consequently on virulence on *L. monocytogenes*. In addition, a concentration of 60 µg/mL of chloroform extract, showed an interesting modulation of *S. epidermidis* growth curve.

Consequently, further studies were performed on *L. monocytogenes* and *S. epidermidis*. Nine different fractions derived from the chromatographic separation of the chloroform extract were analysed (A₂ to I₂). Fraction C₂ exhibited an interesting effect against both bacteria (Figure 17). For *L. monocytogenes*, the impact on bacterial growth and virulence was evident at a concentration of 12 µg/mL, with a marked extension of the lag phase. This delay suggests that the fraction interferes with the initial adaptation of bacterial cells to the growth environment, likely by affecting early cellular processes such as membrane integrity, nutrient uptake, or metabolic activation (Stewart et al., 2018; Rolfe et al., 2012).

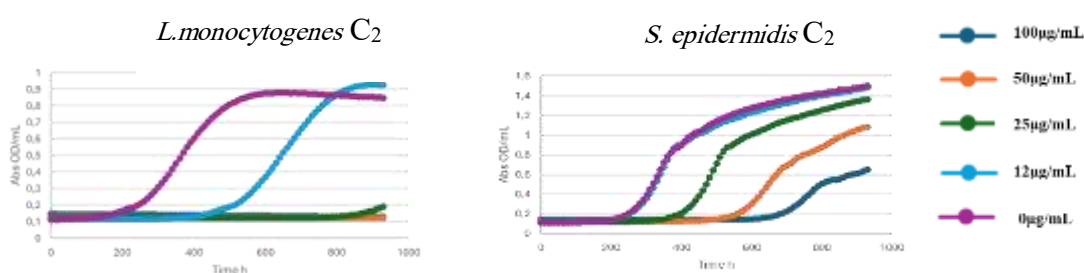


Figure 17. *L. monocytogenes* and *S. epidermidis* growth curves in the presence of fraction C₂. Growth was monitored over 940 minutes using a Tecan microplate reader at 600 nm (OD₆₀₀). Each strain was exposed to different concentrations of the compound (100, 50, 25, 12, and 0 µg/mL). The untreated control (0 µg/mL) served as a reference for normal grow

In addition to *L. monocytogenes*, *S. epidermidis* also exhibited concentration-dependent alterations in its growth pattern upon incubation with fraction C₂ (Figure 17). Unlike *L. monocytogenes*, the growth trend of *S. epidermidis* varied proportionally with the compound concentration, suggesting that components of fraction C₂ may modulate cellular functions rather than exert a purely bactericidal effect. In light of these findings, the subsequent investigation was directed toward *S. epidermidis*. This species is a component of the normal human microbiota but can act as an opportunistic pathogen. Its capacity to form biofilms and develop resistance to conventional antibiotics makes it a clinically relevant target. Therefore, identifying molecules capable of combating *S. epidermidis* is of great importance (Gomes et al., 2014). On this basis, molecules isolated from the active fraction C₂ were evaluated for their activity against *Staphylococcus epidermidis*. In particular, the following compounds were tested: **5**, **7**, **8**, **13**, **22**, **23**, **24**. Moreover,

compound **14** was tested for close structure similarity to compound **13** but only a slight activity was observed.

Among these, compound **13** exhibited promising results (Figure 18). It was tested at concentrations of 50, 25, 12, and 6 $\mu\text{g/mL}$, showing a MIC_{100} of 12 $\mu\text{g/mL}$. A clear alteration in bacterial growth was observed at 6 $\mu\text{g/mL}$, indicating an inhibitory effect. Compound **13** displayed a dose-dependent impact on *S. epidermidis*, with complete inhibition at higher concentrations and growth modulation at lower ones.

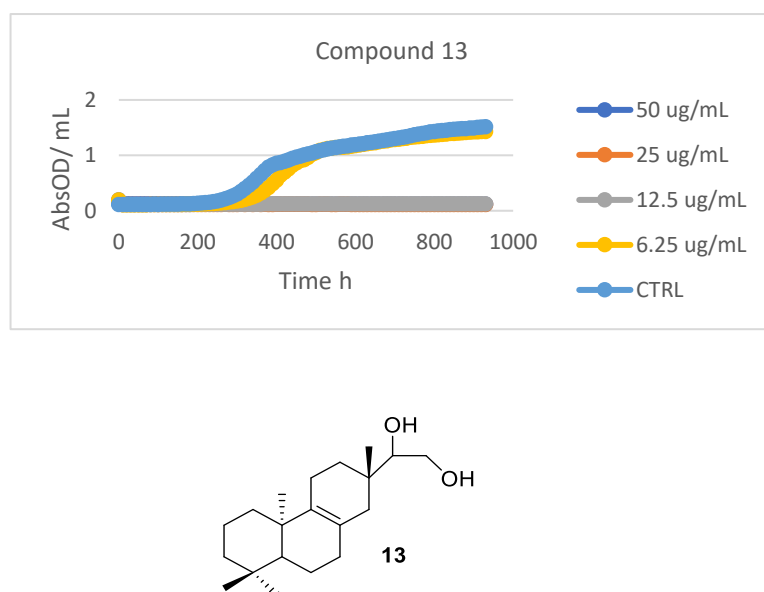


Figure 18. *S. epidermidis* growth curves in the presence of compound **13**. Growth of *S. epidermidis* was monitored over 940 minutes using a Tecan microplate reader at 600 nm (OD_{600}). It was exposed to different concentrations of the compound (50, 25, 12.5, 6.25, and 0 $\mu\text{g/mL}$). The untreated control (0 $\mu\text{g/mL}$) served as a reference for normal growth.

Figure 19, shows the growth curves of *S. epidermidis* in the presence of ursolic acid **22** and oleanolic acid **23**. Both **22** and **23** exhibited significant inhibitory effects, indicating a capacity to suppress bacterial growth. These findings suggest that **22** and **23** play also a role in the antimicrobial activity of the active fraction C₂, possibly through membrane disruption or interference with essential metabolic pathways. Further studies will be necessary to elucidate their mechanism of action.

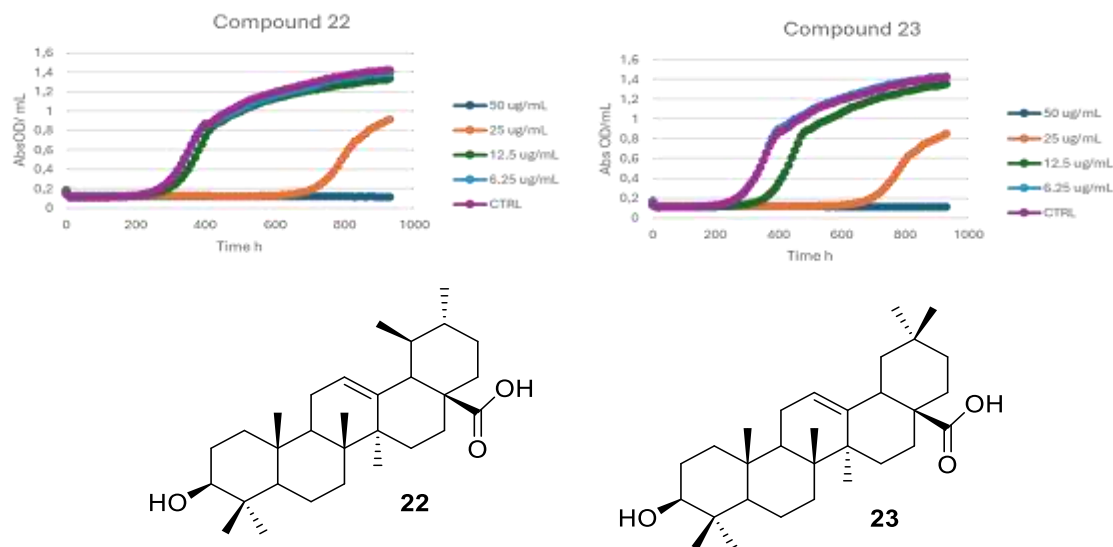


Figure 19. *S. epidermidis* growth curves in the presence of compound ursolic acid (**22**) and oleanolic acid (**23**). Growth of *S. epidermidis* was monitored over 940 minutes using a Tecan microplate reader at 600 nm (OD_{600}). Each strain was exposed to different concentrations of compounds (50, 25, 12.5, 6.25, and 0 $\mu\text{g/mL}$). The untreated control (0 $\mu\text{g/mL}$) served as a reference for normal growth

5.3 b Results and discussion

5.3.1 b Structural elucidation of isolates from leaf surface extract of *Lavandula pubescens* Decne

The phytochemical study of *L. pubescens* leaf surface extract led to the isolation of seven new diterpenes **29–35**, together with four known diterpenes: 15S,16-dihydroxy-7-oxopimar-8(9)-ene (**14**) (Politi et al., 2002), 15,16,17-trihydroxypimar-8(9)-ene (**36**) (Politi et al., 2002); ent-isopimarane-8 α , 15, 16-triol (**37**) (Kubo et al., 2023), 15,16-dihydroxy-ent-pimarane-8(9)-en 7,11-dione (**38**) (Politi et al., 2002). Furthermore, known flavonoids, cirsimaritin (**39**) (Menelaou et al. 2010), xanthomicrol (**40**) (Akit et al., 2023), and gardenin (**41**) (Voirin et al., 1999) were also isolated and identified.

Compound **29**, obtained as colorless oil, had a molecular formula of $\text{C}_{20}\text{H}_{30}\text{O}_2$ according to its HRESIMS (m/z 303.2316 $[\text{M}+\text{H}]^+$). Comparing the NMR data of **29** (Table 8) with those of 3 β -hydroxy-abieta-8,11,13-triene (Urones et al., 1998). revealed that the two compounds exhibited a certain degree of structural similarity. However, differences were also observed. In 3 β -hydroxy-abieta-8,11,13-triene, the quaternary carbons resonating at δ_{C} 148.0 (C-9) and 38.1 (C-10) were markedly shifted downfield in compound **29**, appearing at δ_{C} 155.0 and 82.7, respectively. These shifts strongly suggest that the C-9/C-10 bond has

been cleaved and that a 9,10-epoxy bridge has formed in compound **29**. Additional differences were evident in the ^1H NMR spectrum, where the absence of the 3-OH proton signal further distinguished compound 3 β -hydroxy-abieta-8,11,13-triene from **29**. Finally, **29** displayed a hydroxy-isopropyl substituent at the C-13 position on the aromatic ring, whereas reference compound had an isopropyl group. This data was in well agreement with its molecular formula and further supported by long range correlations from H-5 (δ_{H} 1.64) to C-3, Me-18, Me-19, Me-20, and C-7, Me-20 (δ_{H} 1.02) to C-1, C-5, C-10, from H-14 (δ_{H} 6.99) to C-9, C-12 and C-7, and from H-11 (δ_{H} 6.77) to C-8, C-13 and Me-16 and C-13 in the HMBC spectrum. The NOE correlation observed in the ROESY spectrum between Me-20 (δ_{H} 1.02) and Me-19 (δ_{H} 0.83) led to establish an *ent*-abietane skeleton. Thus, compound **29** was identified as 16-Hydroxy-9,10- epoxy-9,10-seco-*ent*-abieta-8,11,13-triene.

Compound **30** was assigned the molecular formula $\text{C}_{20}\text{H}_{32}\text{O}_4$ by its HRESIMS spectrum, which was acquired in the positive ion mode (m/z 359.2205 $[\text{M} + \text{Na}]^+$). This information, along with the ^{13}C NMR data (Table 8), led to the determination of five indices of hydrogen deficiency and an *ent*-pimarane skeleton for **30**. The ^1H and ^{13}C NMR data (Table 8) of compound **30** showed several significant differences compared to the data for 7 β ,11 β ,15,16-tetrahydroxy-*ent*-isopimar-8(9)-ene (**6**), which was isolated from the leaves chloroform extract of *L. pubescens*. Notably, the ^{13}C NMR spectrum exhibited a signal corresponding to a carbonyl carbon at δ_{C} 203.0 ppm, replacing the C-7 hydroxy methine carbon observed in compound **6**. This substitution strongly suggests oxidation at that specific position. The HMBC correlations Me-20/C-1, C-5, C-9, and H₂-6/C-10, C-5 and H₂-14/C-7, C-9, C-12, C-15, Me-17 confirmed the presence of the carbonyl group at C-7. The relative configuration of **30** was obtained on the basis of the literature survey (Zhao et al., 1988) the values of coupling constant and ROESY data. The ROESY correlations of H-5/Me-18, and Me-17/H-15, showed that these protons were on the same side. Thus, **30** was characterized as 11 β ,15,16-trihydroxy-*ent*-pimar-8(9)-en-7-one.

