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Ph.D. Thesis

NATURAL PRODUCTS CHEMISTRY: ISOLATION, BIOACTIVITY, AND FUNCTIONAL POTENTIAL OF SECONDARY METABOLITES FROM PLANT SOURCES

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PREFACE

The three-year Ph.D. was conducted at the Department of Bioscience and Territory of the University of Molise, under the supervision of Profs. Maria Giovanna Chini, Maria Iorizzi and Gabriella Saviano, starting in November 2022.

The research activity focused on the chemical characterisation and biological evaluation of natural products, with particular attention to plant-derived extracts and essential oils, as well as to the characterisation of their bioactive constituents with potential nutraceutical and pharmacological relevance.

During my Ph.D., I conducted phytochemical investigations on several plant species, mainly from the Mediterranean area, using an integrated analytical approach for the characterisation of complex natural matrices. These studies enabled the identification and description of the main components responsible for the observed biological activities.

The chemical data obtained were integrated with biological studies aimed at evaluating antioxidant properties, as well as the anti-inflammatory and neuroprotective potential of selected plant extracts and essential oils. In selected cases, antifungal activity against wood-degrading fungi was also assessed. In some cases, computational approaches were employed to support experimental findings and to explore possible molecular mechanisms underlying the observed bioactivities, such as the modulation of inflammation-related targets.

Overall, the Ph.D. project was based on a multidisciplinary approach combining phytochemistry, analytical chemistry, biological evaluation and, when appropriate, *in silico* studies. This integrated strategy contributed to a deeper understanding of the chemical and biological properties of natural products and highlighted their potential applications in the nutraceutical, pharmaceutical and cosmetic fields.

PUBLICATIONS

This Thesis covers the following published papers (2022 - 2025):

1. Caprari, C., **Fantasma, F.**, Monaco, P., Divino, F., Iorizzi, M., Ranalli, G., Fasano, F. and Saviano, G. (2023). Chemical Profiles, *in vitro* antioxidant and antifungal activity of four different *Lavandula angustifolia* L. EOs. *Molecules*, *28*(1), 392. https://doi.org/10.3390/molecules28010392
2. **Fantasma, F.**, Samukha, V., Saviano, G., Chini, M. G., Iorizzi, M. and Caprari, C. (2024). Nutraceutical aspects of selected wild edible plants of the Italian Central Apennines. *Nutraceuticals*, *4*(2), 190-231. https://doi.org/10.3390/nutraceuticals4020013
3. **Fantasma, F.**, Samukha, V., Aliberti, M., Colarusso, E., Chini, M. G., Saviano, G., ... & Iorizzi, M. (2024). Essential oils of *Laurus nobilis* L.: From chemical analysis to *in silico* investigation of anti-inflammatory activity by soluble epoxide hydrolase (sEH) inhibition. *Foods*, *13*(14), 2282. https://doi.org/10.3390/foods13142282
4. **Fantasma, F.**, D'Urso, G., Martella, N., Capuano, A., Boccia, E., Samukha, V., De Felice, V., Saviano, G., Trombetta, F., Lauro, G., Segatto, M., Chini, M.G., Bifulco, G., Casapullo A. and Iorizzi, M. (2025). Chemical Profiling of Polyphenolic Fraction of *Cannabis sativa* L. vr. *Kompolti* Industrial Inflorescences: Insights into Cannabidiol Neuroprotective Effects in a Cellular Model of Parkinson's Disease. Plants, 14(10), 1473. https://doi.org/10.3390/plants14101473
5. D'Urso, G., Capuano, A., **Fantasma, F.**, Chini, M. G., De Felice, V., Saviano, G., Lauro, G., Casapullo, A., Bifulco, G. and Iorizzi, M. (2025). The Role of LC-MS in Profiling Bioactive Compounds from Plant Waste for Cosmetic Applications: A General Overview. Plants, 14(15), 2284. https://doi.org/10.3390/plants14152284
6. **Fantasma, F.**, Segatto, M., Colardo, M., Di Matteo, F., Chini, M. G., Iorizzi, M. and Saviano, G. (2025). Phytochemistry and Bioactivity of Essential Oil and Methanolic Extracts of *Origanum vulgare* L. from Central Italy. Plants, 14(16), 2468. https://doi.org/10.3390/plants14162468

7. **Fantasma, F.**, D'Urso, G., Capuano, A., Colarusso, E., Aliberti, M., Grassi, F., Brunese, M. C., Saviano, G., De Felice, V., Lauro, G., Reginelli, A., Chini, M. G., Casapullo, A., Bifulco, G. and Iorizzi, M. (2026). Potential Nutraceutical Properties of *Vicia faba* L: LC-ESI-HR-MS/MS-Based Profiling of Ancient Faba Bean Varieties and Their Biological Activity. Molecules, 31(1), 184. https://doi.org/10.3390/molecules31010184

Integrative scientific production (2023 - 2025):

1. Beatrice, P., Saviano, G., Reguzzoni, M., Divino, F., **Fantasma, F.**, Chiatante, D. and Montagnoli, A. (2023). Light spectra of biophilic LED-sourced systems modify essential oil composition and plant morphology of *Mentha piperita* L. and *Ocimum basilicum* L. *Frontiers in Plant Science*, 14, 1093883. https://doi.org/10.3389/fpls.2023.1093883
2. Notardonato, I., **Fantasma, F.**, Monaco, P., Di Fiore, C., Saviano, G., Giancola, C., Avino, P. and De Felice, V. (2023). The presence of ultra-traces of persistent organic pollutants (POPs) and heavy metals in some areas of Molise. *Toxics*, 11(3), 250. https://doi.org/10.3390/toxics11030250
3. Notardonato, I., **Fantasma, F.**, Monaco, P., Di Fiore, C., Saviano, G., Giancola, C., Avino, P. and De Felice, V. (2023). Trend of antioxidant activity and total phenolic content in wild edible plants as part of the environmental quality assessment of some areas in Central Italy. *Separations*, 10(5), 271. https://doi.org/10.3390/separations10020092
4. Samukha, V., **Fantasma, F.**, D'Urso, G., Caprari, C., De Felice, V., Saviano, G., Lauro, G., Casapullo, A., Chini, M. G., Bifulco, G. and Iorizzi, M. (2024). NMR metabolomics and chemometrics of commercial varieties of *Phaseolus vulgaris* L. seeds from Italy. *Plants*, 13(2), 227. https://doi.org/10.3390/plants13020227
5. Samukha, V., **Fantasma, F.**, D'Urso, G., Colarusso, E., Schettino, A., Marigliano, N., Chini, M. G., Saviano, G., De Felice, V., Lauro, G., Maione, F., Bifulco, G., Casapullo, A. and Iorizzi, M. (2024). Chemical profiling of polar lipids and the polyphenolic fraction of commercial Italian *Phaseolus* seeds by UHPLC-HRMS. *Biomolecules*, 14(10), 1336. https://doi.org/10.3390/biom14101336

6. Russo, M., Martella, N., Gargano, D., **Fantasma, F.**, Marcovecchio, C., Russo, V., Oliva, M. A., Segatto, M., Saviano, G., Di Bartolomeo, S. and Arcella, A. (2024). Lavender essential oil and its terpenic components negatively affect tumor properties in a cell model of glioblastoma. *Molecules*, 29(24), 6044. https://doi.org/10.3390/molecules29246044
7. Cavone, C., Monaco, P., **Fantasma, F.**, Rizzo, P., Tarracchini, C., Petraro, S., Ventura, M., Milani, C., Celico, F., Naclerio, G. and Bucci, A. (2025). Natural hydrocarbon-contaminated springs as a reservoir of microorganisms useful for bioremediation. Sustainability, 17(4), 1568. https://doi.org/10.3390/su17073083

SCIENTIFIC CONTRIBUTIONS AT CONFERENCES

Poster Presentations

1. **Fantasma F.**, Fortini P., Chini M.G., De Felice V., Iorizzi M., Saviano G. "A comparative study on chemical composition and antioxidant activities of essential oil and methanol extracts from seeds of *Daucus carota* L. subsp. *carota* growing in the central Apennines (Italy)".
   Applied Sciences, Biology and Territory. Termoli, December 20th, 2022.
2. Samukha V., **Fantasma F.,** Saviano G., De Felice V., Fortini P., Chini M.G., Iorizzi M. "Identification and Characterization of Constituents of *Malus domestica* and *Pyrus communis* of Molise Region, Evaluation of Bioactivity of Secondary Metabolites and Development of Nutraceuticals, Cosmeceuticals, Food Supplements and Cosmetics".
   Applied Sciences, Biology and Territory. Termoli, December 20th, 2022.
3. Samukha V., **Fantasma F.**, D'Urso G., Lauro G., Casapullo A., Bifulco G., Chini M.G. and Iorizzi M. "Development and optimization of the method for metabolomics analysis of legumes: the case study of commercial *Phaseolus vulgaris* varieties". *GIDRM Day – Quantitative and Non-Targeted NMR for the Protection of Health and Food*. Bari, June 16th, 2023.
4. **Fantasma F.**, Samukha V., Chini M.G., De Felice V., Saviano G. and Iorizzi M. "Evaluation of polyphenolic content and environmental pollutants in *Laurus nobilis* L. leaves from wild plants grown in different geographical areas of central Italy". *Science for the Planet*. Campobasso, June 28th - 30th, 2023.
5. Samukha V., **Fantasma F.**, Chini M.G., Saviano G., De Felice V. and Iorizzi M. "Green technologies for the extraction of phytoconstituents from natural sources". *Science for the Planet*. Campobasso, June 28th - 30th, 2023.
6. **Fantasma F.**, Samukha V., Di Matteo F., Chini M.G., De Felice V., Iorizzi M., Saviano G. "Comparative Analysis on Chemicals Profile of Essential Oil from Wild and Commercial *Origanum vulgare* Leaves and Flowers by GC-MS". *Scienza delle Separazioni 2023*. Termoli, Italy, October 12th - 13th, 2023.
7. Samukha V., **Fantasma F.**, D'Urso G., Lauro G., Casapullo A., Bifulco G., Chini M.G., Iorizzi M. "Analysis of Lipophilic Extracts of Commercial *Phaseolus vulgaris* L. Seeds Varieties by UHPLC-Mass Spectrometry and Multivariate Analysis".
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   31st International Symposium on the Chemistry of Natural Products & 11th International Congress on Biodiversity. Naples, October 15th - 19th, 2023.
9. **Fantasma F.**, Samukha V., Chini M.G., De Felice V., Iorizzi M., Saviano G. "Study of secondary metabolites from food and medicinal plants, *Laurus nobilis* L. and *Origanum vulgare* L., to identify new compounds of pharmaceutical and nutraceutical interest".

Applied Sciences, Biology and Territory. Isernia, December 18th, 2023.

10. Samukha V., **Fantasma F.**, D'Urso G., Caprari C., De Felice V., Saviano G., Lauro G., Casapullo A., Chini M.G., Bifulco G. and Iorizzi M. "Metabolomic profile of commercial *Phaseolus vulgaris* L. varieties by NMR spectroscopy and mass spectrometry".
    Applied Sciences, Biology and Territory. Termoli, December 20th, 2022.
11. **Fantasma F.**, Samukha V., Chini M.G., De Felice V., Iorizzi M., Saviano G. "Study of secondary metabolites from food and medicinal plants, *Laurus nobilis* L. and *Origanum vulgare* L., to identify new compounds of pharmaceutical and nutraceutical interest".
    PhD Expo – 2nd Edition. Campobasso, January 24th, 2024.
12. Samukha V., **Fantasma F.**, D'Urso G., Caprari C., De Felice V., Saviano G., Lauro G., Casapullo A., Chini M.G., Bifulco G. and Iorizzi M. "NMR metabolomics and chemometrics of commercial *Phaseolus vulgaris* L. seeds varieties from Italy".
    PhD Expo – 2nd Edition. Campobasso, January 24th, 2024.
13. **Fantasma F.**, Samukha V., Aliberti M., Colarusso E., Chini M.G., Saviano G., De Felice V., Lauro G., Casapullo A., Bifulco G., Iorizzi M. "Phytochemical analysis of *Laurus nobilis* L. leaves essential oil: rationalization of antioxidant and anti-inflammatory activities by combined *in vitro* and *in silico* approach".
    International Congress on Natural Products Research. Kraków, July 13th - 17th, 2024.
14. Samukha V., **Fantasma F.**, D'Urso G., De Felice V., Saviano G., Lauro G., Casapullo A., Bifulco G., Chini M.G., Iorizzi M. "NMR metabolomics and chemometrics of *Malus domestica* (Suckow) Borkh. varieties from Molise region of Italy".
    International Congress on Natural Products Research. Kraków, July 13th - 17th, 2024.
15. **Fantasma F.**, Samukha V., Aliberti M., Colarusso E., Chini M.G., Saviano G., De Felice V., Lauro G., Casapullo A., Bifulco G. and Iorizzi M. "Investigation analysis of the phytochemical composition of *Laurus nobilis* L. leaves essential oil: a study of the antioxidant and anti-inflammatory properties using a combination of *in vitro* and *in silico* methods".
    XXVIII National Congress of the Società Chimica Italiana. Milan, August 26th - 30th, 2024.
16. Samukha V., **Fantasma F.**, D'Urso G., De Felice V., Saviano G., Lauro G., Casapullo A., Bifulco G., Chini M.G., Iorizzi M. "Metabolomic analysis of *Malus domestica* (Suckow) Borkh. varieties from Molise region (Italy) by NMR and mass spectrometry".
    XXVIII National Congress of the Società Chimica Italiana. Milan, August 26th - 30th, 2024.
17. **Fantasma F.**, Samukha V., D'Urso G., Chini M.G., Grassi E., Saviano G., De Felice V., Lauro G., Grassi R., Bifulco G., Casapullo A. and Iorizzi M. "Preliminary Phytochemical and Metabolomic Analysis of Black and Purple *Vicia faba* L. Seeds:

In Vitro Antioxidant Activity and Mineral Composition of Cotyledons and Seed Coats".
PhD Expo – 3rd Edition. Campobasso, December 10th, 2024.