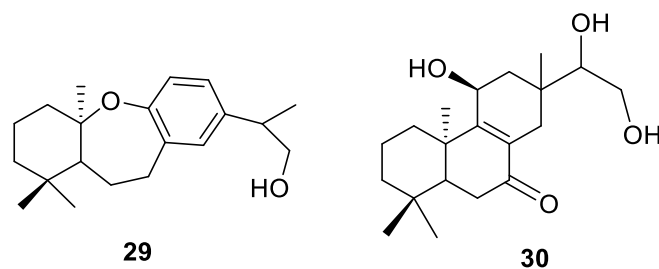


Figure 20. Chemical structure of compounds **29** and **30**

Table 8. ^1H and ^{13}C NMR Spectroscopic Data for Compounds **29** and **30**

position	29		30	
	δ_{C} , type	δ_{H}	δ_{C} , type	δ_{H}
1	42.6, CH ₂	1.92 ^b 1.71 ddd (16.0, 13.0, 4.5)	36.1, CH ₂	1.98 m 1.78 ^b
2	21.2, CH ₂	1.63 ^b 1.63 ^b	19.4, CH ₂	1.78 ^b 1.62 ^b
3	43.4, CH ₂	1.43 m 1.35 ^b	42.6, CH ₂	1.53 ^b 1.31 ^b
4	36.0, C	-	32.2, C	-
5	59.9, CH	1.64 m	51.4, CH	1.86 dd (12.0, 6.5)
6	22.2, CH ₂	1.94 ^b 1.44 m	36.2, CH ₂	2.51 2.50
7	33.4, CH ₂	2.78 ddd (11.0, 7.0, 3.0) 2.74 ddd (14.0, .0, 3.0)	203.0, C	-
8	137.2, C	-	132.3, C	-
9	155.0, C	-	170.2, C	-
10	82.7, C	-	40.0, C	-
11	124.5, CH	6.77 d (8.0)	64.0.0, CH	4.53 br t (3.5)
12	126.6, CH	6.96 dd (8.0, 2.0))	44.3, CH ₂	2.05 dd (15.0, 2.5) 1.43 ^b
13	149.9, C	-	34.0, C	-
14	129.5, CH	6.99 br d (2.0)	29.4, CH ₂	2.41 d (16.5) 2.17 d (16.5)
15	43.0, CH	2.84 m	78.9, CH	3.59 dd (9.0, 3.5)
16	69.8, CH ₂	3.65 dd (11.4, 7.0) 3.55 dd (11.4, 7.5)	63.8, CH ₂	3.84 dd (11.0, 3.0) 3.51 dd (11.0, 9.0)
17	18.5, CH ₃	1.27 d (7.0)	25.7, CH ₃	0.88 s
18	35.0, CH ₃	1.03 s	32.7, CH ₃	0.94 s
19	23.6, CH ₃	0.83 s	21.6, CH ₃	1.0 s
20	21.6, CH ₃	1.02 s	18.8, CH ₃	1.14 s

^a Spectra were recorded in methanol-*d*₄ at 600 MHz (^1H) and 150 MHz (^{13}C); chemical shifts are given in ppm; *J* values are in parentheses and reported in Hz; assignments were confirmed by COSY, 1D-TOCSY and HSQC experiments. ^b Overlapped signal

The structural features of compounds **31**, **32** and **33** suggest that they originate from a pimarane-type framework, in which the C-8/C-9 bond has broken to form an enol ether moiety. Although such a rearranged pimarane scaffold is absolutely uncommon, a related example has previously been reported for steroid derivatives from a marine sponge (D'Auria et al., 1991; Costantino et al., 1994).

Compound **31**, obtained as a colorless powder, was assigned the molecular formula C₂₀H₃₂O₃ by its HRESIMS *m/z* of 343.2245 [M + Na]⁺, equating to five double-bond equivalents. The ^{13}C NMR data (Table 9) showed 20 carbon signals, indicative of four

methylys, six methylenes, three methines of which two sp^2 , three quaternary carbons, one hydroxymethylene, one hydroxy methine, and two non-protonated sp^2 carbon-bearing a hydroxy group. The ^1H NMR spectrum showed signals due to one hydroxymethilene group at δ_H 3.42 (1H, d, $J = 11.6, 9.0$ Hz) and δ_H 3.76 (1H, d, $J = 11.6, 2.6$ Hz), one hydroxymethine at δ_H 3.34, and two olefinic proton signals in the ring system appeared at δ_H 4.91 (1H, dd, $J=13.5, 5.5$ Hz) and 4.70 (1H, br t, $J=3.0$ Hz). A combination of 1D-TOCSY and COSY experiments provided evidence for the presence of the spin system H-1/H-3, H-5/H-7, and H-11/H-12., H-15-H-16. The elucidation of all basic carbon skeletons from the above subunits was obtained by a series of ^1J (HSQC) and ^3J (HMBC) correlations, which also allowed the assignment of resonances in the ^{13}C -NMR spectrum to the pertinent carbon (Table 9). The NMR data suggested that **31** possessed a pimarane riarranged structure. The presence in the ^{13}C NMR spectra of the two downfield olefinic carbon signals at 153.2 (s) and 169.5 (s) was indicative of the attachment of these quaternary carbons to an oxygen atom, thereby forming a dienol ether structure (Costantino et al., 1994). The HMBC spectrum revealed long-range couplings between H-11 and C-9, C-13; between H-12 and C-7, C-9, C-14, Me-17, C-15; and between H-14 and C-8, C-7, C-17, C-15. There were also long-range couplings between H-15 and C-12, C-14. Furthermore, long-range couplings were observed for H-5 to C-3 and H-6 to C-4, C-10, C-8 and C-7. Thus, the two double bonds were assigned to the regions between C-7 and C-8, and between C-9 and C-11 respectively, with the oxygen atom located between C-8 and C-9. The ROESY correlations of Me-20 with Me-18, showed that these methyls were on the same side. On the other hand, the correlations between H-5/Me-19 allowed us to confirm the α orientation of Me-20. Furthermore, the downfield shift of H-5 to δ 2.68 indicates that the oxygenated functionality and H-5 are located on the same face of the molecule, supporting the assignment of the 8,9-oxide bridge in the β configuration. Thus, compound **31** was elucidated as 15,16-dihydroxy 8 β ,9 β -oxido-8,9-secopimaran- 8,9(11),22-diene.

Compound **32** gave a molecular ion at m/z 359.2181 $[M+Na^+]$ in HRESIMS analysis. Based on this and the ^{13}C NMR data, it was possible to deduce the molecular formula $C_{20}H_{32}O_4$. This revealed that it was an analogue of **31**, in which a carbon-carbon double bond had been hydrogenated, and an additional epoxy functional group was present. **32** had one epoxy group and lacked the dienol ether. In the ^{13}C NMR spectrum, only two olefinic carbon

signals were observed, which was in accordance with the calculated five degrees of unsaturation. In contrast to **31**, the HMBC data for **32** indicated that C-9 and C-7 correspond to a di-hydroxylated not protonated carbon and a methylene group, respectively. Moreover, several key long-range correlations were detected, including those between Me-17 and C-12, C-15, and the epoxide carbon at C-14, between H-14 and both C-9 and C-7, and between H-11 and C-9 and C-13. These findings show there is no olefinic bond between C-7 and C-8 and confirm the epoxy groups at C-8 and C-14. The stereochemical configuration in compound **32** was consistent with that of compound **31**, as evidenced by its ^1H NMR, COSY, and ROESY spectra. H-11 showed NOE correlations with Me-20 and Me-17 locating this group on the same face of the molecule. Therefore, the structure of compound **32** was determined to be 15,16-dihydroxy-8,14- α -epoxy-8 β ,9 β -oxido-8,9-secopimarane-9(11)-ene.

The positive HRESIMS of compound **33**, obtained as an amorphous white powder, gave a sodiated molecular ion at m/z 359.2197 $[\text{M} + \text{Na}]^+$ in accordance with the molecular formula $\text{C}_{20}\text{H}_{32}\text{O}_4$, indicating an index of hydrogen deficiency of five. A comparison of the NMR data for **33**, (Table 9) and those for **32** indicated that both compounds had similar skeletons. Compound **33** exhibited the epoxy proton signal at δ_{H} 2.91 (1H, multiplet), compared to δ_{H} 2.58 in the **32**. Analysis of the COSY, HSQC, and HMBC spectra confirmed the placement of the epoxy group between C-8 and C-7, rather than between C-8 and C-14. NOE correlations between H-5 and Me-18, Me-19 and Me-20, H-7 and Me-20, H-11 and Me-20, were observed. The structure of compound **33** was then elucidated as 15,16-dihydroxy-8,7- α -epoxy-8 β ,9 β -oxido-8,9-secopimarane-9(11)-ene.

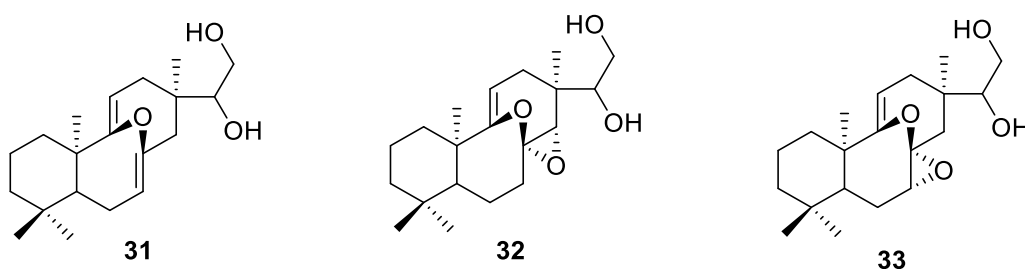


Figure 21. Chemical structure of compound **31**, **32** and **33**

Table 9. ^1H and ^{13}C NMR Spectroscopic Data for Compounds **31**, **32** and **33**

position	31			32			33	
	δ_{C} , type	δ_{H}		δ_{C} , type	δ_{H}		δ_{C} , type	
1	41.9, CH_2	1.57 ^b		41.2, CH_2	1.58 ^b		40.0, CH_2	1.46 ^b
		1.46 ^b			1.28 br dd (12.5, 7.0)			1.27 ^b

2	205, CH ₂	1.70 m 1.50 ^b	20.7, CH ₂	1.75 ^b 1.53 ^b	21.0, CH ₂	1.70 ddd (13.0, 11.0, 8.0, 2.0 1.47 ^b 1.43 ^b 1.21 ^b
3	43.6, CH ₂	1.77 ^b 1.28 ddd (18.0, 13.0, 3.5)	42.6, CH ₂	1.58 ^b 1.48 ^b	42.7, CH ₂	
4	32.6, C	-	33.5, C	-	33.8, C	-
5	47.2, CH	2.68 dd (13.2, 3.0)	51.7, CH	2.01 dd (13.0, 6.0)	42.4, CH	1.97 br d (12.5)
6	26.6, CH ₂	2.36 br ddd (18.0, 6.0, 3.0) 2.00 br dddd (18.0, 14.0, 13.2, 3.5)	22.7, CH ₂	1.78 m 1.39 ^b	25.5 CH ₂	2.27 br dd (15.0, 3.0) 1.82 ddd (15.0, 11.5, 2.5)
7	113.0, CH	4.70 br t (3.0)	38.4, CH	2.19 ^b 1.50 ^b	63.6, CH	2.97 br t (2.5)
8	154.0, C	-	89.0, C	-	86.0, C	-
9	171.2, C	-	166.4, C	-	164.3, C	-
10	41.0, C	-	42.0 C	-	41.0, C	-
11	106.4, CH	4.91 dd (8.5, 5.5)	112.5, CH	5.31 br t (7.0)	111.3, CH	5.20 dd (8.5, 5.0)
12	33.5, CH ₂	2.77 dd (13.5, 5.5) 1.60 ddd (13.0, 8.5, 1.5)	28.4, CH ₂	2.31 dd (12.5, 7.0) 1.58 ^b	33.6, CH ₂	2.70 dd (14.0, 5.5) 1.79 ^b
13	38.0, C	-	35.0, C	-	38.9, C	-
14	43.3, CH ₂	2.45 br d (12.5) 1.96 dd (12.5, 1.5)	68.8, CH ₂	2.58 s	43.7, CH ₂	2.58 d (12.5) 1.20 dd (12.5, 2.5)
15	81.8, CH	3.34 ^b	78.0, CH	3.68 dd (9.0, 2.5)	81.9, CH	3.31 dd (8.5, 3.0)
16	63.7, CH ₂	3.76 dd (11.6, 2.6) 3.42 dd (11.6, 9.0)	63.7, CH ₂	3.83 dd (11.0, 3.0) 3.56 dd (11.0, 9.0)	63.7, CH ₂	3.73 dd (11.5, 3.0) 3.42 dd (11.5, 8.5)
17	19.6, CH ₃	0.84 s	20.0, CH ₃	1.03 s	20.4, CH ₃	0.99 s
18	34.6, CH ₃	0.99 s	34.0, CH ₃	1.0 s	34.2, CH ₃	0.95 s
19	21.6, CH ₃	0.91 s	21.9, CH ₃	0.94 s	22.2 CH ₃	0.92 s
20	19.9, CH ₃	1.22 s	19.5, CH ₃	1.24 s	18.2 CH ₃	1.18 s

^a Spectra were recorded in methanol-d₄, at 600 MHz (¹H) and 150 MHz (¹³C); chemical shifts are given in ppm; J values are in parentheses and reported in Hz; assignments were confirmed by COSY, 1D-TOCSY and HSQC experiments. ^b Overlapped signal

The HRESIMS spectrum of **34** showed a molecular composition of C₂₁H₃₆O₃ (*m/z* 359.2587 [M + Na]⁺) and involved four indices of hydrogen deficiency. One of these could be accounted for one double bond, and the lack of other unsaturated carbons implied a tricyclic structure. The ¹H NMR spectrum (Table 10) displayed the characteristic signals of four methyl singlets, i.e. δ_H 0.84 (3H, s), 0.94 (3H, s), 0.93 (3H, s), and 1.00 (3H, s); one methoxy group at δ_H 3.86, two diastereotopic protons of an oxygenated methylene, i.e. δ_H 3.70 (1H, dd, J = 9.0 and 7.0 Hz), and δ_H 3.47; two oxygenated methines δ_H 3.46 and 3.42 (1H, m). The placement of the hydroxyl group at C-7 was deduced from the following

observations: the chemical shift of C-5 and C-6 were observed at 47.3 and 23.6 ppm, respectively, were shifted upfield and downfield respectively compared to the unsubstituted pimarane (Seca et al., 2008) (Table 10). These assignments were confirmed by the HMBC correlations from H-5 to C-3, C-18, C-19, C-20, C-7 and C-1, and H₂-14 to C-7, C-9, C-12 and C-15. Key HMBC correlations from H₂-12 to C-14 and C-15 and from H-15 to C-13, C-14, and C-16 completed the substitution pattern with two hydroxy groups at C-15 and C-16. Thus, **34** was identified as 7β-methoxy-,15,16-dihydroxy-*ent*-pimar-8(9)-ene.

The molecular formula of **35** was determined as C₂₀H₃₄O₄ from their HRESIMS (*m/z* 361.2376 [M + Na]⁺). In the HRESIMS/MS a major fragment at *m/z* 323.21 [M + H – 17]⁺ due to the loss of a hydroxy group suggested the presence of a peroxide group in the structure. The ¹H and ¹³C NMR (Table 10) displayed resonances typical of a pimarane with one double bond, two oxygenated methines, and an oxygenated methylene. HMBC cross peaks of Me-20 to C-1, C-5, C-9, and C-10, of H-14 to C-9 and C-12, suggested that the double bond was located at C-8,C-9; cross peaks of H-6 to C-7 established the C-7 hydroxy group position (Seca et al., 2008). The other two hydroxy groups were linked to C-15 and C-16 from the COSY spectrum and the HMBC correlations between H₂-16 and C-13 and H-15 and C-13, C-14 and C-12. However, the proton and carbon chemical shifts of H-7 (δ_H 4.09 br s) and C-7 (δ_C 85.1) were too high to be attributed to an -OH group and more consistent with the presence of an -OOH (Parisi et al., 2023). The presence of the hydroperoxy group in **35** was confirmed by HRESIMS. The relative configuration of **35** was obtained from coupling constant of H-7 (Table 10) and ROE correlations between Me-20 and Me-19, and H-5 and Me-18 and comparison with literature data (Politi et al., 2002). On the basis of the above results, the structures of **35** were determined as 7α- hydroperoxy 15,16-dihydroxy-*ent*-pimar-8(9)-ene.

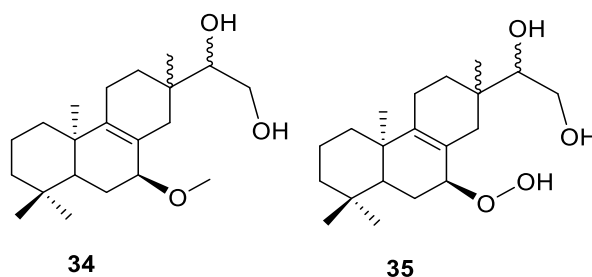


Figure 22. Chemical structure of compounds **34** and **35**

Table 10. ¹H and ¹³C NMR Spectroscopic Data for Compounds **34**, and **35**

position	34		35	
	δ_c , type	δ_H	δ_c , type	
1	37.5, CH ₂	1.80 ddd (13.0, 8.0, 3.0) 1.10 ddd (16.0, 13.0, 4.0)	37.8, CH ₂	1.8° m 1.10 ddd (16.0, 13.0, 3.0)
2	19.5, CH ₂	1.68 m 1.51 ^b	20.5, CH ₂	1.68 ^b 1.53 ^b
3	42.5, CH ₂	1.47 ^b 1.20 ddd (68.0, 13.0, 5.0)	42.9, CH ₂	1.47 ^b 1.24 ^b
4	33.0, C	-	33.9, C	-
5	47.3, CH	1.42 ^b	47.2, CH	1.48 ^b
6	23.6, CH ₂	2.04 m 1.53 ^b	24.5, CH ₂	2.32 br d (14.0) 1.54 ^b
7	80.7, CH, CH	3.62 m	85.1, CH	4.09 br d (2.0)
8	124.7, C	-	123.5, C	-
9	142.8, C	-	147.1, C	-
10	36.7, C	-	39.5 C	-
11	21.1, CH ₂	2.10 br t (8.0) 2.02 br d (14.0)	22.2, CH ₂	2.10 ^b 2.06 ^b
12	32.6, CH ₂	1.58 ^b 1.36 m	32.6, CH ₂	1.50 ^b 1.36 ^b
13	34.5, C	-	35.9, C	-
14	39.1, CH ₂	2.45 d (16.0) 1.56 ^b	39.4, CH ₂	2.51 br d (16.0) 1.64 ^b
15	76.6, CH	3.46 ^b	78.0, CH	3.44 ^b
16	63.6, CH ₂	3.70 dd (9.0, 7.0) 3.47 ^b	63.9, CH ₂	3.73 dd (10.5, 2.0) 3.50 ^b
17	20.3, CH ₃	0.84 s	20.1, CH ₃	0.84
18	33.0, CH ₃	0.94 s	33.0, CH ₃	0.99 s
19	21.7, CH ₃	0.93 s	21.5, CH ₃	0.92 s
20	18.5, CH ₃	1.00 s	19.0, CH ₃	1.01 s
OMe	56.4	3.86 s		

^a Spectra were recorded in methanol-d₄, at 600 MHz (¹H) and 150 MHz (¹³C); chemical shifts in ppm; J values in parentheses and in Hz; assignments were confirmed 2D-NMR experiments. ^b Overlapped signal

5.3.2b Anti-inflammatory activity of extracts, fractions and isolates

The potential in vitro cytotoxicity of *Lavandula pubescens* leaf extracts, leaf surface extract and its derived fractions (D₄ and E₄) and isolates (**14** and **36**) were evaluated on THP-1-activated macrophages, using the MTT assay. This step allowed to assess the safety of these components and to determine the optimal conditions to perform the subsequent experiments. THP-1-activated macrophages were, therefore, exposed to increased concentrations of the extracts (100 µg/mL), fractions (25 µg/mL) and compounds (6.25 µg/mL

to 50 µg/mL) and cell viability was evaluated at 24 h. The results obtained demonstrated that components were nontoxic towards THP-1-activated macrophages (Figure 23 A-D).

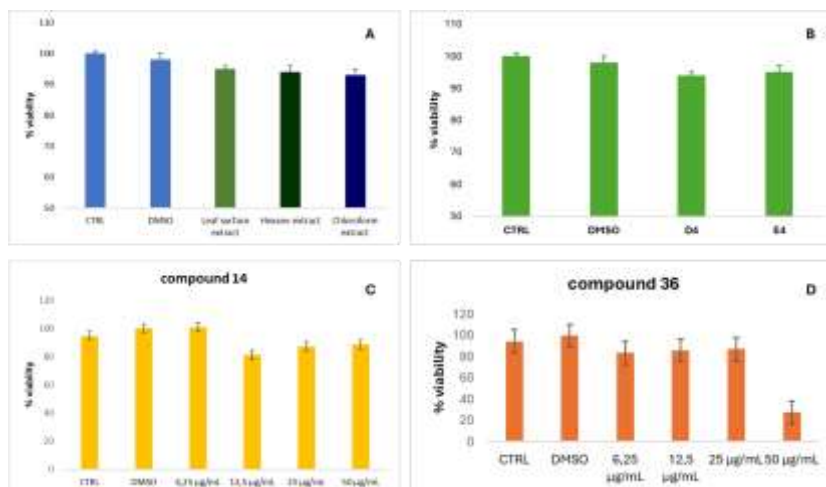


Figure 23. THP-1 viability measured by MTT assay (Mean SEM of 6 independent measurements) following different treatments with extracts (100 µg/mL) (A), fractions (25 µg/mL) (B), compound **14** (C) and compound **36** (D) (6.25 µg/mL to 50 µg/mL).

The modulation of THP-1-activated stimulated with lipopolysaccharide (LPS), by the *L. pubescens* hexane, chloroform and leaf surface extract was investigated. As shown in Figure 24, IL-6 secretion was inhibited by all *L. pubescens* extracts, in particular the chloroform extract showed a dose-dependent inhibition of IL-6 secretion. Moreover, leaf surface extract is able to reduce IL-6 secretion in a dose-dependent manner. Furthermore, the anti-inflammatory activity of leaf surface extract appears most promising despite the activity of *L. pubescens* extracts. Based on these results, the anti-inflammatory activity of fractions from leaf surface extract was studied. As Figure 25 (A) displayed, fractions D₄ and E₄ emerged as the most active in THP-1-activated macrophages after stimulation with LPS at a concentration of 25 µg/mL. In light of these results, the study focused on the main compounds of both fractions D₄ and E₄, compounds **14** (Figure 25 B) and **36** (Figure 25 C). Results in Figure 25 showed that both compounds **14** and **36** reduced IL-6 secretion; in particular, compound **36** was found to reduce IL-6 secretion in a dose-dependent manner, thereby reducing the inflammatory process mediated by LPS stimulation.

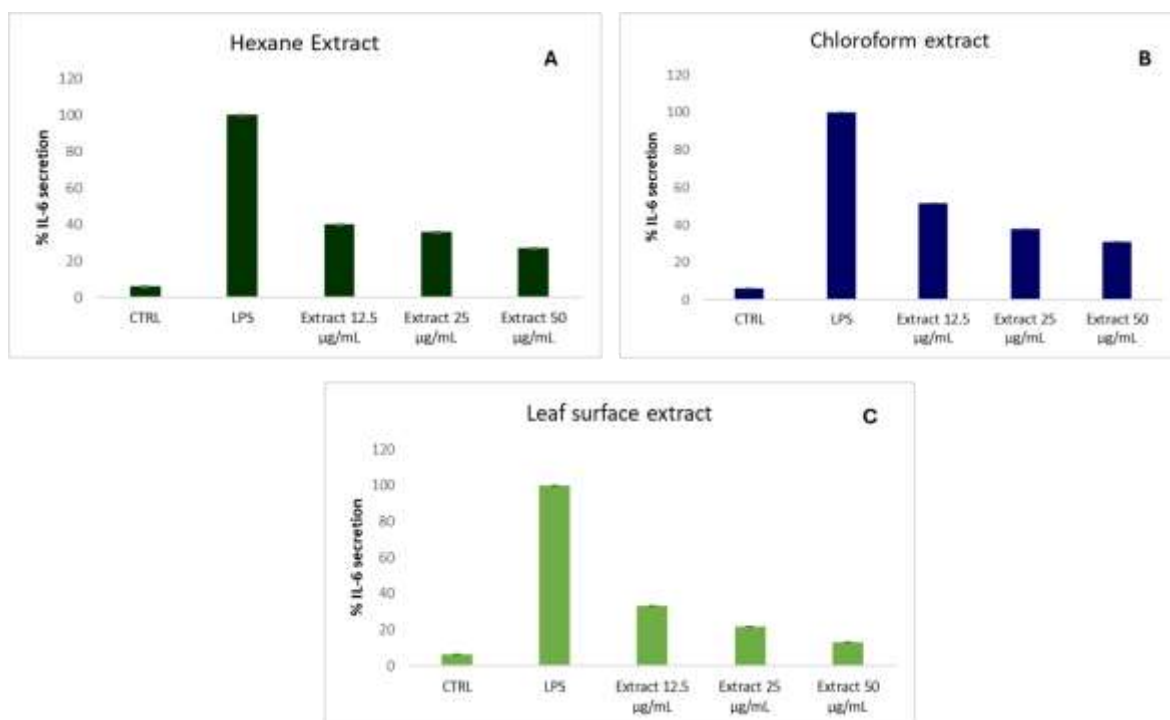


Figure 24. Analysis of IL-6 secretion in THP-1 cell line. THP-1 were stimulated with 0.1 μg/mL LPS for 3 h and then incubated with Hexane extract (A), Chloroform extract (B), and Leaf surface extract (C) (12.5 μg/mL to 50 μg/mL) for 6 h. ELISA were performed to measure the concentration of IL-6 released after LPS stimulation.