18. **Fantasma F.**, D'Urso G., Martella N., Capuano A., Boccia E., Samukha V., Chini M.G., De Felice V., Saviano G., Trombetta F., Lauro G., Segatto M., Bifulco G., Casapullo A. and Iorizzi M. "Chemical analysis of the polyphenolic fraction in infusions of industrial hemp var. *Kompolti*: antioxidant capacity, metal analysis and *in vitro* neuroprotective properties of cannabidiol".
73rd International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA). Naples, Italy, August 31th – September 3th, 2025.

Oral Presentations

1. **Fantasma F.**, Di Matteo F., Colardo M., Segatto M., Chini M.G., Iorizzi M. and Saviano G. "Phytochemical analysis of essential oil and methanolic extract from leaves of wild *Origanum vulgare* L. from central Italy, *in vitro* antioxidant activity and on HepG2 cell line".
Il contributo dei giovani chimici in Campania. Caserta (CE), Italy, July 29th, 2024.
2. **Fantasma F.**, Samukha V., Gioiosa A., De Felice V., Saviano G., Chini M.G. and Iorizzi M. "Green Gold from Nature: Chemical and Biological Insights into *Cannabis sativa* L. var. Kompolti Infusions".
Science for the Planet 2025. Campobasso, Italy, 22th - 25th June 2025
3. **Fantasma F.**, D'Urso G., Martella N., Capuano A., Boccia E., Samukha V., Chini M.G., De Felice V., Saviano G., Trombetta F., Lauro G., Segatto M., Bifulco G., Casapullo A. and Iorizzi M. "Chemical analysis of the polyphenolic fraction in infusions of industrial hemp var. Kompolti: antioxidant capacity, metal analysis and *in vitro* neuroprotective properties of cannabidiol".
Il contributo dei giovani chimici in Campania – 3rd Edition. Salerno, Italy, July 3th - 4th, 2025.

STATEMENT OF ORIGINALITY

I hereby declare that this thesis, submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy (Ph.D.) in Biology and Applied Science, is my own unaided work and has not been previously submitted for a degree or diploma at any other University.

To the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due reference is made in the text. I have not used sources other than those indicated, and all direct and indirect sources are acknowledged as references.

The results presented in this dissertation have been published in international peer-reviewed journals and/or conference proceedings.

Any digital tools used during the preparation of this thesis were employed exclusively for language editing and stylistic improvement.

The scientific content, data, analyses, interpretations, and conclusions are entirely the author's own.

14 January 2026

Signature
(Francesca Fantasma)

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ABSTRACT

The work presented in this Ph.D. thesis investigates plant-derived secondary metabolites from different plant matrices, including aromatic and medicinal plants, wild edible species, and nutraceutical crops, with a focus on their chemical characterization and functional potential in different biological and applicative contexts. The experimental design and the results obtained have been published in international peer-reviewed journals and were developed within a multidisciplinary research framework integrating chemical-analytical and biological approaches.

Volatile fractions, mainly in the form of essential oils, and non-volatile fractions obtained by hydroalcoholic extraction were investigated using complementary analytical techniques.

The chemical characterization of essential oils from *Lavandula angustifolia*, *Origanum vulgare*, and *Laurus nobilis* (Chapters 1–3) was performed by GC–MS, coupled, in selected cases, with multivariate and chemometric analysis to support the interpretation of compositional variability and bioactivity-related trends.

Non-volatile fractions were characterized by LC-based techniques, enabling the definition of polyphenolic and phytochemical profiles of different plant matrices. In particular, the phytochemical composition of the aqueous extract of *Cannabis sativa* var. Kompolti was analysed. The biological evaluation on cannabidiol (CBD) was carried out in a cellular model relevant to Parkinson's disease, this provided information on antioxidant, anti-inflammatory, and neuroprotective mechanisms (Chapter 4).

Alongside targeted and class-oriented chemical analyses, this thesis also includes an untargeted metabolomic investigation based on high-resolution mass spectrometry. In Chapter 5, LC-ESI-HR-MS/MS-based metabolomic profiling was applied to different *Vicia faba* varieties to comprehensively describe their metabolic composition and to support the interpretation of antioxidant and anti-inflammatory activities in relation to multiple metabolite classes rather than single marker compounds.

Biological potential was evaluated through *in vitro* assays, cell-based models, and, when appropriate, supported by *in silico* approaches aimed at exploring possible molecular mechanisms underlying the observed activities (Chapters 1–5). The experimental results are framed within a broader scientific context through two review chapters focused on bioactive

secondary metabolites in edible plants (Chapter 6) and on LC–MS-based strategies for the characterization and valorization of plant resources and plant waste for nutraceutical and cosmetic applications (Chapter 7).

Overall, this thesis highlights the importance of an integrated methodological approach capable of linking chemical and metabolic profiles to biological responses, emphasizing the functional role of the phytocomplex and the interactions among different classes of secondary metabolites. The results contribute to the understanding of the potential applications of plant-derived natural products in nutraceutical, cosmetic, agri-food, and pharmaceutical fields.

RATIONALE AND OBJECTIVES OF THE PHD RESEARCH

Natural products have historically played a central role in the discovery and development of therapeutic agents and continue to represent a valuable source of bioactive compounds for pharmaceutical, nutraceutical, and cosmetic research. A significant proportion of currently approved drugs, or their structural analogues, originate from natural sources, highlighting the relevance of plant secondary metabolites as privileged chemical scaffolds for the identification of new pharmacologically active molecules [1,2].

In recent years, renewed interest has emerged in plant-derived compounds for the treatment of complex and multifactorial diseases, where multitarget activity and the modulation of multiple biological pathways may represent a strategic advantage over single-target approaches [2,3].

In this context, plant secondary metabolites have attracted attention not only for their traditional applications, but also for their potential role in neurodegenerative disorders, such as Parkinson's and Alzheimer's disease. Increasing evidence suggests that complex natural mixtures and their bioactive constituents may exert neuroprotective effects through antioxidant, anti-inflammatory, and cytoprotective mechanisms, supporting their investigation as complementary or innovative approaches in pharmaceutical research [4–6].

Plants represent an extremely rich and diverse source of secondary metabolites, which play a fundamental role in plant physiological and ecological processes. These compounds are involved in defense mechanisms, interactions with the environment, and communication with other organisms, contributing to plant adaptation to different environmental conditions [7,8].

Beyond their biological role in *planta*, secondary metabolites have gained increasing relevance for a wide range of applications in nutraceutical, cosmetic, agri-food, and bioprotection fields, where they are considered promising sources of functional ingredients meeting current demands for efficacy, safety, and sustainability [2,9,10].

Plant secondary metabolites comprise a chemically heterogeneous group of compounds, belonging to different structural classes and characterized by high qualitative and quantitative variability. This complexity makes plant matrices intrinsically dynamic systems, in which the final chemical profile results from the interaction of genetic background, environmental conditions, and physiological state [7,11].

As a consequence, the biological activity associated with plant extracts and essential oils cannot be interpreted solely on the basis of single isolated compounds. Still, it should be considered as the result of the combined action of multiple metabolites acting within the phytocomplex [7]. This concept highlights the limitations of purely reductionist approaches and emphasizes the importance of interactions among different classes of metabolites in modulating biological responses [7,12].

In light of the increasing awareness of biological and chemical complexity in living systems, the last decades have seen the development and consolidation of the so-called *omics* sciences, which aim to describe biological systems at different molecular levels in a comprehensive and integrative manner. Genomics, transcriptomics, proteomics, and metabolomics represent complementary approaches that contribute to the understanding of molecular organization and functional dynamics [13,14]. Among these, metabolomics has gained particular relevance in the study of plant-derived matrices, as it provides a direct snapshot of the biochemical phenotype resulting from the interaction between genetic background and environmental factors [13,15].

In the field of natural products research, omics-based strategies offer a valuable framework to move beyond the analysis of single compounds and to better capture the intrinsic complexity of plant metabolomes. In particular, metabolomic approaches based on high-resolution mass spectrometry enable the simultaneous investigation of multiple classes of secondary metabolites, providing a comprehensive overview of chemical composition and supporting the interpretation of biological activities in relation to complex metabolic patterns rather than isolated marker compounds [13,14,16].

The work presented in this Ph.D. thesis is based on a series of studies published in international peer-reviewed journals and was developed within a multidisciplinary research framework integrating chemical characterization, biological evaluation, and, in selected cases, computational analysis. The experimental work is organized into thematic chapters addressing different plant matrices and research questions. Chapters 1–3 focus on the chemical characterization and biological evaluation of essential oils from *Lavandula angustifolia*, *Origanum vulgare*, and *Laurus nobilis*, investigating compositional variability and functional properties such as antioxidant, antimicrobial/antifungal, and anti-inflammatory activity. In the case of *Laurus nobilis*, multivariate statistical and chemometric

analysis was applied to GC–MS datasets to support the interpretation of chemical variability and bioactivity-related trends [17,18].

Chapter 4 is dedicated to the phytochemical characterization of *Cannabis sativa* extracts and to the investigation of their biological effects of cannabidiol in a cellular model relevant to Parkinson's disease. In this chapter, chemical profiling is integrated with cell-based assays and mechanistic considerations related to oxidative stress and inflammation pathways, providing insights into the neuroprotective potential of selected plant-derived compounds [19–21].

Alongside targeted and class-oriented chemical analyses, the thesis also includes analytical approaches inspired by metabolomic workflows. In particular, an untargeted LC-ESI-HR-MS/MS-based metabolite profiling was applied in Chapter 5 to different *Vicia faba* varieties. This metabolomic approach enabled a comprehensive description of complex plant metabolic patterns and supported the interpretation of antioxidant and anti-inflammatory activities in relation to multiple metabolite classes, rather than single marker compounds [22,23].

The conceptual and methodological background of LC–MS platforms, including targeted and untargeted strategies for the study of complex plant matrices, is further discussed in the review chapter (Chapter 7) dedicated to LC–MS applications for the characterization and valorization of plant resources and plant waste [13,24,25].

The thesis also includes a Review chapter focused on bioactive secondary metabolites in edible plants (Chapter 6), which contextualizes the experimental findings within the broader scientific literature and highlights emerging nutraceutical perspectives [2,9,10]. Together, the experimental and review chapters provide an integrated overview of the chemical and functional potential of plant-derived secondary metabolites.

During the Ph.D. programme, I also carried out a short Research Project abroad at the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca (Romania), focusing on natural products chemistry, with particular reference to the study of honey and pollen. This experience, which was mainly formative, contributed to broadening methodological and analytical skills, although it does not directly fall within the results presented in this thesis.

Taking these considerations into account, the overall aim of this Ph.D. thesis is to contribute to the understanding of the role and functional potential of plant-derived secondary metabolites through an integrated approach combining chemical characterization and biological evaluation. Specifically, the objectives of the work were to:

- characterize the phytochemical profile of different plant matrices, considering both volatile and non-volatile fractions, using complementary analytical techniques (Chapters 1–5);
- evaluate the biological potential of extracts and essential oils through *in vitro* assays, cell-based models, and, when appropriate, support mechanistic interpretation through *in silico* approaches (Chapters 1–4);
- investigate the relationship between chemical composition and biological activity, taking into account the complexity of the phytocomplex and the interactions among different classes of metabolites;
- apply untargeted LC-ESI-HR-MS/MS-based metabolite profiling, together with multivariate and metabolomics-oriented data analysis approaches, to describe complex chemical and metabolic patterns and support a comprehensive interpretation of functional properties in nutraceutical and aromatic plant matrices (Chapters 3 and 5);
- explore the applicative implications of the results obtained in the field of nutraceutical, cosmetic, agri-food, and bioprotection contexts, and critically frame the findings within the current literature through dedicated review chapters (Chapters 6 and 7).