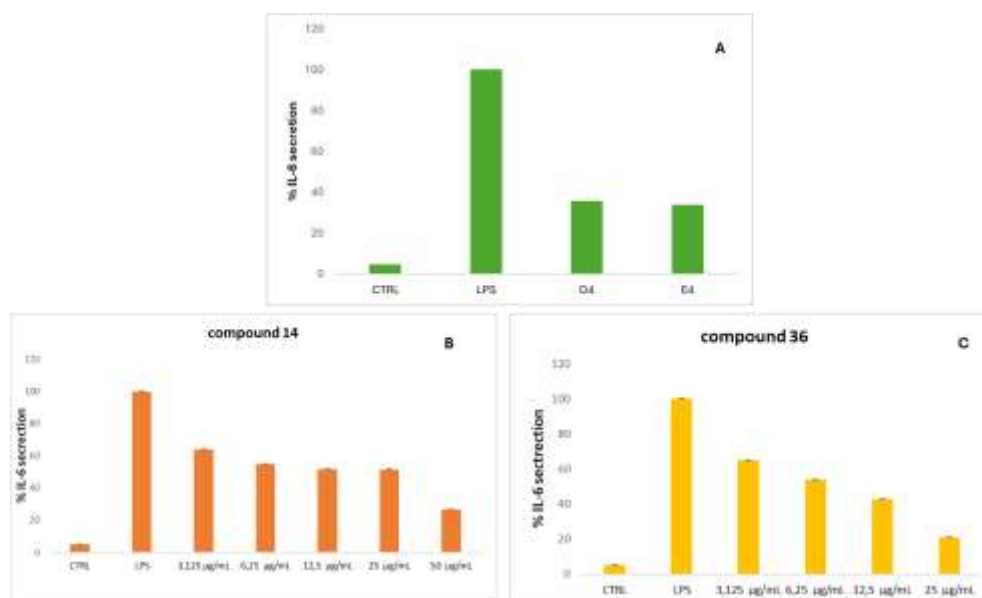


Figure 25. Analysis of IL-6 secretion in THP-1 cell line. THP-1 were stimulated with 0.1 μg/mL LPS for 3 h and then incubated with fractions D₄ and E₄ (25 μg/mL) (A) and compound 14 (B) and 36 (C) (3.125 μg/mL to 50 μg/mL) for 6 h were performed to measure the concentration of IL-6 released after LPS stimulation.

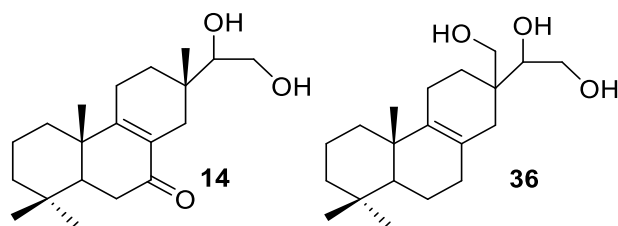


Figure 26, Structures of compounds **14** and **36**

5.4 Conclusion

The phytochemical investigation of *Lavandula pubescens* revealed that this species represents an exceptionally rich source of diterpenoids. Seven new diterpenes previously unreported in the literature were isolated from the surface extract of *L. pubescens*, obtained from the aerial parts, along with seven known compounds. The anti-inflammatory potential of the extract, of some of the fractions, and of two of the isolated diterpenes 15,16-dihydroxy-7-oxopimar-8(9)-ene (**14**) and 15,16,17-trihydroxypimar-8(9)-ene (**36**) was highlighted. In addition, the phytochemical study of *L. pubescens* aerial parts led to the isolation of nine new diterpenes (**1-9**), and six new dimeric monoterpenes (**15-20**). The *L. pubescens* chloroform extract and its active fraction C₂ showed antimicrobial activity, mainly against *S. epidermidis* and *L. monocytogenes*. Compounds 15,16-dihydroxy-isopimaran-8(9)-ene (**13**), ursolic and oleanolic acid (**22-23**) were demonstrated the main active components. The results of this thesis project highlight the potential of *L. pubescens*, whose pruning waste could be used as a source of bioactive molecules, combining its ornamental use in urban parks, the ecological role of the native species in its natural habitat, and its health benefits.

5.5 Appendix

Compound **3** has three unknown stereocenters, therefore 8 stereoisomers are possible. However, NMR data show a *trans* junction of the fused six membered ring system, which allow to consider only the four *trans* stereoisomers for the computations. Moreover, giving that **a** and **c**, **b** and **d** are enantiomeri pairs, computations can be carried out on only two the diastereomers **3-a** and **3-d** epimeric at the exocyclic stereocenter.

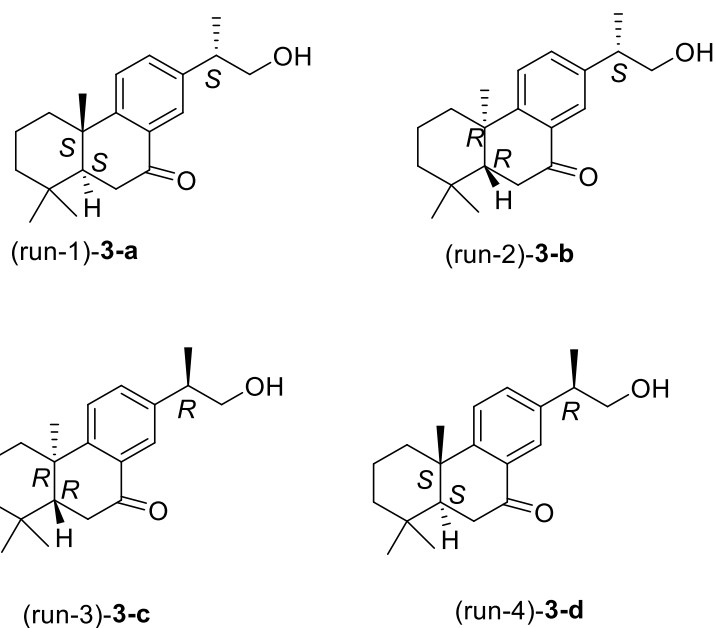


Figure A1. Structures of compound **3** used for calculations.

Table A1. Conformers Boltzmann distribution of **3-a**

DFT/B3LYP/def2-TZVP (CPCM=Methanol)		
Conformers	ΔG (Kcal/mol)	% pop
1	0	16.59
2	0.031	15.76
3	0.299	10.02
4	0.304	9.93
5	0.338	9.37
6	0.37	8.89
7	0.525	6.84
8	0.57	6.33
9	0.642	5.61
10	0.829	4.09
11	0.954	3.32
12	1.319	1.79
13	1.443	1.45

Table A2. Conformers Boltzmann distribution of **3-d**.

DFT/B3LYP/def2-TZVP (CPCM=Methanol)		
Conformers	ΔG (Kcal/mol)	% pop
1	0	14.92
2	0.001	14.90

3	0.006	14.77
4	0.262	9.58
5	0.268	9.49
6	0.313	8.79
7	0.448	7.00
8	0.509	6.32
9	0.522	6.18
10	0.906	3.23
11	0.97	2.90
12	1.214	1.92

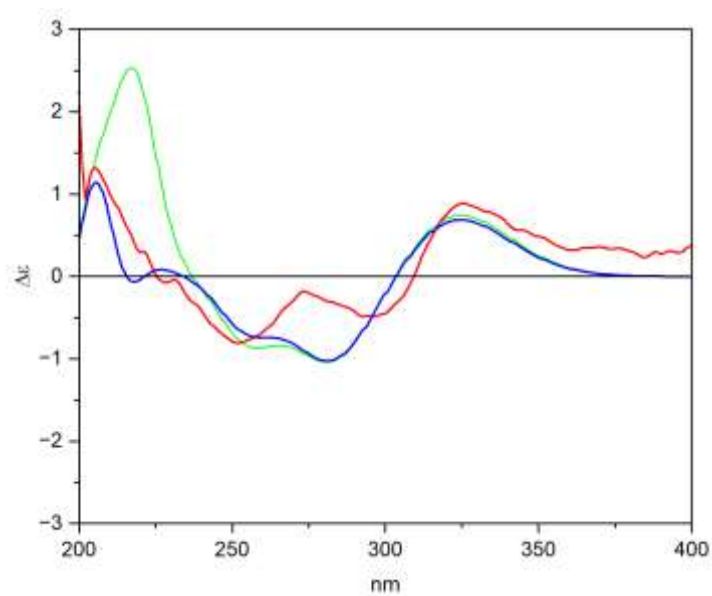


Figure A2. Experimental (red line) and computed ECD spectra for lpe2174-a (blue line) and lpe2174-d (green line). The computed spectra are shifted by +15nm and divided by 5 to facilitate the visual comparison.

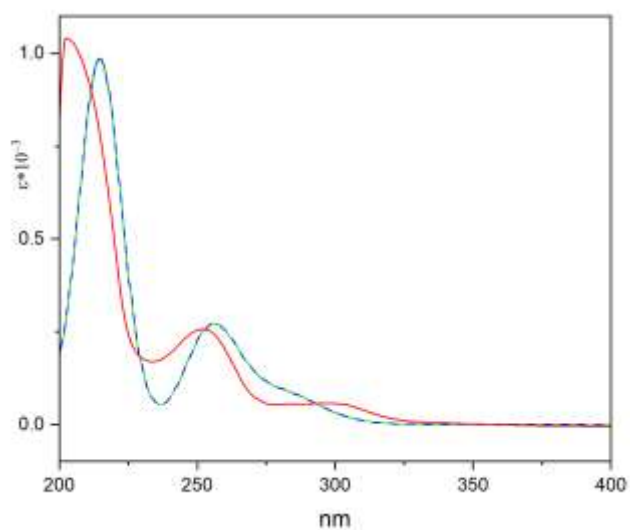


Figure A3. Experimental (red line) and computed Uv-vis spectra for **3-a** (solid blue line) and **3-d** (dashed green line). The computed spectra are shifted by +15nm to facilitate the visual comparison.

The experimental ECD of **3** displayed four main cotton effect (CE): a positive CE at 326 nm, two negative CEs at 296 nm and 256 nm and the last positive one at 205 nm. In Figure 2 and 3 the comparison between the experimental and computed ECD and UV spectra for **3-a** and **3-d** is reported. The two diastereoisomers differ for the AC of the C-x, located on the side chain bonded to the phenyl group. From the molecular orbital (MO) analysis, it is possible to determine the origin and nature of the transitions involved in the generation of the ECD spectra (Figure A4 and A5). Out of the 100 states taken into account for the TDDFT calculation, only the first 7 played a role in the simulation (Figure A4, A6, and A7). The first three bands are well reproduced in both **3-a** and **3-D** by the transitions 1, 2 and 3, which are nearly identical in energy and intensity in the two diastereomers (Figure A4, A6, and A7). Transition 1 is a HOMO-2 $\rightarrow$ LUMO transition with a main $n\text{-}\pi^*$ character and therefore is mainly located on the carbonyl group; transitions 2 and 3 are the HOMO and HOMO-1 $\rightarrow$ LUMO transitions, representing the two orthogonal $\pi\text{-}\pi^*$ transitions of the phenyl ring and being mainly affected by the AC of the two junction carbons. All the other electronic transitions of the two epimers also coincide in energy, but some of them, in particular transitions 4 and 7, involving orbital on the benzylic stereocenter, show different intensity in the two diastereomers. In fact, the HOMO-3 $\rightarrow$ LUMO transition 4 shows a stronger rotational strength in **3-d** than in **3-a**, while the opposite holds for the HOMO-1 $\rightarrow$ LUMO+1 transition 7, which have a stronger rotational strength in **3-a** (Figure A7). This behavior is reflected in the different appearance of the ECD spectra of the two epimers in the 200-220 nm range. There, **3-d** shows a quite intense band, determined by the stronger transition 4, while the ECD spectrum of **3-a** shows a much better agreement with the experimental. (Figure A6). This result than allows indicating the AC of the **3-a** diastereomer as that of the isolated natural product.

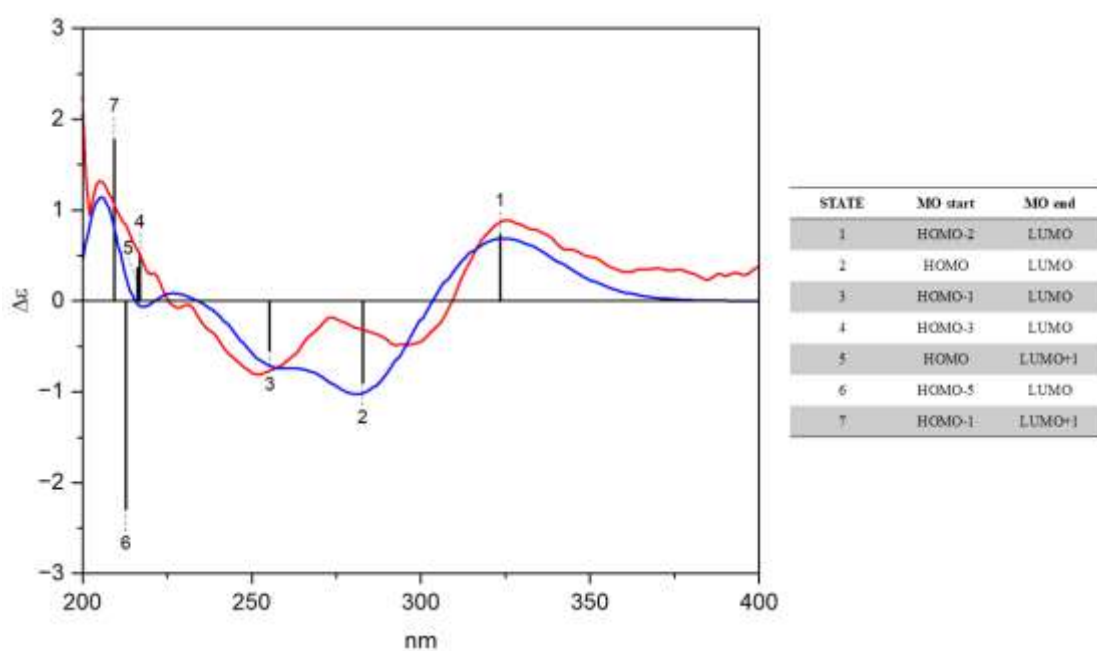


Figure A4. Experimental (red line) and computed ECD spectra for lpe2174-a (blue line) and the computed Rotational strength for **3-a** (black bars). The computed spectra are shifted by +15nm and divided by 5 to facilitate the visual comparison.