CHAPTER 1 *Lavandula angustifolia* L.

1.1 Background and rationale

Essential oils (EOs) are complex mixtures of volatile secondary metabolites synthesized by numerous plant species and accumulated in various organs, including flowers, leaves, seeds, and bark. In recent years, there has been considerable growth in scientific and practical interest in EOs thanks to their documented antimicrobial, antioxidant, and antifungal properties. These properties make EOs potential eco-sustainable alternatives to traditional synthetic biocides, which are often characterized by high toxicity and poor biodegradability [26–28].

In this context, the genus *Lavandula* (family Lamiaceae) is one of the most widely researched sources of essential oils with biological and industrial applications. *Lavandula angustifolia* L., in particular, is widely used in pharmaceuticals, cosmetics, and phytotherapy thanks to its sedative, anxiolytic, anti-inflammatory, and antimicrobial properties [29,30]. Numerous studies have also highlighted the effectiveness of lavender essential oil against a wide spectrum of pathogenic microorganisms and its significant antioxidant activity [28,31].

However, the chemical composition of *L. angustifolia* essential oils can vary significantly depending on genetic, environmental, and technological factors. These include geographical area of cultivation, soil and climate conditions, phenological stage at harvest, and extraction methodology [32–36]. This variability is primarily reflected in quantitative fluctuations in monoterpenes and oxygenated monoterpenoids, including linalool and linalyl acetate. These have been identified as phytochemical markers responsible for the main aromatic and biological characteristics of lavender essential oil [37–40].

The dynamic nature of the terpene profile thus necessitates detailed chemical characterization of essential oils to establish reliable correlations between phytochemical composition and biological activities. In particular, compositional variability has been demonstrated to significantly influence the antimicrobial and antifungal efficacy of EOs, as evidenced by numerous studies on aromatic species [28,41].

The present study analyses essential oils obtained from four populations of *Lavandula angustifolia* from different geographical areas. The oils were obtained from three populations located in central-southern Italy (LaCC, LaPE, LaPS) and one in southern France (LaPRV).

The study aims to evaluate the influence of geographical origin on the chemical composition and biological properties of the extracted oils. In particular, antioxidant and antifungal activities are investigated *in vitro* against phytopathogenic and wood-deteriorating fungi, as well as the effectiveness of essential oils on historical wooden substrates, with a view to developing sustainable biocide strategies for cultural heritage conservation [42–44].

1.2 Experimental design

This section outlines the experimental design adopted for the study of *Lavandula angustifolia* L..

Detailed experimental procedures are reported in Chapter 10.

1.2.1 Plant material

The *Lavandula angustifolia* L. accessions analyzed in this study originate from four discrete geographical areas, distinguished by varying soil and climate conditions, with the objective of evaluating the impact of geographical and environmental variability on the composition of the essential oil. As is well documented, a variety of factors have been shown to have a significant impact on the phytochemical variability of essential oils. Such factors include soil type, altitude, climate, and agronomic practices, which have been found to influence the yield, metabolic profile, and biological activities of the oils [32–35].

The identification of the plants was conducted in accordance with the taxonomic criteria documented in the extant literature [45,46]. The plant material, consisting of flowering tops, was collected at the same phenological stage, an essential condition to ensure the comparability of terpene profiles between different accessions [34]. Subsequently, the samples were dried in a ventilated environment away from direct light until they reached a constant weight. They were then ground and stored in airtight containers at room temperature until the extraction phase. This was done in order to limit the loss of volatile compounds responsible for the biological activities of essential oils.

1.2.2 Essential oil extraction

The essential oils were extracted using hydrodistillation in a Clevenger apparatus, a technique that has been extensively utilized for the isolation of volatile compounds from

aromatic matrices and is recognized for its ability to preserve the integrity of monoterpenoids without the use of organic solvents [27,32].

For each accession, 100 g of dried plant material was utilized, in accordance with established experimental protocols [34]. The essential oil was subject to a distillation process for a total duration of three hours, which was deemed sufficient to achieve a representative and stable yield of the phytochemical profile [32,47].

1.2.3 GC–MS analysis of essential oils

The chemical characterization of essential oils was performed using gas chromatography coupled with mass spectrometry (GC–MS), the reference technique for the qualitative and quantitative analysis of volatile compounds present in essential oils [38,48].

The compounds under investigation were identified by means of a comparison of the mass spectra with the NIST libraries, as well as by a comparison of the experimental retention indices with those reported in the literature [48–50]. The relative percentages of the individual constituents were calculated by normalising the areas of the chromatographic peaks, a methodology that is commonly employed to describe the composition of essential oils [38].

1.2.4 *In vitro* antifungal assay

The antifungal activity of essential oils was evaluated by testing their ability to inhibit mycelial growth in five fungal species: *Sclerotium rolfsii*, *Botrytis cinerea*, *Fusarium verticillioides*, *Phanerochaete chrysosporium*, and *Trametes cingulata*. These microorganisms were selected for their relevance in both phytopathology and the biodeterioration processes of lignocellulosic materials [51–53].

The fungal strains were grown on Potato Dextrose Agar (PDA) medium and then exposed to different concentrations of essential oil incorporated into the growth medium, according to standardized protocols widely used for evaluating the antifungal activity of essential oils [53–55].

The plates were subjected to an incubation period of seven days, after which the radial growth of the colonies was measured at regular intervals. The antifungal activity was

expressed as a percentage of mycelial growth inhibition (MGI), calculated according to the following formula:

$$\mathbf{MGI\ (\%) = [(d_c - d_t) / d_c] \times 100}$$

where dc represents the average diameter of the colony in the untreated control and d_t that observed in the treatment with essential oil [54].

The fungicide *Thiram* was used as a positive control, given its proven effectiveness against phytopathogenic fungi [52]. All tests were performed in triplicate to ensure the statistical significance of the results.

1.2.5 Antioxidant activity (DPPH assay)

The antioxidant activity of essential oils was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) spectrophotometric assay, a method which is widely used to evaluate the radical scavenging capacity of essential oils and aromatic matrices [56,57].

Aliquots of each essential oil were then incubated with a methanolic solution of DPPH (27 μg/mL) in the dark for a period of 30 minutes. The decrease in light absorption was measured at a wavelength of 517 nm using a UV-Vis spectrophotometer.

The results were expressed as a percentage of radical inhibition as a function of essential oil concentration, and the IC_{50} value was calculated as an index of antioxidant efficacy [56]. Ascorbic acid was utilized as a positive control. It is imperative to note that all measurements were performed in triplicate.

1.2.6 Application on wooden substrates

In order to evaluate the effectiveness of essential oils in real-life conditions, a case study was conducted on a historic wooden artefact (an olive wood stamp dated 1876, Villa Badessa, Pescara). In the initial phase of the experiment, small wood samples were subjected to a process of sterilization. Following this, the samples were treated with solutions containing known concentrations of essential oil. These solutions were applied to the surface of the samples in a uniform manner using a micropipette. This procedure served to simulate a conservation intervention on contaminated wood material [44,51].

Following desiccation, the samples were placed in direct contact with the selected fungal strains and then incubated in controlled climatic chambers. The mycotic growth was monitored both visually and by periodic measurements of the colonization area. The effectiveness of the treatment was expressed as a percentage reduction in the colonized area in comparison with the untreated control.

1.2.7 Statistical analysis

The data obtained from chemical analyses and biological assays were subjected to multivariate statistical analysis in order to explore the relationships between the different accessions based on the chemical composition of the essential oils. This methodological approach facilitated the identification of patterns of similarity and difference between the samples, and the subsequent identification of the main compositional contributions associated with geographical variability. This, in turn, provided interpretative support for the results obtained from chemical and biological analyses [8,58].

1.3 Results

1.3.1 Chromatographic profile of essential oils

The hydrodistillation of the flowering tops of *Lavandula angustifolia* was found to be a successful method for the extraction of essential oils from all the accessions analyzed, with yields varying according to the geographical origin of the plant material. The yields obtained, expressed as a percentage of the initial dry weight, fall within the ranges commonly reported for the species. This suggests that significant influences are exerted by soil, climate, and environmental factors on biosynthesis and the accumulation of volatile metabolites [32–35].

Gas chromatography coupled with mass spectrometry (GC–MS) analysis revealed a characteristic chromatographic profile for all accessions, dominated by the presence of monoterpenes and oxygenated monoterpenoids, typical of *L. angustifolia* essential oil. The total chromatograms (TIC) manifest a high degree of compositional complexity, characterized by the presence of numerous well-resolved peaks. This finding is indicative of the substantial phytochemical richness of the oils that were analyzed.
The overlaid TIC chromatograms of the four accessions are shown in *Figure 1.1*.

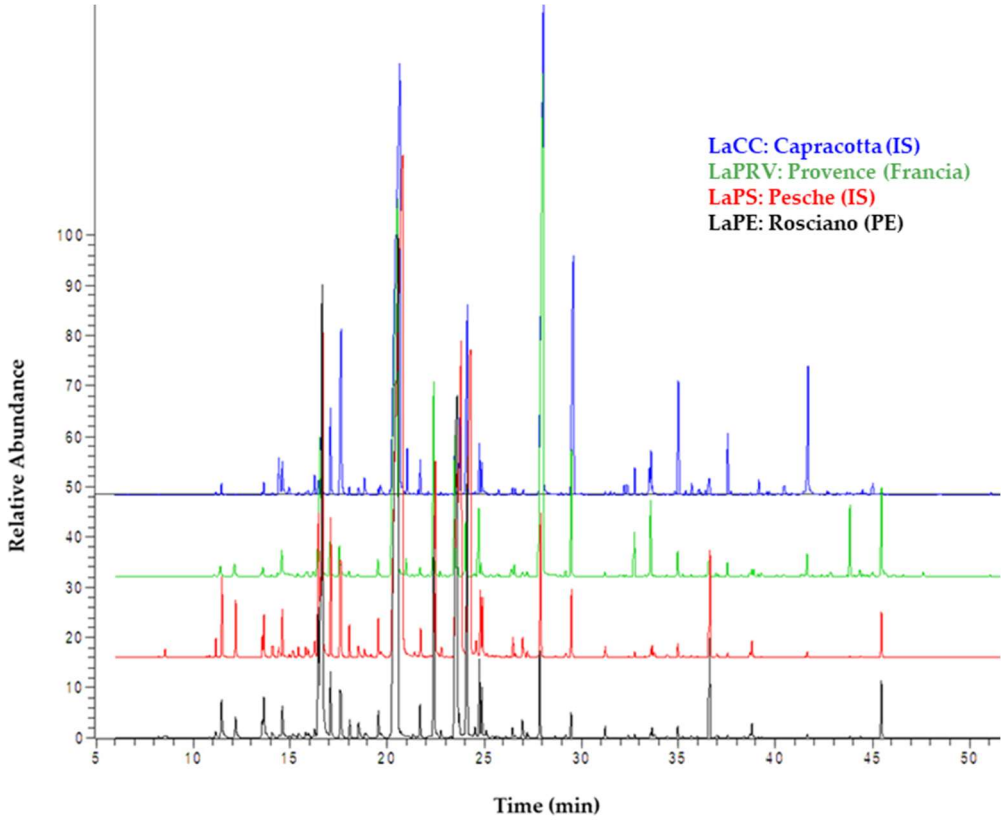


Figure 1.1 - Overlaid GC–MS TIC chromatograms of lavender EO samples

1.3.2 Chemical composition of the essential oil

The integration of qualitative and quantitative analysis methodologies enabled the identification of over seventy volatile compounds for each sample, predominantly classified within the classes of monoterpenes, oxygenated monoterpenoids, sesquiterpenes, and sesquiterpenoids.

For clarity, only compounds present at ≥ 0.5% in at least one accession are reported in *Table 1.1*, while extended datasets, tables, and supplementary figures supporting the results discussed in this chapter are available in the Supplementary Materials of the corresponding peer-reviewed publication (see Appendix).

In all accessions, linalool is the major constituent, with percentages ranging from approximately 30% to 40% of the total, thus confirming its status as a distinctive chemical marker of *L. angustifolia* essential oil. Alongside linalool, linalyl acetate constitutes one of the principal components, although with marked variations between accessions, reflecting the influence of geographical origin on phytochemical composition.

Table 1.1 - Chemical composition (% relative abundance) of essential oils obtained from the four *Lavandula angustifolia* accessions, as determined by GC–MS analysis. Only compounds present at ≥ 0.5% in at least one accession are reported.

N.	Compound	Exp RI	Ref RI	Area % ± SD LaCC	Area % ± SD LaPE	Area % ± SD LaPS	Area % ± SD LaPRV
3	*α*-Pinene	936	939	0.22 ± 0.01	1.26 ± 0.07	1.14 ± 0.02	0.36 ± 0.02
4	Camphene	950	954	0.03 ± 0.01	0.69 ± 0.04	0.81 ± 0.02	0.5 ± 0.01
6	*β*-Pinene	977	979	0.22 ± 0.03	1.18 ± 0.01	0.57 ± 0.04	0.25 ± 0.06
8	3-Octanone	990	983	0.75 ± 0.03	0.08 ± 0.01	0.13 ± 0.01	0.11 ± 0.01
9	Myrcene	993	990	0.78 ± 0.06	0.79 ± 0.02	0.58 ± 0.02	0.96 ± 0.05
16	Limonene	1030	1029	0.25 ± 0.02	3.83 ± 0.41	3.43 ± 0.14	0.75 ± 0.03
17	1,8-Cineole	1032	1031	1.18 ± 0.08	13.48 ± 0.44	7.0 ± 0.12	4.26 ± 0.1
18	(*Z*)-*β*-Ocimene	1042	1037	1.72 ± 0.04	1.7 ± 0.27	1.94 ± 0.06	0.91 ± 0.04
19	(*E*)-*β*-Ocimene	1053	1050	3.45 ± 0.05	1.06 ± 0.01	1.31 ± 0.04	0.91 ± 0.02
23	Terpinolen	1089	1088	0.11 ± 0.01	0.63 ± 0.03	0.54 ± 0.01	0.43 ± 0.02
24	Linalool	1107	1096	34.6 ± 0.43	33.54 ± 0.21	39.73 ± 0.43	30.02 ± 0.26
25	Octen-3-yl acetate	1119	1112	0.73 ± 0.01	-	-	0.35 ± 0.01
26	allo-Ocimene	1133	1132	0.5 ± 0.11	0.34 ± 0.33	0.33 ± 0.09	0.19 ± 0.04
28	Camphor	1147	1146	0.47 ± 0.01	5.68 ± 0.1	3.87 ± 0.03	6.2 ± 0.03
29	Borneol	1169	1169	1.05 ± 0.02	13.65 ± 0.18	16.83 ± 0.34	4.43 ± 0.07
30	Lavandulol	1173	1169	1.98 ± 0.04	0.27 ± 0.02	-	0.29 ± 0.08
31	Terpinen-4-ol	1180	1177	5.42 ± 0.02	8.2 ± 0.16	9.98 ± 0.06	1.25 ± 0.01
32	*α*-Terpineol	1192	1188	0.88 ± 0.02	1.48 ± 0.03	0.86 ± 0.06	1.61 ± 0.05
33	Hexil butanoate	1194	1192	0.51 ± 0.04	0.85 ± 0.03	0.75 ± 0.07	0.27 ± 0.02
40	Linalyl acetate	1265	1257	24.34 ± 0.34	1.8 ± 0.1	2.41 ± 0.05	31.07 ± 0.13
42	Lavandulyl acetate	1296	1290	6.51 ± 0.02	0.49 ± 0.01	0.98 ± 0.01	3.2 ± 0.03
45	Neryl acetate	1368	1361	0.45 ± 0.02	0.06 ± 0.01	0.07 ± 0.01	0.96 ± 0.03
49	*trans*-Myrtanol acetate	1384	1386	0.78 ± 0.05	0.08 ± 0.01	0.1 ± 0.01	1.72 ± 0.06
54	(*E*)-Caryophyllene	1420	1419	2.78 ± 0.04	0.26 ± 0.01	0.21 ± 0.01	0.63 ± 0.02
61	(*E*)-*β*-Farnesene	1458	1456	0.33 ± 0.01	1.99 ± 0.04	1.61 ± 0.01	0.33 ± 0.01
65	*γ*-Muurolene	1481	1479	1.21 ± 0.03	0.07 ± 0.01	0.08 ± 0.01	0.32 ± 0
75	Caryophyllene oxide	1584	1583	2.84 ± 0.14	0.07 ± 0.01	0.02 ± 0.01	0.55 ± 0.01
78	*α*-Muurolol	1643	1646	-	0.04 ± 0	0.02 ± 0	1.73 ± 0.05
81	*α*-Bisabolol	1686	1685	0.11 ± 0.02	1.23 ± 0.06	0.68 ± 0.02	2.23 ± 0.12

SD: standard deviation; Exp. RI: experimental retention index; Ref. RI: literature data.

Further significant differences were observed for other oxygenated monoterpenoids, such as borneol, camphor, terpinen-4-ol, and eucalyptol (1,8-cineole), whose relative abundance varies considerably between samples. These compositional variations can be attributed to environmental and climatic factors, which have been demonstrated to modulate the expression of terpene biosynthetic pathways [34].

The analysis of the chemical class indicates that oxygenated monoterpenoids constitute the predominant fraction in all accessions, with hydrocarbon monoterpenes representing a secondary fraction, and sesquiterpenes and sesquiterpenoids comprising a smaller percentage of the total. This distribution is consistent with the literature on the essential oil

of *L. angustifolia*, and is particularly relevant in relation to the biological properties of the oils analyzed.

1.3.3 Explorative data analysis of chemical composition

In order to assess the degree of diversity and similarity between the chemical profiles of the essential oils analyzed, an exploratory analysis of the data was conducted based on Shannon's entropy index, Pielou's equidistribution index, and the Percent Model Affinity (PMA) index.

The index values, shown in *Table 1.2*, highlight differences in the complexity and distribution of components among the different accessions.

Table 1.2 - Similarity and diversity indices calculated for the chemical composition of essential oils from the four *Lavandula angustifolia* accessions. Similarity between samples was evaluated using the Percent Model Affinity (PMA) index, while chemical diversity was assessed through Shannon's entropy and Pielou's equidistribution indices. Values are reported as mean ± standard deviation.

EOs	**Similarity**				**Diversity**	
	LaCC	**LaPE**	**LaPS**	**LaPRV**	**Shannon entropy**	**Pielou index**
LaCC	1	0.535 ± 0.001	0.552 ± 0.001	0.719 ± 0.001	2.376 ± 0.007	0.539 ± 0.002
LaPE		1	0.866 ± 0.001	0.592 ± 0.001	2.463 ± 0.010	0.559 ± 0.002
LaPS			1	0.565 ± 0.001	2.266 ± 0.006	0.514 ± 0.001
LaPRV				1	2.310 ± 0.007	0.524 ± 0.002

In particular, some populations show greater compositional heterogeneity, while others are characterized by a more homogeneous profile, suggesting a different relative distribution of volatile metabolites.

The similarity analysis based on the PMA indicates a variable degree of chemical affinity between the accessions, allowing samples with more similar compositional profiles to be distinguished from those that are more differentiated.

The differences observed are mainly associated with the different relative abundance of oxygenated monoterpenoids, in accordance with the results of the qualitative and quantitative analysis of essential oils.

1.3.4 *In vitro* antifungal activity

Mycelial growth inhibition tests demonstrated that all the essential oils analyzed exhibited antifungal activity against the tested species, although with varying degrees of effectiveness depending on both accession and fungal strain considered.

The antifungal activity was generally concentration-dependent, and a differential sensitivity among the tested fungi was observed. In particular, some phytopathogenic and wood-deteriorating strains showed a high susceptibility to *L. angustifolia* essential oils, whereas others exhibited a more moderate response.

The observed differences may be attributed to the compositional variability of the essential oils, particularly to variations in the relative abundance of oxygenated monoterpenoids, which are recognised for their antifungal properties. The quantitative results of the *in vitro* assays are reported in *Table 1.3*.

Table 1.3 - *In vitro* antifungal activity of *Lavandula angustifolia* essential oils against selected phytopathogenic and wood-deteriorating fungi, expressed as percentage reduction of the colonized area. Results are reported for the most effective essential oil concentration tested for each fungal strain. Visual assessment of mycelial growth is also reported.

Tested fungus	EO concentration (%)	Colonized area (mm²) Control	Colonized area (mm²) Treated	Colonization reduction (%)	Visual outcome
Botrytis cinerea	0.5%	120	45	62.5%	Reduced growth with irregular margins
Fusarium verticillioides	1%	180	20	88.9%	Almost absent growth
Trametes cingulata	1%	200	90	55.0%	Partial colonization
Phanerochaete chrysosporium	2%	240	0	100%	No visible growth

1.3.5 Antifungal activity on wooden substrate

Consistent with the *in vitro* antifungal assays, the application of essential oil solutions on wooden samples resulted in a marked reduction of fungal colonization compared to untreated controls.

The antifungal efficacy of the essential oils was evaluated under conditions that more closely resemble a real-life conservation context through an application test on wooden substrates. The case study was conducted on a historic olive wood artefact representative of cultural heritage objects (*Figure 1.2*).



(a) (b)

Figure 1.2 - Olive wood stamp for the Offering Bread (a, b: double-sided) used during Easter celebrations in the Catholic community of Villa Badessa (Rosciano, Pescara, Italy), Greek-Byzantine rite. The handmade artefact, dated to 1876, is preserved in the Permanent Historical–Ethnoanthropological Exhibition of Villa Badessa and was selected as a representative historical wooden substrate for the application tests.

Following surface treatment with essential oil solutions and subsequent incubation with selected fungal strains, a clear inhibition of fungal growth was observed on treated samples when compared to the corresponding untreated controls.

In particular, certain essential oil accessions exhibited a higher capacity to limit fungal proliferation over time, suggesting a sustained antifungal effect on the lignocellulosic substrate. Visual inspection, supported by quantitative measurements of the colonized area, indicated that the treatment not only reduced overall mycelial growth but also affected the morphology and spatial distribution of fungal colonies on the wood surface.

Overall, these results confirm that the antifungal activity observed *in vitro* is retained when essential oils are applied to a complex substrate such as wood, supporting their potential use as natural and eco-sustainable biocidal agents for the protection and conservation of historical wooden artefacts.

1.3.6 *In vitro* antioxidant activity

The antioxidant activity of essential oils, as measured by the DPPH assay, showed considerable variation among the different accessions. The IC_{50} values reported in *Table 1.4* indicate that certain populations exhibit higher radical scavenging activity, while others show lower efficacy.

Table 1.4 - IC_{50} values (mg mL^{-1}) and scavenging activity range (%) of four EOs lavender analyzed.

Samples	IC_{50} (mg/mL)	% Scavenging activity
LaPS	49.63 ± 0.42	13.08 – 93.04
LaPE	48.00 ± 0.35	28.40 – 94.24
LaPRV	33.53 ± 0.23	29.98 – 94.87
LaCC	26.26 ± 0.21	35.29 – 98.70

These differences can be attributed to variations in the chemical composition of the essential oils, particularly to the presence and relative abundance of compounds known for their antioxidant activity, such as linalool, linalyl acetate, and other oxygenated monoterpenoids. Overall, the results highlight the antioxidant potential of *L. angustifolia* essential oils and their possible applications in nutraceutical and preservative fields.

1.4 Discussions

The findings of this study underscore the pivotal role of geographical origin in shaping both the chemical composition and the biological properties of essential oils from *Lavandula angustifolia* populations. In all the accessions analyzed, the phytochemical profile was dominated by oxygenated monoterpenoids, in particular linalool and linalyl acetate, in accordance with the extensive literature on this species [37–40]. However, the marked quantitative differences observed among samples confirm the strong metabolic plasticity of *L. angustifolia*, which has been widely reported in relation to pedoclimatic and environmental factors [32–35].

The compositional variability observed, especially for compounds such as borneol, camphor, terpinen-4-ol, and eucalyptol, represents a key element for interpreting the biological activities recorded. Numerous studies have demonstrated that these monoterpenoids contribute synergistically to the antimicrobial and antifungal activity of essential oils through mechanisms including membrane permeability alteration, inhibition of key enzymatic pathways, and disruption of microbial cellular metabolism [28,54,59].

In this context, the differences in antifungal efficacy among the analyzed accessions can be attributed not only to the presence of the major constituents, but also to the relative balance and interactions among the components within the volatile mixture.

The *in vitro* antifungal assays revealed a generalized inhibitory effect against all the tested fungal strains, albeit with differential sensitivity among species. This behaviour is consistent with that reported for other essential oils rich in oxygenated monoterpenoids and reflects the complex interactions between multicomponent chemical mixtures and biological targets [53–55,60].

Notably, the activity observed against lignicolous and biodeteriogenic fungi highlights the potential application of *L. angustifolia* essential oils as natural biocidal agents in the field of cultural heritage conservation, where there is an increasing demand for sustainable and low–environmental-impact strategies [43,51,61].

The application tests performed on a wooden substrate further supported this hypothesis, demonstrating that the antifungal activity of the essential oils can be retained under conditions that more closely resemble a real conservation context than conventional *in vitro* assays on culture media. The ability of essential oils to interact with the lignocellulosic substrate and exert a sustained inhibitory effect represents a crucial factor for their potential use in conservation treatments, either as alternatives to or in combination with conventional synthetic biocides [51,61].

With regard to antioxidant activity, the IC_{50} values obtained using the DPPH assay indicated variable radical scavenging capacities among the different accessions, in accordance with the compositional differences observed. Although essential oils generally represent a secondary source of antioxidants compared to other plant-derived matrices, the presence of oxygenated monoterpenoids and minor compounds with redox activity contributes to the overall antioxidant potential of the mixture [11,62]. This property may be relevant both in the nutraceutical field and in the context of material protection, where oxidative processes play a significant role in degradation phenomena.