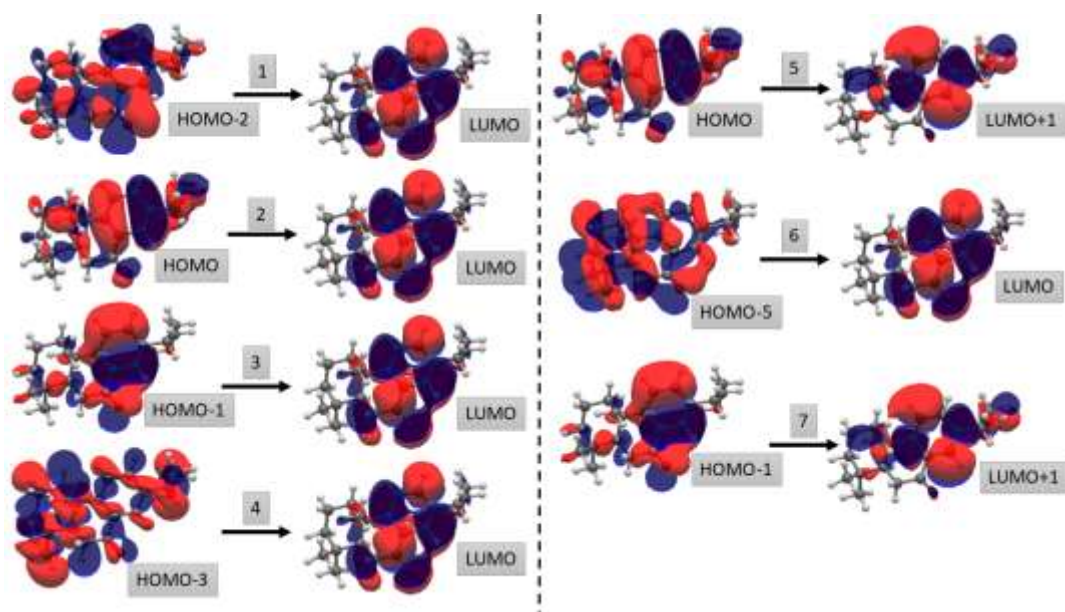


Figure A5. Computed Mos of the first 7 transition states of **3-a** C1, the most populated conformer.

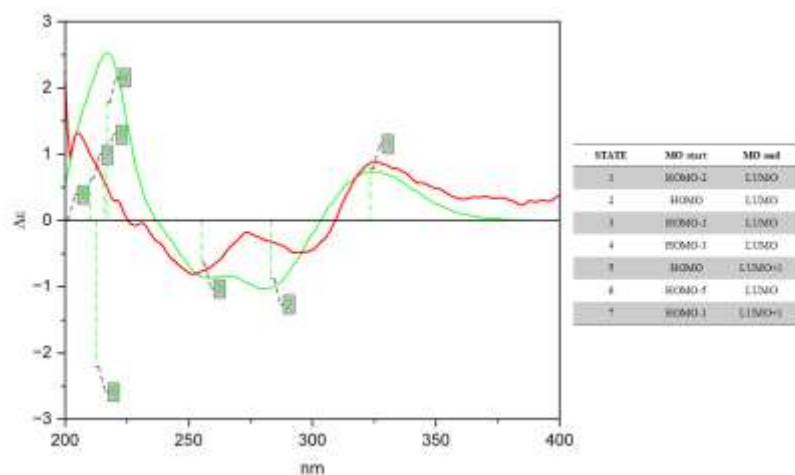


Figure 6. Experimental (red line) and computed ECD spectra for **3-d** (green line) and the computed Rotational strength for **3-d** (green bars). The computed spectra are shifted by +15nm and divided by 5 to facilitate the visual comparison.

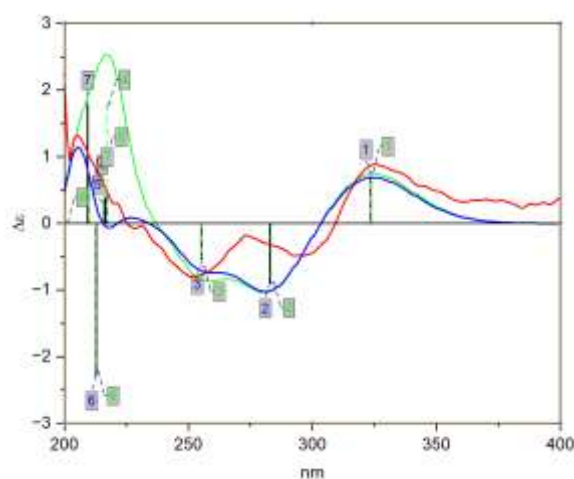


Figure A7. Experimental (red line) and computed ECD spectra for **3-a** (blue line) and **3-d** (green line). The computed spectra are shifted by +15nm and divided by 5 to facilitate the visual comparison.

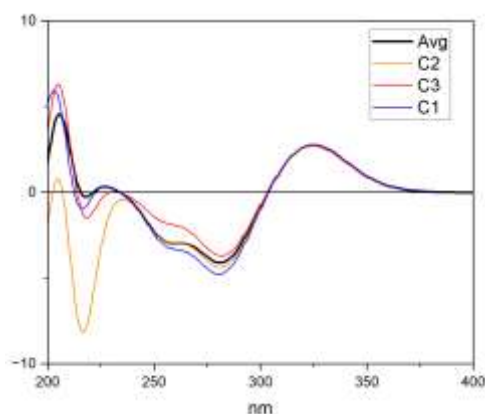


Figure A8. Computed ECD spectra for the most populated conformers of **3-a**: average (black line), conformer 1 (blue line), conformer 2 (red line), conformer 3 (orange line).

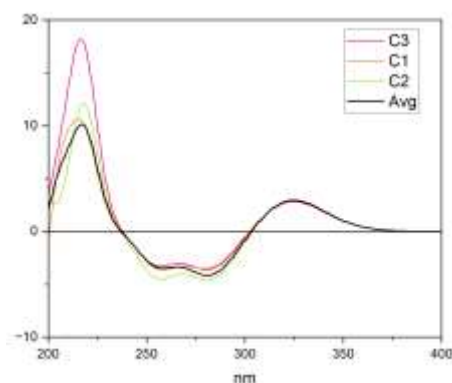


Figure A9. Computed ECD spectra for the most populated conformers of **3-d**: average (black line), conformer 1 (orange line), conformer 2 (green line), conformer 3 (pink line).

Computational details.

Starting from **3-a** a conformational search has been performed with ORCA 6.0.1 program (Neese, 2022) at GOAT level of theory (Bannwarth et al., 2019) providing 15 conformers with a Boltzmann population above 1%. Those conformations were further optimized at DFT/B3LYP/def2-TZVP/CPCM (methanol) level of theory obtaining a final number of 13 conformers with a >1% population. All conformers are real minima; no imaginary vibrational frequencies have been found and the free energy values have been calculated and used to get the Boltzmann population of conformers at 298.15 K. Those conformations were used to perform ECD calculation at TDDFT/CAM-B3LYP/def2-TZVP/CPCM (methanol) level of theory (Neese & Olberich, 2022). TDDFT calculations employing the long-range corrected CAM-B3LYP functional (Yanai et al., 2004) provided good reproduction of Cotton effects observed in the experimental ECD spectra. The theoretical UV and ECD spectra were obtained as average over the conformers Boltzmann populations. The solvent used for calculations with the CPCM formalism was Methanol (Garcia-Rates & Neese, 2020). The ECD spectra were obtained from calculated excitation energies and rotational strengths, as a sum of Gaussian functions centered at the wavelength of each transition, with a parameter σ (width of the band at $\frac{1}{2}$ height) of 0.5 eV. To guarantee origin independence and to evaluate the quality of the molecular wave functions employed, theoretical ECD spectra were obtained both in the length and velocity representation, using the lowest 100 states. The velocity/length calculated spectra were almost coincident, indicating a good level of calculation. Therefore, in all figures only the velocity-form predicted spectra are reported. The ORCA_mapspc module has been used to generate all the spectra.

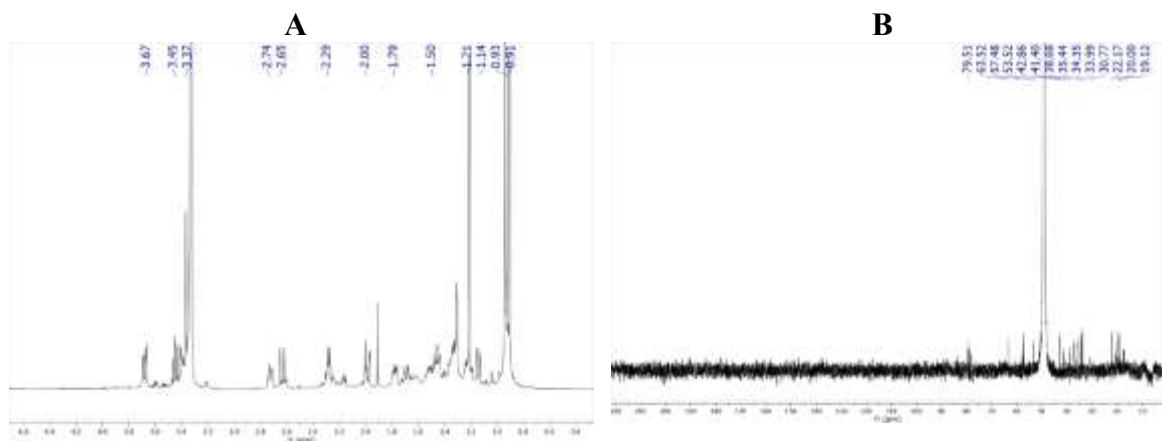


Figure A10. ^1H NMR (A) ^{13}C NMR (B) spectra of compound 1

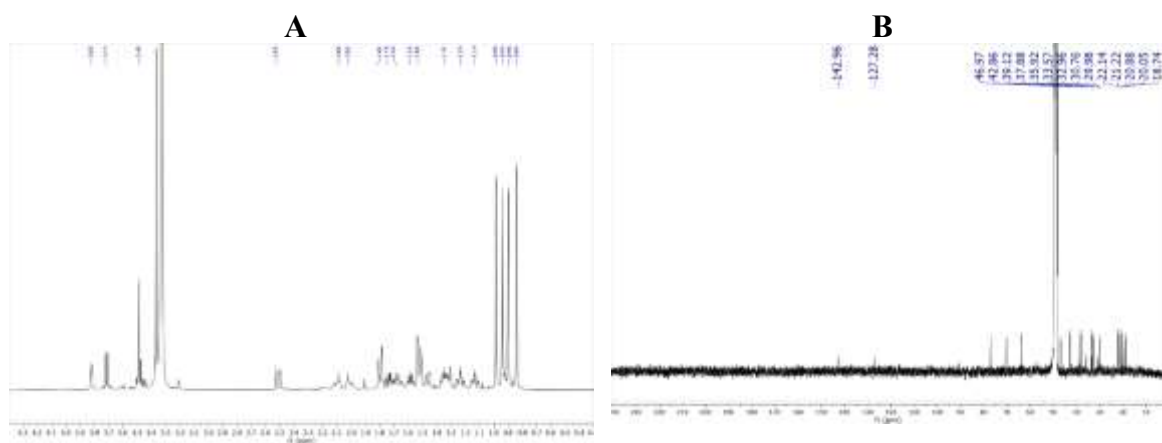


Figure A11. ^1H NMR (A) ^{13}C NMR (B) spectra of compound 2

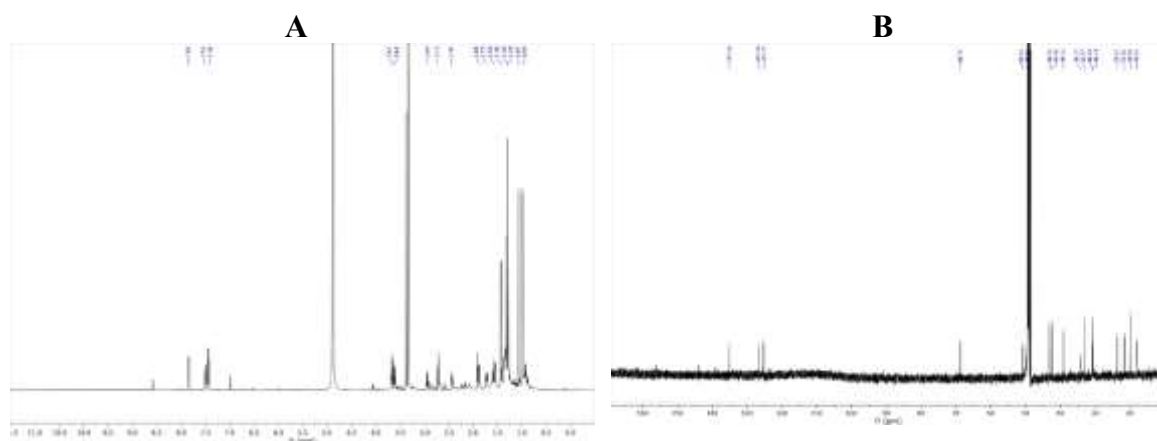


Figure A12. ^1H NMR (A) ^{13}C NMR (B) spectra of compound 3

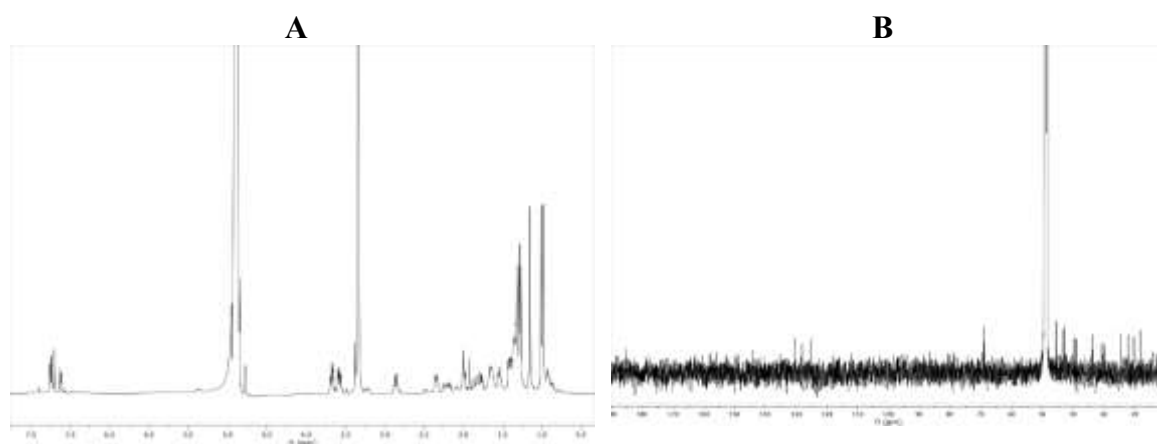


Figure A13. ^1H NMR (A) ^{13}C NMR (B) spectra of compound 4

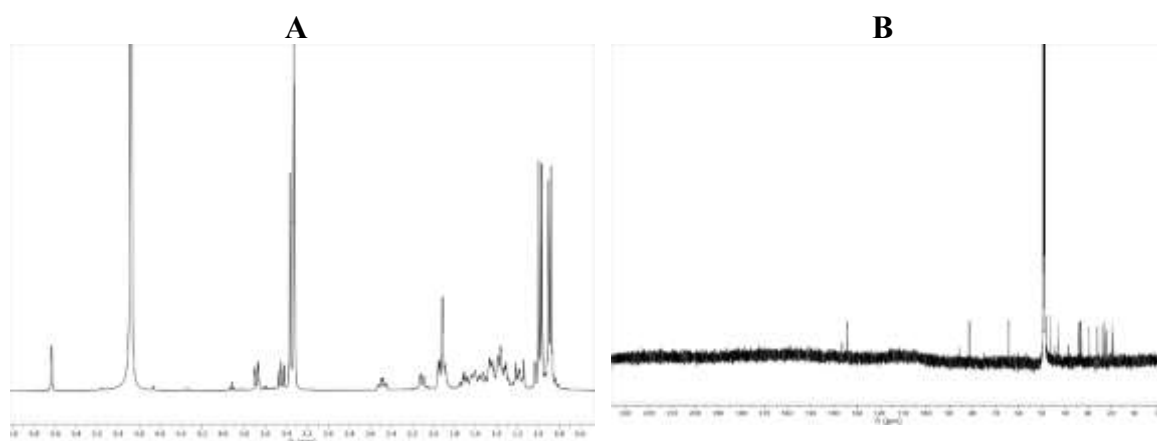


Figure A14. ^1H NMR (A) ^{13}C NMR (B) spectra of compound 5

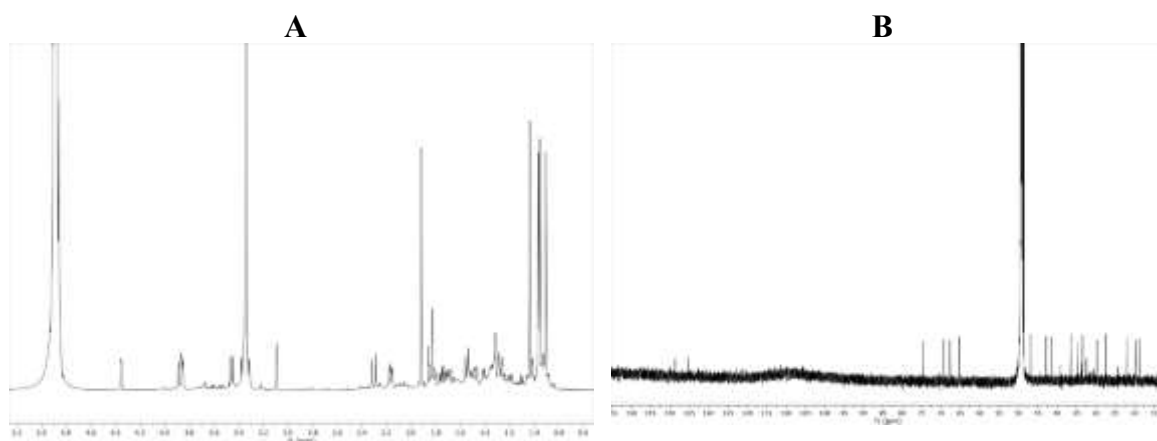


Figure A15. ^1H NMR (A) ^{13}C NMR (B) spectra of compound **6**

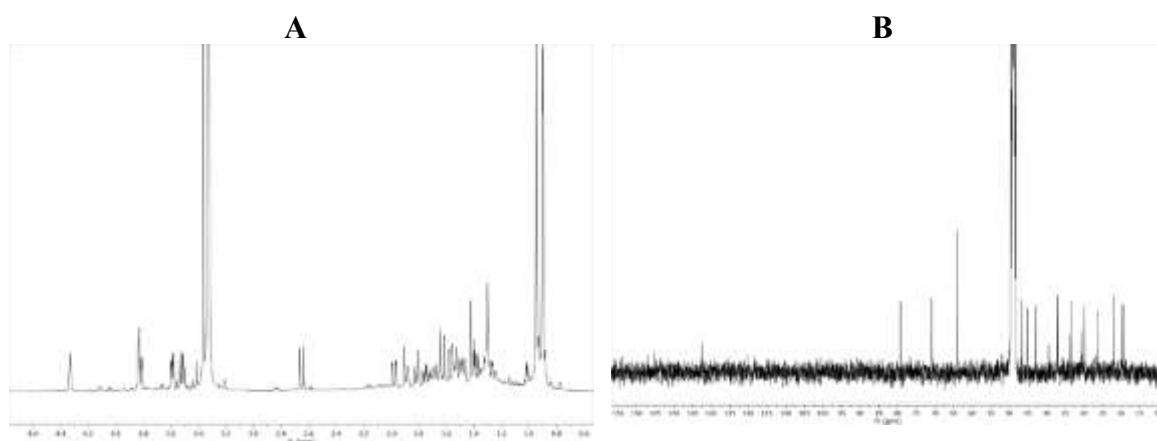


Figure A16. ^1H NMR (A) ^{13}C NMR (B) spectra of compound **7**

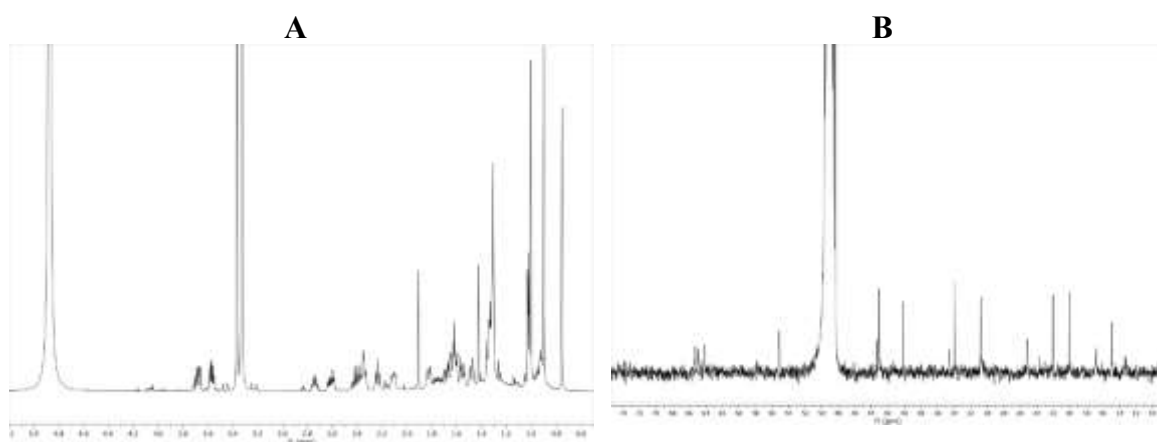


Figure A17. ^1H NMR (A) ^{13}C NMR (B) spectra of compound **8**

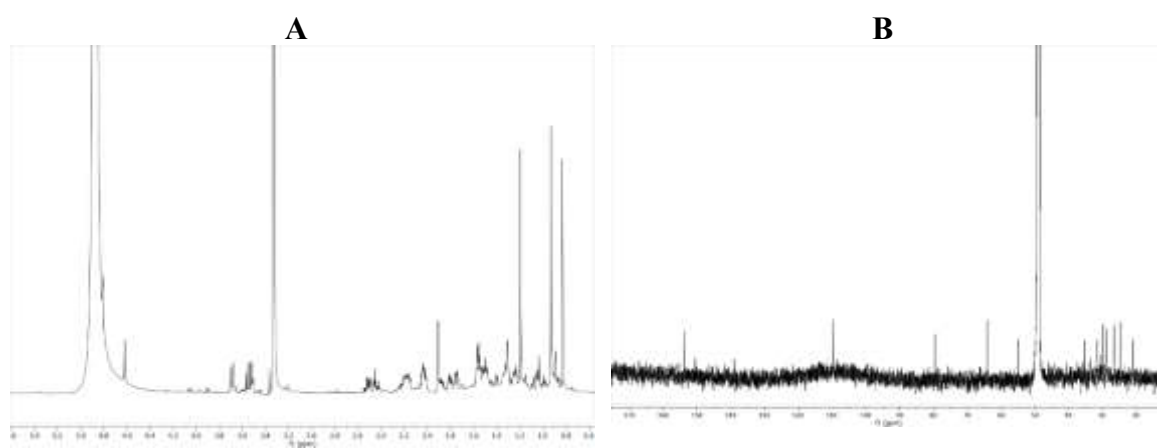


Figure A18. ^1H NMR (A) ^{13}C NMR (B) spectra of compound **9**

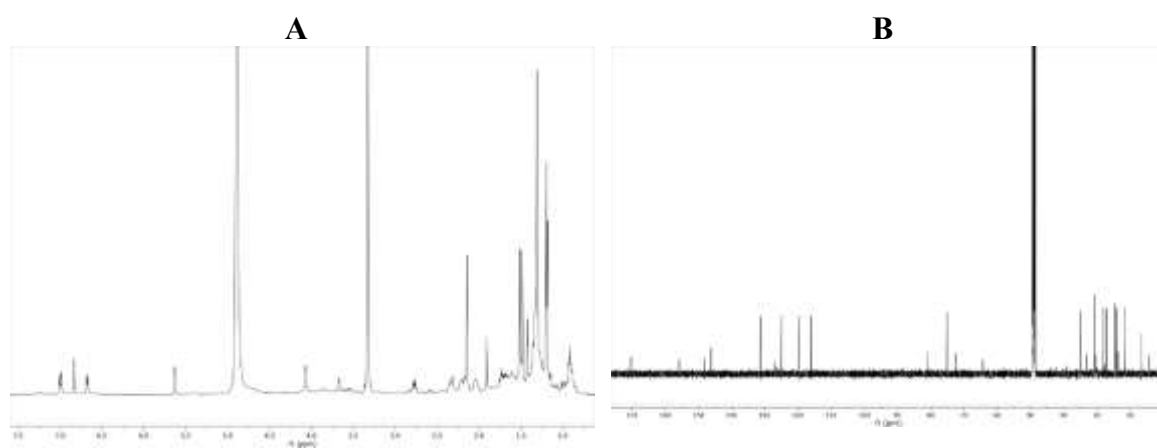


Figure A19. ^1H NMR (A) ^{13}C NMR (B) spectra of compound **15**

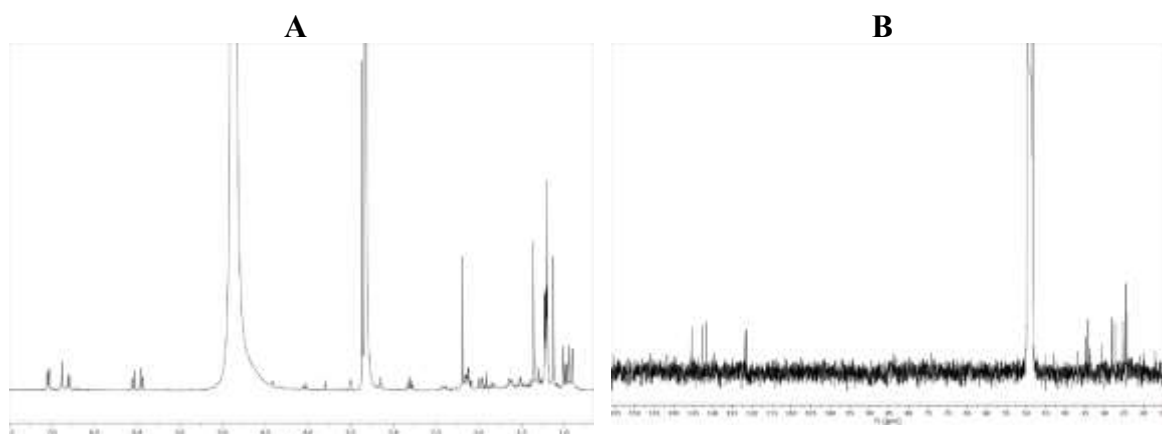


Figure A20. ^1H NMR (A) ^{13}C NMR (B) spectra of compound **16**

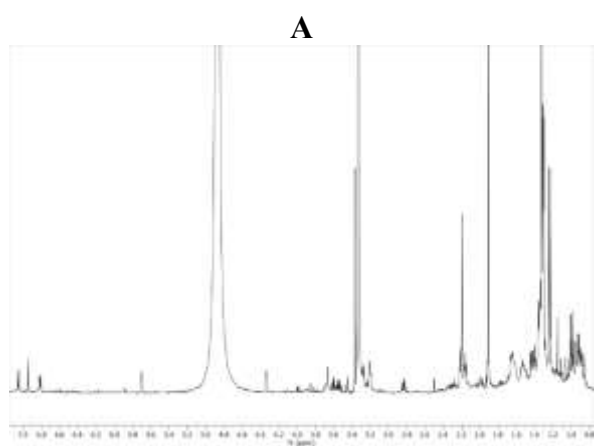


Figure A21. ^1H NMR (A) spectrum of compound **17**

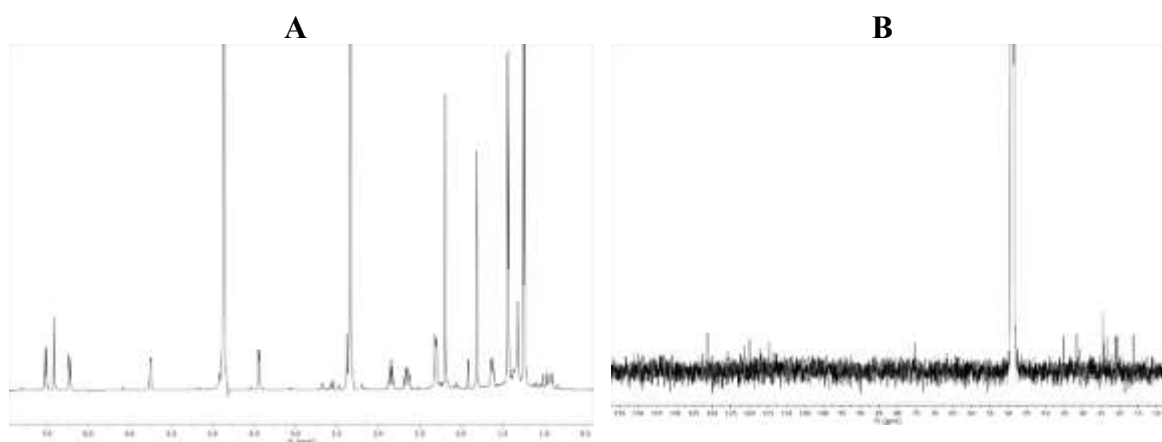


Figure A22. ^1H NMR (A) ^{13}C NMR (B) spectra of compound **18**

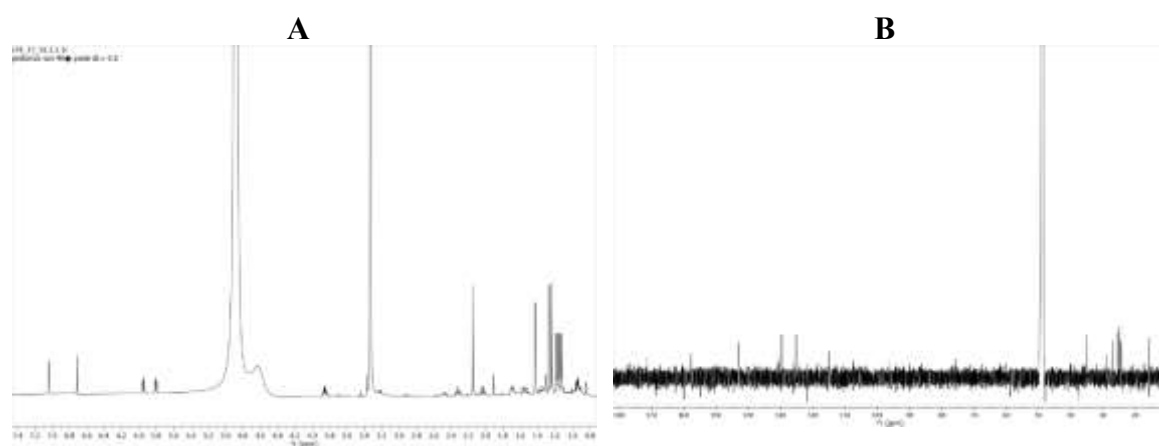


Figure A23. ^1H NMR (A) ^{13}C NMR (B) spectra of compound 19

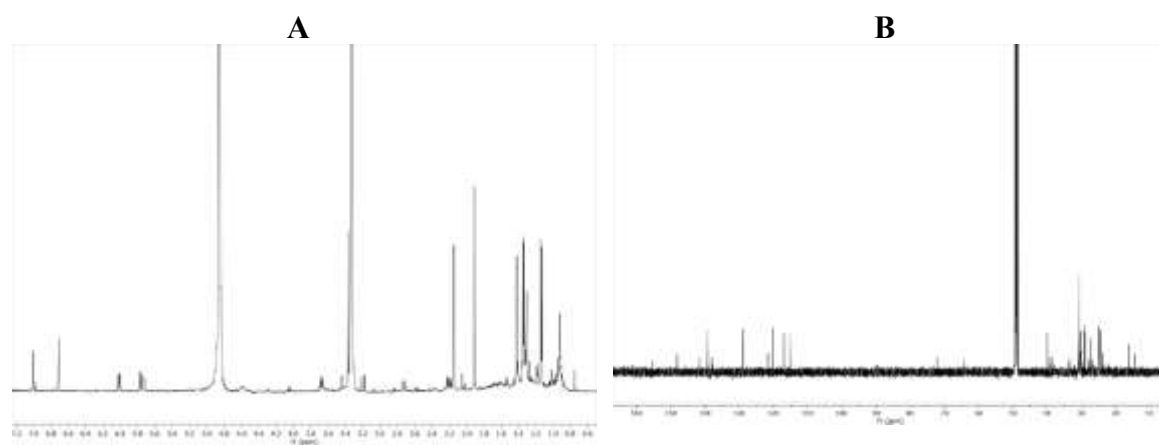


Figure A24. ^1H NMR (A) ^{13}C NMR (B) spectra of compound 20

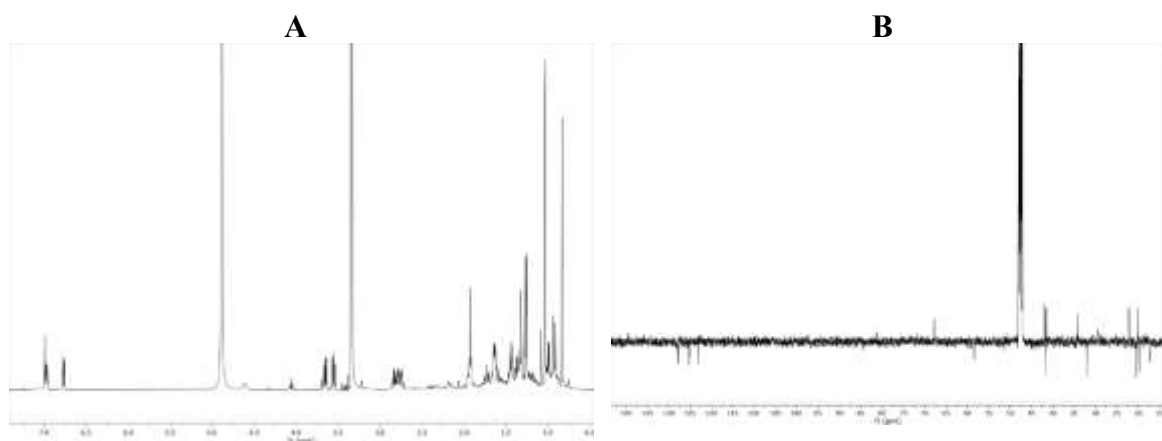


Figure A25. ^1H NMR (A) ^{13}C NMR (B) spectra of compound 29

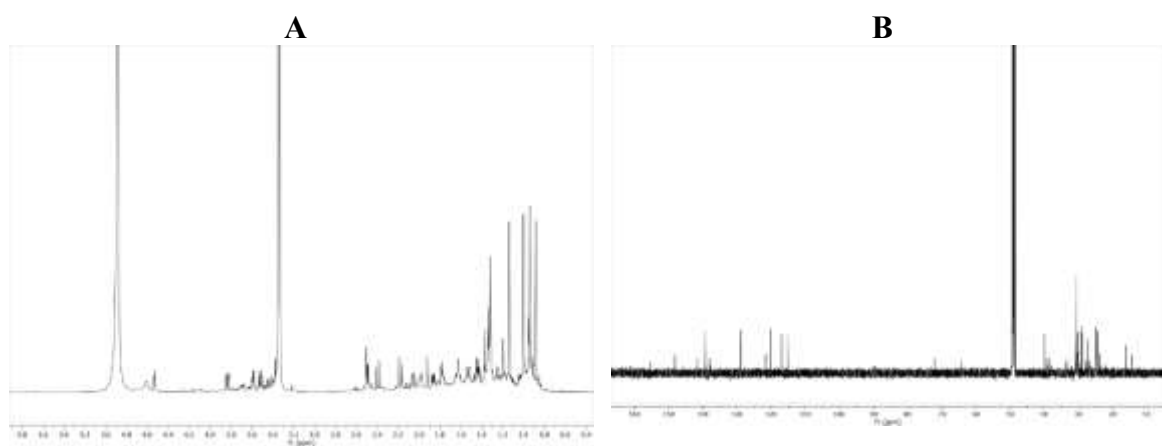


Figure A26. ^1H NMR (A) ^{13}C NMR (B) spectra of compound 30

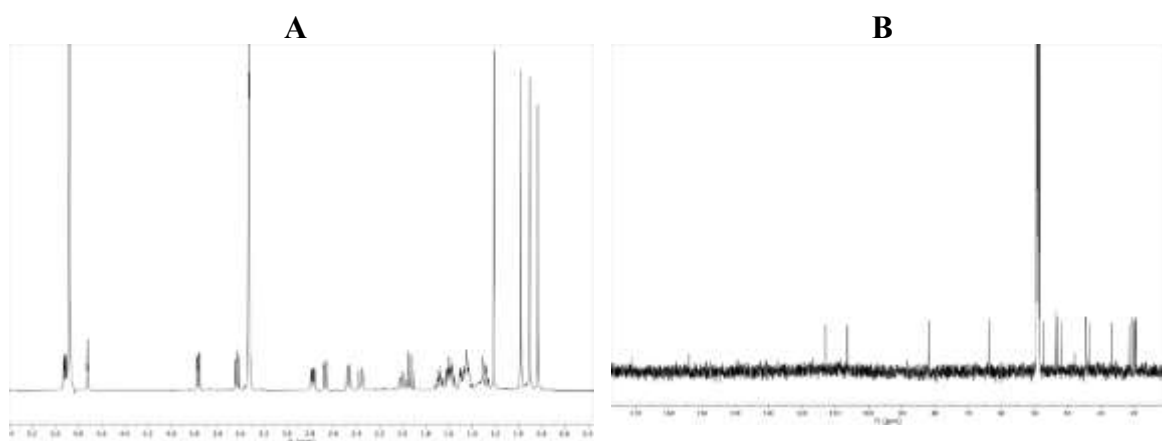


Figure A27. ^1H NMR (A) ^{13}C NMR (B) spectra of compound 31

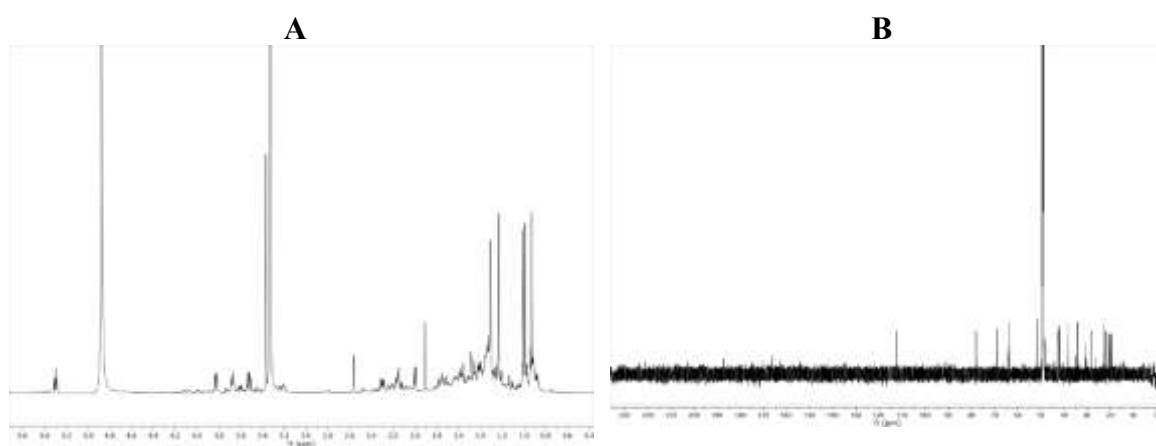


Figure A28. ^1H NMR (A) ^{13}C NMR (B) spectra of compound **32**

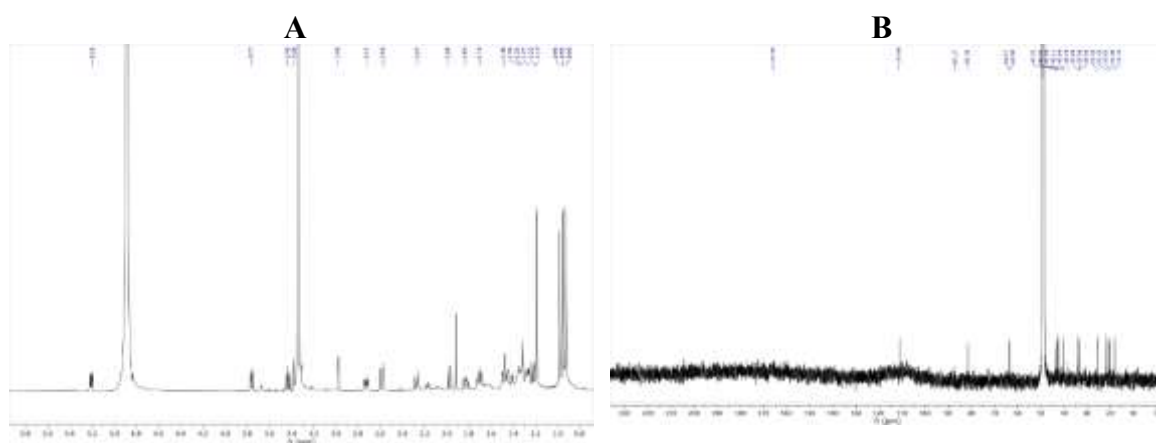


Figure A29. ^1H NMR (A) ^{13}C NMR (B) spectra of compound **33**

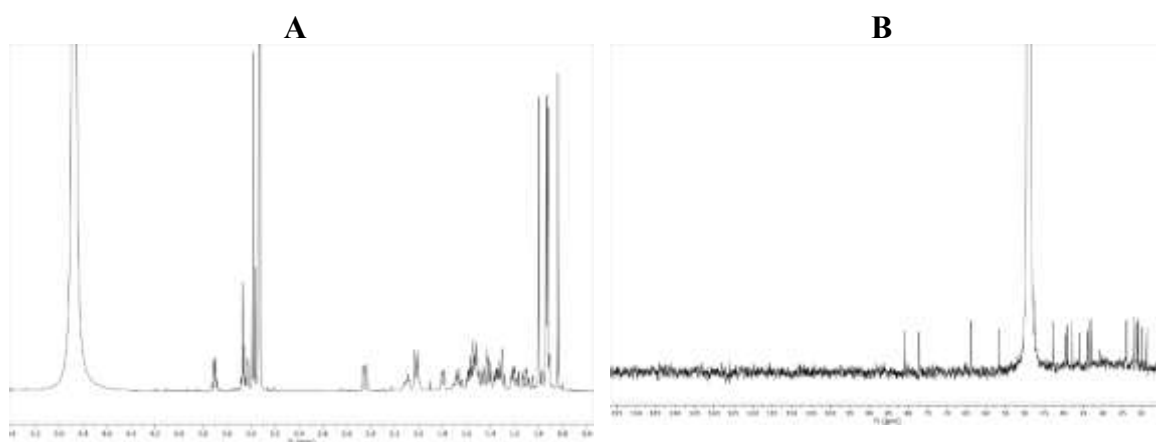


Figure A30. ^1H NMR (A) ^{13}C NMR (B) spectra of compound **34**

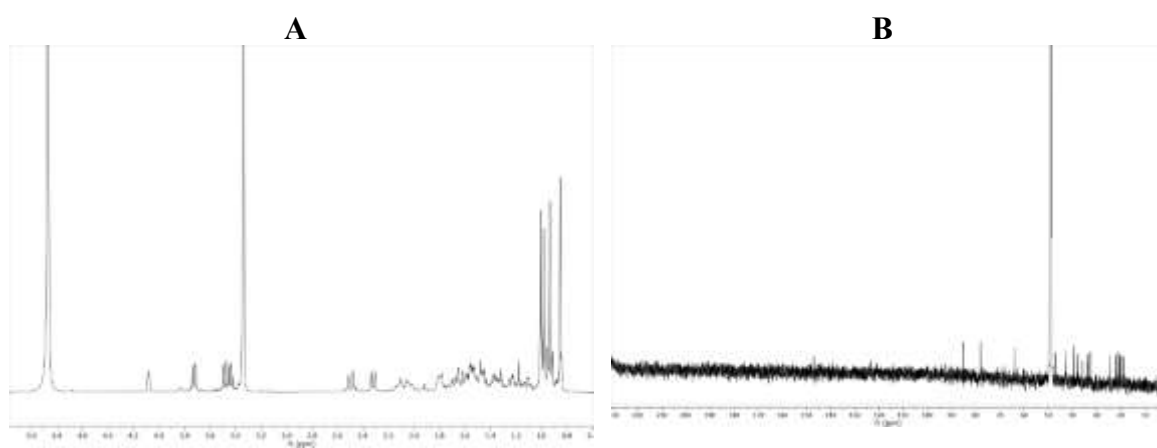


Figure A31. ^1H NMR (A) ^{13}C NMR (B) spectra of compound 35

Chapter 6

Conclusions and future prospective

This PhD project was carried out an in-depth investigation on the valorization of wild edible and medicinal plants, combining phytochemical investigation with biological studies. By applying advanced approaches together with bioactivity-guided fractionation and chemotaxonomic observations, this study shows that many understudied species can provide valuable natural compounds with relevant bio-activities. The study on the *Crataegus* ssp. confirmed that flowers and fruits contain a wide range of C- and O-glycosylated flavonoids. These constituents displayed antioxidant activity, antimelanogenic effects, and inhibition of α -amylase and α -glucosidase. The results suggest that *Crataegus* species could be promising candidates for nutraceutical or cosmeceutical applications. They also expand our knowledge of the chemical diversity within the genus.