Overall, the results of this study demonstrate that the biological quality of *Lavandula angustifolia* essential oils cannot be assessed solely on the basis of their major constituents. Rather, it requires an integrated evaluation of the complete phytochemical profile and the synergistic interactions among its components. This perspective is particularly relevant from an applicative standpoint, as the selection of the accession and geographical origin can have a decisive impact on the final effectiveness of essential oil–based products.

1.5 Remarks

This chapter explored the chemical and biological characterization of *Lavandula angustifolia* essential oils from different geographical areas. The results demonstrated that the origin of the plant material significantly influences the phytochemical profile and, consequently, the functional properties of the extracted oils. In all the accessions analyzed, oxygenated monoterpenoids, in particular linalool and linalyl acetate, were confirmed as the main constituents of the essential oils, although with significant quantitative differences among populations.

The observed compositional variability was directly reflected in the biological activities evaluated. *In vitro* antifungal assays revealed a generalized inhibitory effect against the tested strains, with differentiated responses depending on both the accession and microorganism considered. These findings were further corroborated by the application tests on wooden substrates, which highlighted the ability of essential oils to interfere with fungal colonization even under conditions that more closely resemble real-life applications. This supports their potential use in the bioprotection of wood materials and in the conservation of cultural heritage.

The antioxidant activity, evaluated using the DPPH assay, showed variable radical scavenging capacities among the different accessions, in agreement with the differences observed in the chemical composition of the essential oils. Although antioxidant activity alone does not represent the primary criterion for application, it contributes to defining the overall functional value of *L. angustifolia* essential oils in nutraceutical and phytotherapeutic contexts.

Overall, the results demonstrate that the quality and biological of *Lavandula angustifolia* essential oils depend not only on the presence of major chemical markers, but also on the relative balance of the volatile components within the mixture, which is strongly influenced by geographical origin.

These findings underline the importance of selecting specific accessions according to the intended application and support the potential of essential oils as natural and sustainable tools in the fields of nutraceuticals, agriculture, and cultural heritage conservation.

CHAPTER 2 *Origanum vulgare* L.

2.1 Background and rationale

Origanum vulgare L., commonly known as oregano, is a herbaceous species belonging to the Lamiaceae family. It is widely distributed in the Mediterranean basin and has a long history of use in both cooking and traditional medicine for the treatment of inflammatory and infectious disorders [63–65].

The scientific community has recently demonstrated a heightened level of interest in this species, primarily due to its distinctive chemical composition, which is characterized by the presence of volatile compounds (essential oil) and a substantial polyphenolic phytocomplex. These components are responsible for the predominant biological activities attributed to oregano, encompassing antioxidant, antimicrobial, anti-inflammatory, and antiviral properties [66–70].

In particular, the essential oil of *O. vulgare* has a terpene profile dominated by the monoterpene phenols carvacrol and thymol. These molecules are considered functional markers for the oil's antimicrobial and antioxidant action, thanks to their ability to interfere with the bacterial membrane [71] and neutralise reactive oxygen species thanks to the presence of electron-donor phenolic groups [72,73].

This volatile fraction is accompanied by the contribution of polyphenols, among which phenolic acids and flavonoids stand out, known for their high antioxidant capacity and their role in counteracting oxidative damage to lipids, proteins, and DNA [72–75]. Of particular interest are rosmarinic acid, caffeic acid, and glycosylated flavonoids.

Another distinctive feature of the *Origanum* genus is its marked chemodiversity, attributable to the qualitative and quantitative variability of the biosynthetic pathways responsible for the formation of cymyl, sabinyl, and acyclic compounds. This diversity is influenced by genetic and environmental factors, including latitude, soil and climate conditions, harvest period, and phenological stage of the plant. These factors allow *O. vulgare* to be classified into different chemotypes, mainly with a prevalence of carvacrol, thymol, or linalool [66,67,76–78]. This has significant implications for essential oil yield and secondary metabolite profile [79].

Despite the fact that a number of studies have investigated the characterization of *O. vulgare* essential oil on a European scale, information on hydroalcoholic extracts from Italian matrices remains limited [76,80,81].

In view of the points that have previously been enumerated, it is the aim of this chapter to:

i. characterize and compare the volatile composition of the essential oil obtained from wild plants collected in Lazio (WEO_OR) with a commercial essential oil (CEO_OR) using GC-MS;
ii. define the polyphenolic profile of the methanolic extract (ME_OR) using UHPLC-DAD;
iii. evaluate the antioxidant activities of the samples through DPPH, ABTS, and FRAP assays;
iv. investigate the biological effects on a HepG2 cell model subjected to oxidative stress.

2.2 Experimental design

This section outlines the experimental design adopted for the study of *Origanum Vulgare* L.

Detailed experimental procedures are reported in Chapter 10.

2.2.1 Plant material and sample preparation

The harvesting of the aerial parts of *Origanum vulgare* L. occurred during the peak balsamic period in the Lazio region (central Italy). This phenological phase is characterized by the maximal levels of secondary metabolites, particularly volatile and phenolic compounds [76–79]. Following harvesting, the plant material was subjected to a drying process in a ventilated environment, in the shade and at room temperature, under conditions that preserved the integrity of the thermolabile compounds characteristic of the species [68,74]. Subsequently, the inflorescences were separated manually, as this portion represents the matrix with the highest content of essential oils and polyphenols [65,74,82].

The raw material obtained was utilized to prepare two distinct types of experimental samples.

a) Essential oil from wild plants (WEO_OR)

50 g of dried leaves and inflorescences were subjected to hydrodistillation using a Clevenger apparatus for a period of 3 hours. The essential oil collected was then dehydrated on anhydrous sodium sulfate and stored in amber glass bottles at 4 °C until analysis, in order to avoid oxidation and photodegradation [48,83].

b) Methanol extract (ME_OR)

A quantity of 0.45 g of dried oregano powder, comprising both leaves and inflorescences, was extracted with 9.0 mL of a methanol/water solution (80:20, v/v). The mixture was stirred for a period of 45 minutes at room temperature and then incubated in the dark for a further 15 minutes. Following centrifugation at 5000 rpm for 15 minutes, the resultant upper layer was collected and stored in the dark at 4 °C until analysis [72,74,84].

This sampling strategy was adopted in order to allow a comparative evaluation of the chemical composition and biological activity of the different samples analyzed in the following sections.

2.2.2 Chemical characterization

The volatile fraction of the essential oil was characterized using GC-MS, a technique that is widely used to define the terpene fingerprint of *Origanum* species due to its high analytical sensitivity [48].

The polyphenolic component of the methanolic extract (ME_OR) was determined using UHPLC-DAD, the method of choice for the analysis of phenolic metabolites in *Origanum* due to its high accuracy and resolution [80,81,84].

The employment of a combination of highly specific chromatographic techniques has enabled the accurate differentiation of both volatile and phenolic components, thereby facilitating a comparative characterization of materials from diverse origins. This approach is considered essential for correlating the phytochemical composition of oregano with its biological properties [66–68,80] and represents the methodological standard in the most recent studies dedicated to the integrated analysis of essential oils and plant extracts [67,80,81].

2.2.3 Antioxidant activity assays

The antioxidant activity of the samples was evaluated using three *in vitro* assays based on complementary mechanisms - radical neutralization (DPPH, ABTS) and electron transfer (FRAP) - in order to obtain a complete characterization of the redox potential of the samples [81,84,85].

2.2.3.1 DPPH radical scavenging assay

The DPPH assay, based on the reduction of the stable radical ion 2,2-diphenyl-1-picrylhydrazyl, was performed by incubating the samples with a DPPH solution in the dark for 30 minutes. The decrease in extinction coefficient at 517 nm was utilized to calculate the IC_{50} value, defined as the concentration required to reduce radical activity by 50% [63,66,68].

2.2.3.2 ABTS radical cation decolorization assay

For the ABTS assay, the $ABTS^{+}$ radical was generated by reaction with potassium persulfate for 16 hours and then diluted to an absorption of 0.70 ± 0.02 at 734 nm. The incorporation of the sample serves to reduce the intensity of the chromophore radical, thereby enabling the activity to be determined in Trolox equivalents (TE). This method is particularly sensitive to hydrophilic phenolic compounds and oxygenated monoterpenoids present in essential oils [62,86,87].

2.2.3.3 FRAP assay

The reducing capacity was measured using the FRAP assay, which is based on the reduction of the ferric-tripyridylthiazine (Fe^{3+}- TPTZ) complex to ferrous (Fe^{2+}- TPTZ). This results in the development of a blue chromophore that can be quantified at 593 nm. The value obtained was expressed in Trolox equivalents (mg TE g^{-1}), a parameter that reflects the sample's ability to transfer electrons to oxidising species [81,84,88].

The integration of the three assays enabled an integrated assessment of the antioxidant activity of the samples using approaches based on distinct mechanisms - radical neutralization for DPPH and ABTS, electron transfer for FRAP - thus allowing the

phytochemical composition of oregano to be correlated with its biological potential [66,80,81,84,88].

2.2.4 Cell-based biological assays (HepG2 cells)

The biological effects of the samples were evaluated using the HepG2 cell line, derived from human hepatocellular carcinoma, widely used as an experimental model for studying liver metabolism, cytotoxicity, and responses to oxidative stress [85,89,90]. This line has a stable biochemical profile and high inter-experimental reproducibility, characteristics that make it suitable for investigating the cellular mechanisms associated with exposure to plant extracts and antioxidant compounds.

The cells were cultured in DMEM medium supplemented with fetal bovine serum (10% FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL), in a humidified atmosphere with 5% CO_2 at 37 °C.

The biological tests were designed to evaluate two main aspects:

- the possible cytotoxicity of the samples;
- their protective capacity against damage induced by oxidative stress.

2.2.4.1 Cell viability (MTT assay)

The analysis of cell viability was conducted through the implementation of the MTT assay, which is predicated on the reduction of tetrazolium salt to formazan, a process that is directly proportional to mitochondrial metabolic activity. HepG2 cells were exposed to escalating concentrations of WEO_OR, CEO_OR, and ME_OR for a duration of 24 hours, and the degree of absorption at a wavelength of 570 nm was documented to ascertain the proportion of viable cells in comparison to the control group [85,89]. This approach is frequently employed to ascertain the absence of direct toxic effects associated with bioactive compounds.

2.2.4.2 Induction of oxidative stress

In order to simulate oxidative stress conditions, cells were exposed to hydrogen peroxide (H_2O_2, 0.5 mM for 1 h). Hydrogen peroxide is an oxidising agent capable of generating reactive oxygen species (ROS) and inducing biomolecular damage [85,90]. In order to evaluate a potential protective effect, the samples were administered two hours before

exposure to H_2O_2, in accordance with established protocols for the analysis of compounds with antioxidant activity [85,89,90].

2.2.4.3 Assessment of oxidative DNA damage

As oxidative stress has been demonstrated to compromise genomic integrity, the second experimental objective was to explore this phenomenon by quantifying DNA damage.
To this end, two complementary methodologies were applied:

- Comet assay, an electrophoretic technique that has been employed for the purpose of highlighting single-strand DNA breaks. This technique is widely utilized for the preliminary evaluation of genotoxic damage;
- FPG-modified comet assay, this is a variant of the test that uses formamidopyrimidine-DNA glycosylase (Fpg), an enzyme capable of specifically recognizing oxidized purine bases and removing them, thereby allowing discrimination of damage induced by reactive oxygen species.

The combined use of the two approaches makes it possible to distinguish between primary genotoxic damage and oxidative base lesions, allowing the effects of the samples to be correlated with different cellular defense or vulnerability mechanisms [85,90].

Additional methodological and instrumental details are reported in the corresponding published paper.

2.3 Results

2.3.1 Yield and composition of essential oil (GC-MS)

The hydrodistillation of the aerial parts of *O. vulgare* collected in Lazio yielded an essential oil with a yield of 0.3% w/w, a value in line with that reported for wild populations of the genus *Origanum* subject to soil and climate variability [76–79].

This yield serves to confirm the close dependence of the volatile component on environmental factors and the balsamic harvesting period. These have been recognized as determining factors in the terpene biosynthesis of the species [66,67].

GC-MS analysis enabled the identification of approximately 40 compounds, accounting for over 96% of the total essential oil content. This finding corroborates the hypothesis that the matrix is chemically complex. In both samples examined - essential oil from wild plants (WEO_OR) and commercial essential oil (CEO_OR) - a marked prevalence of phenolmonoterpenes was observed, albeit with significant quantitative differences.

In particular, carvacrol was the dominant component in both oils, with percentages of 19.17% in WEO_OR and 75.95% in CEO_OR. Thymol, a structurally similar component, was detected exclusively in CEO_OR (4.39%), while it was absent in WEO_OR. Representative GC–MS chromatograms of WEO_OR and CEO_OR are reported in *Figure 2.1*.

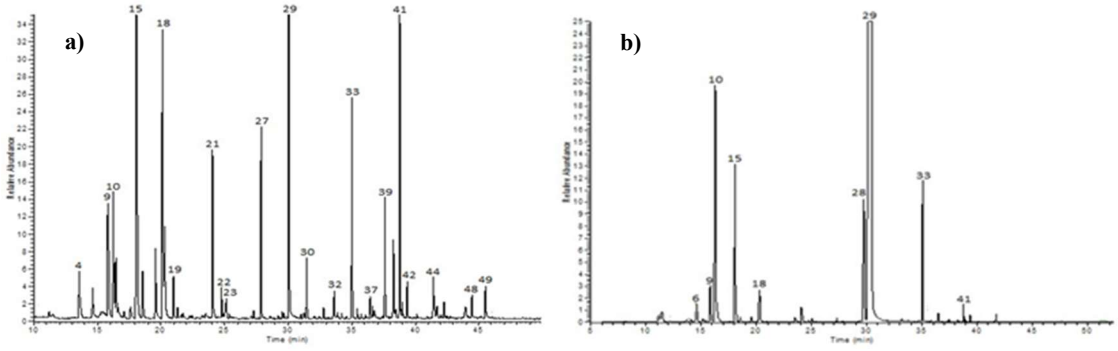


Figure 2.1 - GC-MS TIC chromatogram of a) wild essential oil (WEO_OR) and b) commercial essential oil (CEO_OR) of the plant species *Origanum vulgare*. The numbers shown on each peak correspond to those in *Table 2.1*, allowing the identification of the compounds associate with each peak.

The quantitative distribution of the main constituents of both oils is reported in *Table 2.1*.

Table 2.1 - Chemical composition of the wild essential oil (WEO_OR) and commercial essential oil (CEO_OR) of the plant species *Origanum vulgare*.

N.	Compound	Exp RI	Ref RI	Area %±SD WEO_OR	Area %±SD CEO_OR	Abbr.
2	α-Pinene	935	939	0,16 ± 0,02	0,51 ± 0,01	BM
4	β-Pinene	975	979	1,94 ± 0,14	0,09 ± 0,01	BM
6	Myrcene	993	990	0,99 ± 0,02	0,58 ± 0,03	AM
9	α-Terpinene	1016	1017	3,72 ± 0,13	1,18 ± 0,03	MM
10	*p*-Cymene	1024	1024	3,47 ± 0,13	6,81 ± 0,17	MM
12	Limonene	1030	1029	1,49 ± 0,18	-	MM
15	γ-Terpinene	1061	1059	17,25 ± 0,60	3,70 ± 0,16	MM
16	*p*-Mentha-3,8-diene	1070	1072	1,26 ± 0,10	0,12 ± 0,01	MM
17	Terpinolene	1089	1088	1,91 ± 0,04	0,14 ± 0,01	MM
18	Linalool	1100	1096	9,7 ± 0,36	0,78 ± 0,04	AMO
19	Octen-3-yl-acetate	1116	1112	0,89 ± 0,08	-	OT
21	Terpinen-4-ol	1179	1177	3,97 ± 0,05	0,58 ± 0,02	MMO
22	α-Terpineol	1192	1188	0,65 ± 0,03	0,02 ± 0,02	MMO
27	Linalyl acetate	1260	1257	4,18 ± 0,15	-	AMO
28	Thimol	1298	1290	-	4,39 ± 0,06	MMO
29	Carvacrol	1305	1299	19,17 ± 0,32	75,95 ± 0,40	MMO
30	δ-Elemene	1339	1338	1,19 ± 0,14	-	MS
32	Geranil acetate	1387	1381	0,50 ± 0,08	-	AMO
33	β-Caryophyllene	1420	1419	4,79 ± 0,11	2,28 ± 0,03	BS
39	Germacrene D	1483	1485	2,88 ± 0,15	-	MS
40	γ-Amorphene	1498	1495	2,02 ± 0,20	-	BS
41	Germacrene A	1510	1509	7,11 ± 0,19	0,29 ± 0,02	MS
43	δ-Cadinene	1526	1523	0,77 ± 0,07	0,14 ± 0,01	BS
44	Spatulenol	1581	1578	0,51 ± 0,04	-	BSO
48	Eudesmol 7-epi-α	1659	1663	0,60 ± 0,06	-	BSO
49	Cedren-13-ol 8	1687	1689	0,85 ± 0,08	-	BSO

Abbreviations: AM: aliphatic monoterpenes; MM: monocyclic monoterpenes; BM: bi- and tricyclic monoterpenes; AMO: aliphatic monoterpenoids; MMO: monocyclic monoterpenoids; MS: monocyclic sesquiterpenes; BS: bi- and tricyclic sesquiterpenes; BSO: bi- and tricyclic sesquiterpenoids, OT: others. SD: standard deviation; Exp. RI: experimental retention index; Ref. RI: literature data

For clarity, *Table 2.1* reports only compounds present at ≥ 0.5% in at least one sample, while extended datasets, tables, and supplementary figures supporting the results discussed in this chapter are available in the Supplementary Materials of the corresponding peer-reviewed publication (see Appendix).

Among the non-oxygenated monoterpenes, γ-terpinene and p-cymene exhibited contrasting distributions: The proportions observed in WEO_OR were 17.25% and 3.47%, respectively, in comparison to the 3.70% and 6.81% observed in CEO_OR.

These differences delineate a discrete phytochemical profile between the two oils, with the commercial sample exhibiting a pronounced predominance of carvacrol and reduced terpene variability.

The co-presence of γ-terpinene, p-cymene, carvacrol, and thymol is consistent with the cymyl biosynthetic pathway, typical of chemotypes rich in phenolmonoterpenes and documented in several European populations of *O. vulgare* [66,67,83]. The quantitative analysis suggests that the combined influence of botanical origin, soil and climate conditions, and extraction processes [76–78] is a determining factor in the chemodiversity of the *Origanum* genus.

Overall, both samples can be classified as carvacrol–thymol chemotypes. However, the commercial essential oil exhibited a simplified profile with a strong carvacrol-dominant character, whereas the wild sample showed a higher degree of metabolic complexity, which may be relevant for modulating the biological properties and application potential of the oil [62,68,86,87].

2.3.2 Polyphenolic profile of the methanolic extract (UHPLC-DAD)

UHPLC-DAD analysis enabled the identification and quantification of 15 phenolic compounds, predominantly belonging to the classes of phenolic acids and flavonoids, thereby confirming the elevated phytochemical richness of the species [72,74,84].

The investigation revealed that rosmarinic acid was the predominant phenolic constituent, with a concentration of 38.8 mg/g of dry extract. This compound, which has been extensively documented for its antioxidant properties [54,55,73], is a distinctive metabolic indicator of the Lamiaceae family.

In addition to rosmarinic acid, compounds such as caffeic acid and luteolin-7-O-glucoside further contribute to the antioxidant potential of the extract. This supports the matrix's ability to modulate cellular systems subject to oxidative stress [74,82,84].

Representative UHPLC-DAD chromatographic profiles recorded at different wavelengths are shown in *Figure 2.2*.

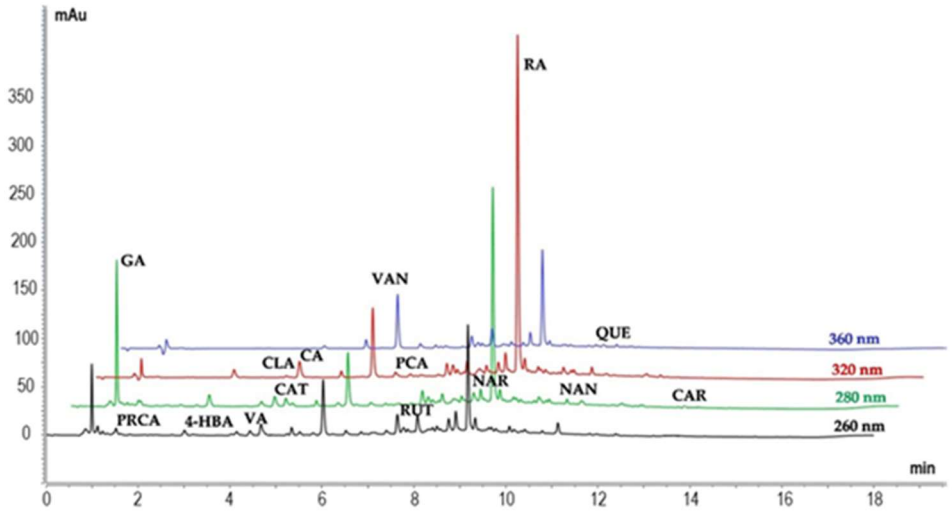


Figure 2.2 - HPLC-DAD profiles at 260, 280, 320, and 360 nm of *O. vulgare* extract.

In total, fifteen compounds were identified and quantified using known standards for calibration.

The content of individual phenolic compounds identified in the methanolic extract is reported in *Table 2.2*.

Table 2.2 - Content of phenolic compounds in ME_OR.

Peak	Phenolic Compounds	Abbr.	RT	λ (nm)	ME_OR ± SD (mg g^{-1} DW)	R^2
1	Gallic acid	GA	1.1	280	13.1 ± 1.3	0.9998
2	Protocatechuic acid	PRCA	1.5	260	1.2 ± 0.4	0.9989
3	4-Hydroxibenzoic acid	4-HBA	3.0	260	0.4 ± 0.1	0.9997
4	Chlorogenic acid	CLA	4.0	320	0.2 ± 0.1	0.9996
5	Catechin	CAT	4.1	280	2.2 ± 0.2	0.9990
6	Vanillic acid	VA	4.4	260	0.9 ± 0.2	0.9991
7	Caffeic acid	CA	4.6	320	1.1 ± 0.1	0.9988
8	Vanillin	VAN	6.0	280	8.1 ± 0.6	0.9989
9	*p*-Coumaric acid	PCA	6.5	320	0.4 ± 0.1	0.9998
10	Rutin	RUT	7.7	260	5.2 ± 0.5	0.9993
11	Naringin	NAR	8.8	280	4.4 ± 0.3	0.9996
12	Rosmarinic acid	RA	9.2	320	38.8 ± 2.8	0.9983
13	Quercetin	QUE	10.4	260	1.7 ± 0.4	0.9986
14	Naringenin	NAN	11.2	280	1.2 ± 0.3	0.9987
15	Carvacrol	CAR	13.2	280	0.3 ± 0.1	0.9997

RT: retention time; R^2: coefficient of determination.

2.3.3 *In vitro* antioxidant activity and polyphenolic content

The results of the DPPH, ABTS, and FRAP assays showed significant differences between the samples analyzed, confirming that antioxidant potential depends on phytochemical composition.

The ME_OR sample demonstrated the most effective antioxidant capacity, as evidenced by the DPPH IC_{50} value of 0.052 mg mL^{-1}, which is considerably lower than that of the other oils. The same extract also exhibited high scavenger activity against the ABTS radical (IC_{50} 0.044 mg mL^{-1}; 3.94 mg TE mL^{-1}) and maximum reducing capacity in the FRAP assay (30.58 mg TE g^{-1}), thus confirming it as the most active sample from an antioxidant point of view.

A comparative overview of the antioxidant activity of essential oils and methanolic extract, as determined by DPPH, ABTS, and FRAP assays, is reported in *Table 2.3*.

Table 2.3 - Antioxidant activity of wild essential oil (WEO_OR), commercial essential oil (CEO_OR), and methanolic extract of *Origanum vulgare* (DPPH, ABTS, and FRAP assays).

SAMPLE	DPPH	ABTS		FRAP
	IC_{50} (mg mL^{-1})	**IC_{50} (mg mL^{-1})**	**(mg TE mL^{-1})**	**(mg TE g^{-1})**
ME_OR	0.052 ± 0.01	0.044 ± 0.006	3.94 ± 0.07	30.58 ± 3.7
CEO_OR	0.45 ± 0,11	0.033 ± 0.015	9.57 ± 0.52	7.33 ± 0.31
WEO_OR	1.54 ± 0.22	0.56 ± 0.07	0.10 ± 0.02	-
Positive control	0.00375 *	0.00347 **		0.00506 **

IC_{50}: concentration of the extract that inhibits 50% of the radical activity. TE: Trolox equivalents. Each value is a mean ± SD of triplicate analysis. * Ascorbic acid; ** Trolox equivalent.

The superiority of ME_OR is attributable to its richness in antioxidant phenols, particularly rosmarinic acid, which is capable of transferring electrons, chelating metals, and neutralizing free radicals [4,72,74].

In accordance with previous studies, essential oils exhibited reduced activity in comparison to the respective polyphenolic extracts, confirming the decisive role of the phenolic fraction in the biological potential of oregano [81,82,84,88].