The reinvestigation of *Cedrela odorata* led to the isolation of two new 8', 9'-bis-neolignans, which showed potential antiangiogenic effects in the *Danio rerio* model of antiangiogenic activity and may offer new possibilities for developing antiangiogenic drugs based on neolignans.

Finally, the comprehensive phytochemical profiling of the leaf-surface exudate and aerial-part extracts of *Lavandula pubescens* resulted in the isolation and spectroscopic characterisation of fifteen previously unreported diterpenoids, as well as six structurally novel dimeric monoterpenes. The anti-inflammatory and antibacterial activities observed, particularly the reduction of IL-6 production in THP-1 macrophages and the inhibition of *Staphylococcus epidermidis*, highlight the biological potential of the plant's extracts and purified compounds. Identifying the active fractions and molecules responsible for these effects further illustrates the strength of a bioactivity-guided approach. Overall, the findings of this thesis demonstrate the potential of wild medicinal plants as sources of structurally diverse compounds with promising biological properties.

Although this project has significantly expanded the chemical and biological knowledge of the investigated species, several research directions remain essential to further advance and translate these findings. The most active compounds, such as the neolignans from *Cedrela odorata* and the isolated diterpenoids from *Lavandula pubescens*, merit deeper studies. Target identification, pathway mapping, and structure–activity relationship

analyses will be important for determining their therapeutic importance. In addition, because of the complexity of plant matrices, synergy or complementary effects of these plant extracts and pure components may be demonstrated, resulting in potential increases in antimicrobial and anti-inflammatory activity.

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Acquired techniques

Use of analytical and semipreparative HPLC and BIOTAGE. the use of LTQ-Orbitrap XL Mass Spectrometer (Thermo Scientific®). The use of database for bibliographic research and of software for data analyses. Analysis of chemical profiles using NMR, GC–MS, LC–MS, and HPTLC.

Publications

- Mirabile, S., D'Angelo, V., Germanò, M.P., **Pouramin Arabi, S.**, Parisi, V., Raimondo, F.M. and Rosa, E., **2023**. Chemical Profile and Health-Promoting Activities of *Crataegus laciniata* (Rosaceae) Flowers. *Plants*, 13(1), p.34. <https://doi.org/10.3390/plants13010034>
- Iobbi, V.; **Pouramin Arabi, S.**; Bisio, A.; D'Angelo, V.; Germanò, M. P.; Braca, A.; De Tommasi, N. 8',9'-Bis-nor-neolignans from *Cedrela odorata* and their antiangiogenic activity. *Phytochemistry Letters* **2024**, 64, 13–18. <https://doi.org/10.1016/j.phytol.2024.09.002>
- Cacciola, A., **Pouramin Arabi, S.**, D'Angelo, V., Donadio, G., Maria Raimondo, F., Santoro, V., Rosa, E., Germanò, M. P., & De Tommasi, N. (n.d.). Polyphenolic characterization, antioxidant and antidiabetic activities of *Crataegus spp.* fruit extracts. 2025, Journal of Food Science:

Attendance at congress/conferences/ schools

- "1°HR MS School "Metabolomics in natural product research, University of Catania, September. 22nd 2023. (Attended).
- Pouramin, S., Parisi, V. Bader A., Rosa E., and De Tommasi N. "**Isolation and biological evaluation of ent-pimarane diterpenoids from *Lavandula pubescens* Decne**" Presented at 1st Intersociety Congress of plant-based products for health; the role of medicinal plants in modern medicine: University of Padua, Jun ,15-17th 2023; Poster 255.
- 3rd HR-MS school " the Spectrometry Division of Mass of the Italian Chemical Society, University of Salerno, 30 th Nov-1 th Dec 2023.
- **Pouramin, S.**, Parisi, V., Bader, A., Rosa, E., and De Tommasi, N. "**Nano-phytosome delivery system loaded with *Crataegus laciniata* (Rosaceae) flowers extract: whitening properties**" Presented at 72 International Congress on Natural Products Research (GA), Krakow, July 13_17,2024; Poster 664.

- Parisi, V., **Pouramin, S.**, Nocera, R., Rosa, E., Bader, A., E., Gazzillo, E., Chini, M.G., Bifulco, G., and De Tommasi, N. **“New Diterpenes with Potential Antiinflammatory Activity from *Lavandula pubescens* Decne”** Presented at 25th Conference on Isoprenoids, Naples, September 17/19/09/2024; oral communication.
- **Pouramin, S.**, Parisi, V., Nocera, R., Rosa, E., Bader, A., and De Tommasi, N. **“Diterpenes with Potential Anti-Inflammatory Properties from *Lavandula pubescens* Decne”** Presented at 2nd Intersociety Congress on Plant-Based Products for Health, The Role of Medicinal Plants in Modern Medicine, Naples, April 10–12, 2025, Federico II Conference Center, Via Partenope 36. Oral Presentation (Speaker).
- **Pouramin, S.**, Parisi, V., Nocera, R., Veneziano, M., Severino, L., Scivicco, M., & Cacciola, N. A. **“*Lavandula dentata* L.: chemical composition and biological activity”** Presented at the 73 International Congress on Natural Products Research (GA), Naples, August 31_September 3, 2025; Poster 352.
- Cacciola, A., D’Angelo, V., Germanò, M.P., Mirabile, S., Raimondo, F.M., **Pouramin Arabi, S.**, Rosa, E., Santoro, V., De Tommasi, N., Chiocchio, I., Mandrone, M., Trincia, S., Sanna, C., Mastinu, A., Abate, G., & Sweeney, E.M. **“Ferruccio Poli Polyphenolic characterization, antioxidant and antidiabetic activities of *Crataegus* spp. fruit extracts”** presented at the 119° Congresso della Società Botanica Italiana X INTERNATIONAL PLANT SCIENCE CONFERENCE (IPSC) TERAMO, 11 - 13 SEPTEMBER 2024; Poster 106.
- Benayache, F., **Pouramin, S.**, Fadel, H., Donadio, G., Nocera, R., Parisi, V., D’Angiolo, M., Braca, A., & De Tommasi, N. **“Antibacterial activity and phytochemical characterisation of aerial parts of *Cedrus atlantica*”** Presented at the 73 International Congress on Natural Products Research (GA), Naples, August 31_September 3, 2025.
- Rosa, R., Mirabile, S., Valeria D’Angelo, Paola Germanò, M., **Pouramin Arabi, S.**, & Parisi, V., **“Pharmaceutical and cosmetic potential of *crataegus laciniata* (Rosaceae) flowers extract”** Presented at 4th MS-NatMed Day, Salerno, Italy, February, 15-16, 2024; Poster 06.