To further support the antioxidant results, the total phenolic and flavonoid contents of the methanolic extract were determined and are reported in *Table 2.4*.

Table 2.4 Total phenolic and flavonoid content of methanolic extract of *Origanum vulgare* (ME_OR).

SAMPLE	Total Polyphenols	Flavonoids	
	(mg g^{-1} GAE)	(mg QUE g^{-1} DM)	(mg CAE g^{-1} DM)
ME_OR	75.49 ± 0.9	147.2 ± 2.1	34.7 ± 0.5

GAE: gallic acid equivalents; QUE: quercetin equivalents; CAE: catechin equivalents. Each value is a mean ± SD of a triplicate analysis.

2.3.4 Biological effects in HepG2 cells

Treatment of HepG2 cells with the methanolic extract of *Origanum vulgare* (ME_OR) in the concentration range of 50–150 μg mL^{-1} did not induce significant cytotoxic effects up to 48 h of exposure, indicating a good safety profile at the tested doses. Cell viability remained comparable to that of untreated control cells, confirming the suitability of these concentrations for subsequent experiments under oxidative stress conditions.

To investigate the potential protective role of ME_OR against oxidative stress, HepG2 cells were pretreated with the extract prior to exposure to hydrogen peroxide (H_2O_2), used as an oxidizing agent. As expected, treatment with H_2O_2 alone resulted in a marked reduction in cell viability.

Pretreatment with ME_OR, however, produced a dose-dependent response. At lower concentrations, the extract partially attenuated oxidative damage, whereas at the highest tested dose (150 μg mL^{-1}) a pro-oxidant effect was observed, characterized by an exacerbation of oxidative stress–related cellular responses.

The evaluation of 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels, a marker of oxidative DNA damage, confirmed this trend. In particular, lower concentrations of ME_OR were associated with reduced DNA oxidation, while higher concentrations led to increased 8-OHdG accumulation, suggesting a shift from antioxidant to pro-oxidant behaviour depending on dose.

In contrast, treatment with the wild essential oil (WEO_OR) at 0.005% did not significantly affect cell viability, either in basal conditions or following oxidative challenge. Notably, pretreatment with WEO_OR significantly reduced H_2O_2-induced 8-OHdG accumulation, indicating a protective effect against oxidative DNA damage without compromising cell viability.

The effects of ME_OR and WEO_OR on HepG2 cell viability and oxidative stress markers are shown in *Figures 2.3* and *2.4*.

Overall, these findings highlight a differential biological behaviour between the methanolic extract and the essential oil, with the former exhibiting a dose-dependent dual antioxidant/pro-oxidant activity and the latter displaying a consistent protective effect under oxidative stress conditions.

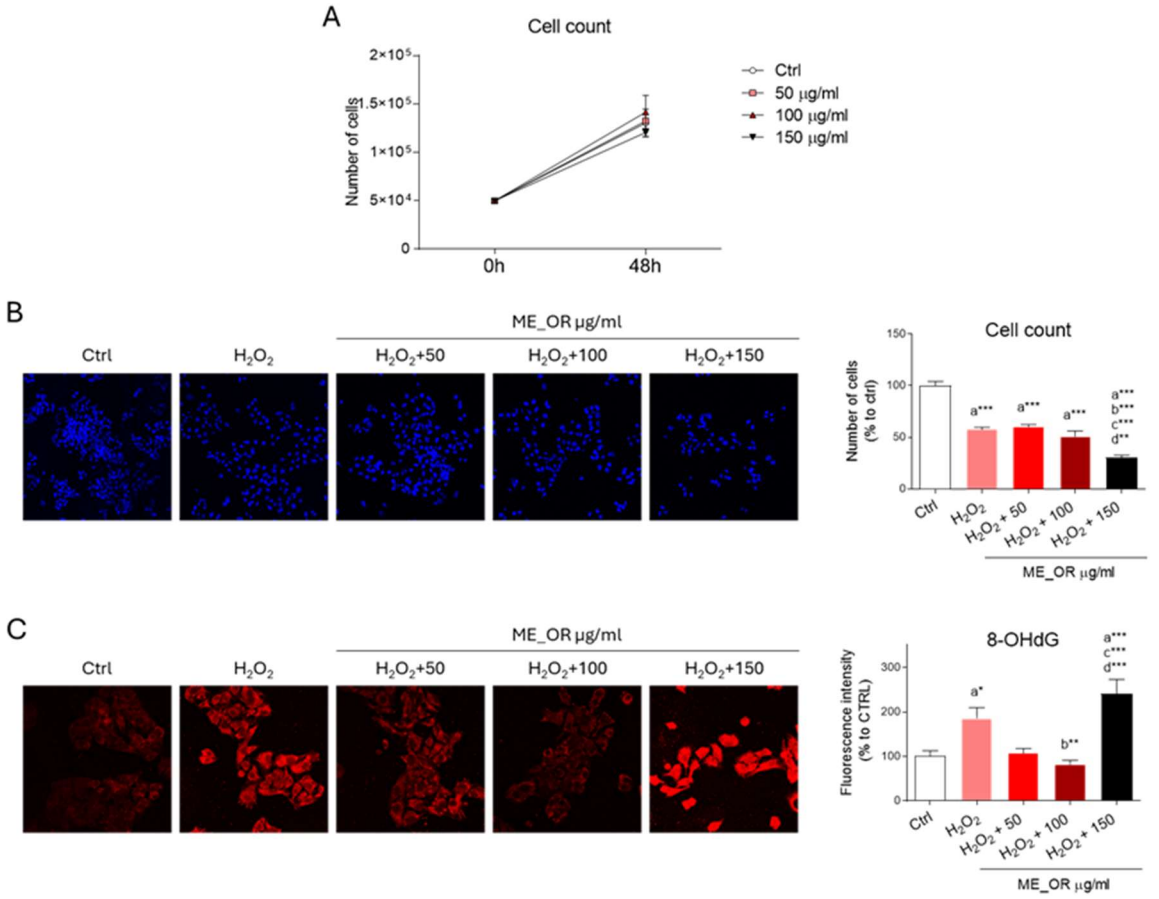


Figure 2.3 - Effects of the methanolic extract of *Origanum vulgare* (ME_OR) on HepG2 cell viability and oxidative stress under H_2O_2-induced conditions. HepG2 cells were treated with ME_OR (50–150 µg mL^{-1}) prior to exposure to H_2O_2. Cell viability and oxidative DNA damage were evaluated as described in the published study.

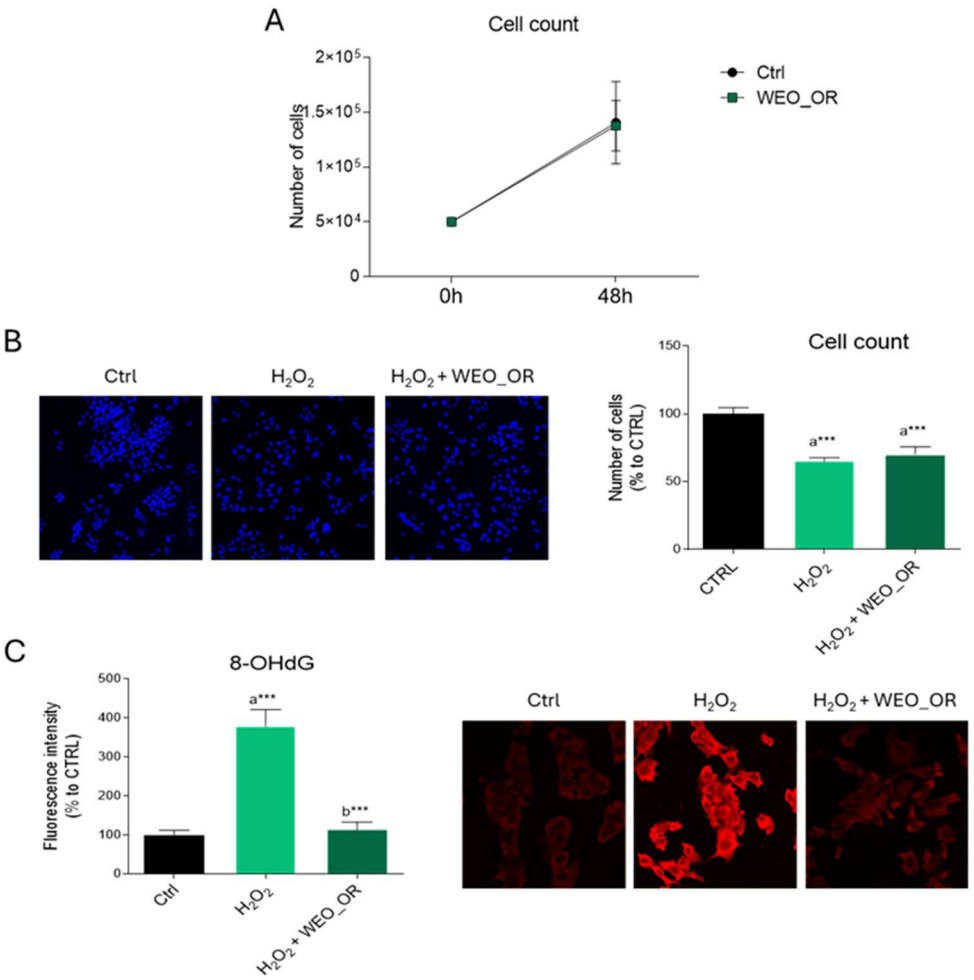


Figure 2.4 - Effects of wild *Origanum vulgare* essential oil (WEO_OR) on HepG2 cell viability and oxidative DNA damage induced by H_2O_2. Cells were pretreated with WEO_OR (0.005%) before oxidative challenge. Protective effects were evaluated by cell viability assays and 8-OHdG detection, as described in the published study.

2.4 Discussion

The results obtained demonstrate that the different extractive fractions of *Origanum vulgare* L. have distinct phytochemical profiles and non-overlapping biological activities, reflecting the metabolic complexity typical of the species. The yield of the essential oil and its composition, characterized by the prevalent presence of phenolimonoterpenes such as carvacrol and thymol, confirm that the samples belong to the carvacrol-thymol chemotype. This finding is in accordance with that reported for Mediterranean populations characterized by high geographical and pedoclimatic variability [66,67,76–79,83]. The quantitative disparities that emerged between WEO_OR and CEO_OR underscore the impact of extraction methodologies and industrial processing on the distribution of the primary terpenes, consistent with the findings documented in recent literature [79,91].

Concurrently, UHPLC-DAD analysis of the methanol extract demonstrated a marked predominance of rosmarinic acid, accompanied by substantial amounts of gallic acid, vanillin, rutin, and naringin. This outcome is consistent with the established physiology of the *Origanum* genus, in which rosmarinic acid is identified as one of the primary metabolites responding to biotic and abiotic stress [4,72,74,82,84]. It provides a compelling explanation for the significantly higher contribution of polyphenols compared to terpenes to the antioxidant activity observed.

The evaluation of antioxidant activity confirms this interpretation: among the samples analyzed, ME_OR showed the best antioxidant performance in assays based on both radical neutralization and electron transfer, clearly distinguishing itself from essential oils [73,81,84,88,92]. In contrast, WEO_OR and CEO_OR showed lower efficacy, in line with the reduced availability of electron-donating phenolic groups.

A particular element of interest emerges from the analysis conducted on HepG2 cells. ME_OR exhibited no direct cytoprotective effects on cell viability under conditions of oxidative stress, and at high doses, even amplified the toxic effects induced by hydrogen peroxide. However, low and intermediate doses of the extract reduced the accumulation of 8-OHdG, suggesting a selective modulation of oxidative DNA damage not attributable to an increase in cell proliferation.

The experimental data, when considered as a whole, provide support for a functional model that incorporates the following elements:

- the essential oil exhibits a terpene matrix, characterized by predominantly antimicrobial bioactivity and moderate antioxidant activity;
- the methanolic extract has been shown to concentrate phenolic compounds, which have the capacity to modulate oxidative stress and the level of oxidative damage to DNA, in a dose-dependent manner;
- it has been hypothesized that the combination of the two fractions may result in either synergistic or antagonistic interactions, in accordance with the phytochemical cooperation hypotheses formulated for the *Origanum* genus [62,68,82,86,87,93].

The results obtained confirm that *Origanum vulgare* L. is a botanical matrix with high phytochemical diversity, characterized by distinct functional profiles depending on the extract fraction considered. The essential oil, both that obtained from wild plants in Lazio and that of commercial origin, shows a composition attributable to the carvacrol-thymol chemotype, although characterized by marked quantitative differences attributable to botanical origin and processing conditions.

Concurrently, the methanolic extract is distinguished by its elevated concentration of phenolic compounds, predominantly rosmarinic acid, which are responsible for its superior antioxidant capacity in comparison to the volatile fraction. However, this activity does not translate into a universal preservation of cell viability. In the HepG2 model, ME_OR did not prevent the reduction in viability induced by H_2O_2 and, at higher concentrations, even amplified the effects of oxidative stress, confirming a dose-dependent behaviour.

In contrast, wild essential oil (WEO_OR) exhibited the capacity to mitigate the accumulation of oxidative damage to DNA without compromising cell viability, thereby underscoring a distinct and complementary functional profile in comparison to the polyphenolic extract.

Overall, the experimental data indicate the following:

- the volatile fraction exhibits moderate antioxidant activity and can selectively modulate oxidative DNA damage;
- the polyphenolic fraction concentrates antioxidant radical scavenging potential, though it does not necessarily have a direct cytoprotective effect on cell viability;

- the two matrices exhibit different biological properties, which are attributable to their respective phytochemical compositions.

These findings suggest potential targeted applications: the essential oil could be used as an aromatic matrix or adjuvant with selective antioxidant properties, while the methanolic extract could be used as an ingredient with a high phenolic content in nutraceutical and cosmetic formulations.

2.5 Remarks

The study highlighted how *O. vulgare* essential oil harvested in central Italy and commercial oil share a basic composition attributable to the carvacrol-thymol chemotype, albeit with significant quantitative differences in the main constituents. This variability reflects the influence of geographical origin and production conditions on the yield and chemical profile of essential oils.

The methanolic extract stood out for its high content of rosmarinic acid, identified as the main phenolic constituent, and for its marked antioxidant activity, superior to that observed for essential oils. *In vitro* biological tests on HepG2 cells also demonstrated the absence of cytotoxicity at low concentrations and a selective, dose-dependent modulation of oxidative DNA damage under H_2O_2-induced stress.

These results confirm the potential of *O. vulgare* extracts as natural sources of bioactive compounds with antioxidant activity and suggest possible applications in the nutraceutical and pharmaceutical sectors.

CHAPTER 3 *Laurus nobilis* L.

3.1 Background and rationale

Chronic inflammation is now recognized as a key predisposing factor for many degenerative and non-communicable diseases, including cardiovascular disease, metabolic disorders, neurodegenerative diseases, and various cancers [94–99]. These conditions are caused by prolonged oxidative stress, which is characterized by the accumulation of reactive oxygen species and impaired endogenous antioxidant systems. This results in damage to biomolecules and tissues [95,99]. The arachidonic acid cascade plays a crucial role in these processes, involving enzymes such as cyclooxygenase (COX) and lipoxygenase (LOX), as well as components of the cytochrome P450 system, in the biosynthesis of mediators that orchestrate the inflammatory response [100].

Of the various metabolic nodes involved, soluble epoxide hydrolase (sEH) is particularly important. Widely distributed in organs such as the liver, kidneys, and central nervous system, this enzyme catalyses the hydrolysis of epoxides derived from polyunsaturated fatty acids. This reduces the concentration of epoxyeicosanoids, which have anti-inflammatory, vasodilatory, and cardioprotective properties [1,100–103]. Consequently, inhibiting sEH activity is a promising pharmacological strategy for modulating the inflammatory response and pathological conditions related to oxidative stress [101–103]. However, the use of several highly potent synthetic inhibitors, mainly belonging to the urea derivative class, is limited by issues of selectivity, bioavailability, and side effects [103,104].

In this scenario, scientific interest has progressively focused on the identification of bioactive molecules of natural origin, particularly from medicinal and aromatic plants. These represent resources of high pharmacological value thanks to the presence of secondary metabolites with antioxidant, anti-inflammatory, and antimicrobial properties [2,12,105].

In the array of plant species under consideration, *Laurus nobilis* L., an evergreen species that is widely distributed in the Mediterranean region, occupies a prominent position. This species has a long history of traditional use in the treatment of gastrointestinal and respiratory disorders, as well as a carminative, antiseptic, and adjuvant agent in rheumatic symptoms [17,18,106]. The essential oil in question is a subject of mounting interest in the food, cosmetic, and pharmaceutical sectors. This is due to the oil high content of terpene and phenylpropanoid compounds, which are known to possess a variety of biological activities.

The phytochemical composition of *L. nobilis* essential oils has been extensively documented, and is dominated by oxygenated monoterpenes such as 1,8-cineole, linalool, and α-terpinyl acetate, as well as phenylpropanoids such as eugenol and methyleugenol. It is believed that these compounds are responsible for the pharmacological activities associated with the species [107,108].

In view of these assumptions, the objective of this chapter is to:

i. characterize the chemical composition of *L. nobilis* essential oils using GC-FID and GC-MS techniques;
ii. evaluate their antioxidant activity through *in vitro* assays;
iii. determine their inhibitory activity against the sEH enzyme;
iv. interpret the experimental results using molecular docking studies to identify the constituents potentially involved in the interaction with the enzymatic target.

This integrated approach facilitates the rigorous correlation of the chemical composition of essential oils with the observed biological properties, thereby contributing to the definition of the potential role of *Laurus nobilis* as a natural source of molecules with anti-inflammatory activity.

3.2 Experimental design

This section outlines the experimental design adopted for the study of *Laurus nobilis* L.

Detailed experimental procedures are reported in Chapter 10.

3.2.1 Plant material and sample preparation

Fresh leaves of *Laurus nobilis* L. were collected in April 2023 in various locations in central and southern Italy, selected based on their altitude, to evaluate the possible influence of geographical factors on the chemical profile of essential oils. Samples were obtained from five sites in the Molise region.

The following locations were identified in the study: Petacciato, 25 m a.s.l. (LNMO1); Campobasso, 700 m a.s.l. (LNMO2); Isernia, 420 m a.s.l. (LNMO3); Carpinone, 630 m a.s.l. (LNMO4); and Capracotta, 1420 m a.s.l. (LNMO5). Two neighboring areas were also identified: Abruzzo (Rosciano, 150 m above sea level) and Campania (Santa Maria Capua Vetere, 35 m a.s.l.), identified as LNAB and LNCA, respectively.

The leaves (800 g per sample) were cleaned manually and subjected separately to hydrodistillation using a Clevenger apparatus for 3 hours, following the official procedure reported by the Council of Europe [109,110].

The essential oils obtained were then subjected to a drying process over anhydrous sodium sulfate, with the objective of eliminating any residual moisture. Subsequent to this, the oils were stored in dark glass vials at a temperature of 4 °C, pending further analysis.

3.2.2 Chemical characterization

The chemical composition of essential oils was analyzed by gas chromatography with flame ionization detector (GC-FID) and gas chromatography coupled to mass spectrometry (GC-MS). For each analysis, 1 μL of essential oil, diluted in n-hexane (1:500 v/v), was injected in split/splitless mode.

In order to ensure the reproducibility of results, each sample was analyzed in triplicate.

The relative quantification of the components was expressed as a percentage of the chromatographic peak areas, obtained in triplicate, without applying correction factors

[111]. The analytical standards of the main monoterpenes and phenylpropanoids present in the essential oil were used to validate the identification of the compounds reported in *Table 3.1*.

3.2.3 Antioxidant activity assays

3.2.3.1 DPPH radical scavenging assay

The antioxidant activity of essential oils was evaluated by the DPPH assay, performed according to a procedure described in the literature [76] with some modifications. Different concentrations of essential oil, diluted in ethanol, were mixed with an ethanolic solution of DPPH· (27 μg/mL) in a 1:1 (v/v) ratio. Subsequently, the reaction mixtures were subjected to incubation in conditions of darkness for a period of 30 minutes, thereby enabling the interaction between the free radical and the antioxidant molecules present within the sample.

The experimental tests were performed in triplicate, and the results are reported as mean ± standard deviation. Ascorbic acid was utilized as a positive control under identical experimental conditions.

3.2.3.2 FRAP assay

The reducing capacity of essential oils was determined using the FRAP method, as reported by Benzie et al. [112], with appropriate modifications.

The addition of FRAP reagent (1.8 mL) to each sample (200 μL) resulted in the formation of a mixture, which was then subjected to incubation at 37 °C for a duration of 30 minutes. Absorbance was measured at a wavelength of 593nm, and the reducing power was correlated with the increase in signal compared to the blank. Ascorbic acid was utilized as a positive reference standard.

Antioxidant activity was measured in accordance with established protocols, employing a calibration curve obtained with Trolox (4–25 μM) as the standard. The results were expressed in millimoles of Trolox equivalent per gram of essential oil.

3.2.4 Soluble epoxide hydrolase (sEH) inhibition assay

The inhibitory activity of essential oils against soluble epoxide hydrolase (sEH) was assessed using an *in vitro* fluorimetric assay. Each sample was tested in triplicate at a final concentration of 10 µg/µL using a specific enzyme kit for screening sEH inhibitors.

The recombinant enzyme was diluted in bis-Tris buffer (25 mM, pH 7) and pre-incubated for 15 minutes at 25°C with the essential oil, the reference compound AUDA (12-(3-adamantan-1-yl-ureido) dodecanoic acid), or the vehicle (2.5% ethanol). The initiation of the reaction was achieved by the addition of the fluorogenic substrate PHOME to a final concentration of 0.25 µM.

Subsequent to the hydrolysis of the substrate by the enzyme, an intermediate is generated through intramolecular cyclization, which rapidly decomposes to form the fluorophore 6-methoxy-2-naphthaldehyde. The formation of this product was monitored by detecting fluorescence at wavelengths of 330 nm and 465 nm, respectively.

The IC_{50} value, defined as the concentration required to inhibit enzyme activity by 50%, was determined for each sample. All experiments were conducted in triplicate, and the results are expressed as mean ± standard error.

3.2.5 *In silico* studies (molecular docking)

In order to further investigate the molecular mechanisms underlying the observed inhibitory activity against sEH, a computational docking study was performed. The crystallographic structure of the enzyme in complex with its ligand was obtained from the Protein Data Bank (PDB ID: 4Y2X) [113] and prepared using standard protocols for protein structure optimization [114,115], including the addition of hydrogen atoms and the assignment of bond orders. The co-crystallized ligand was removed, and the catalytic site was defined based on its coordinates.

The docking area was delineated by centring the search box on the active site of sEH, utilising the ligand originally present in the complex as a reference point. The grid centre coordinates were set to −17.54 (x), −9.70 (y), and 66.48 (z), with internal dimensions of 10 × 10 × 10 Å and external dimensions of 24.58 × 24.58 × 24.58 Å.

The two-dimensional structures of the main chemical constituents identified in essential oils were retrieved from the PubChem database and converted to three-dimensional format [114,116]. The ligands were subjected to protonation state generation at pH 7.4 and energetically minimized using the OPLS 2005 force field.

Docking experiments were performed using Glide software [114,116–119], with the aim of evaluating the binding affinity for each compound to the catalytic site of the sEH. The results were expressed as binding energies and analyzed with reference to the main interactions established between the ligands and the amino acid residues of the enzyme.

3.3 Results

Extended datasets, tables, and supplementary figures supporting the results discussed in this chapter are available in the Supplementary Materials of the corresponding peer-reviewed publication (see Appendix).

3.3.1 Chemical Profile of Essential Oils

A comprehensive gas chromatography-mass spectrometry (GC-MS) analysis was conducted on the essential oils derived from the seven *Laurus nobilis* accessions, resulting in the identification of a total of 78 volatile compounds. The relative abundance of the major constituents is reported in *Table 3.1*.

In all samples, the predominant compound was 1,8-cineole, with concentrations ranging from 31.31% to 43.52%. This compound was consistently detected together with variable amounts of methyleugenol, α-terpinyl acetate, linalool, and sabinene, which together define the characteristic metabolic profile of the analyzed essential oils.

The major volatile constituents (≥ 1.0%) identified in *Laurus nobilis* essential oils are summarized in *Figure 3.1*.

The most abundant class of compounds was represented by oxygenated monoterpenoids, followed by monoterpene hydrocarbons and phenylpropanoids, while other classes were present in lower proportions.

Table 3.1 - Major chemical constituents (≥1.0% in at least one sample) of *Laurus nobilis* essential oils from different geographical origins. Relative abundance is expressed as percentage values. For clarity, only major compounds are reported, while the complete corresponding published paper.

COMPOUND	Area (%)						
	LNMO1	**LNMO2**	**LNMO3**	**LNMO4**	**LNMO5**	**LNCA**	**LNAB**
***α*-Pinene**	4.83	5.17	7.41	3.61	4.80	4.73	4.06
Sabinene	6.70	9.84	10.57	9.12	4.85	10.2	7.56
***β*-Pinene**	3.77	2.96	3.81	2.44	2.47	3.00	2.61
Myrcene	0.38	0.88	0.74	1.02	0.56	1.01	0.7
1.8-Cineole	41.01	43.52	39.9	40.7	31.31	42.39	33.72
Linalool	1.08	3.93	1.09	2.80	11.72	3.44	10.29
Terpinen-4-ol	2.33	1.45	1.46	1.25	1.74	1.58	1.69
***α*-Terpineol**	2.27	1.00	2.55	2.91	0.91	1.31	1.92
***α*-Terpinyl acetate**	10.2	11.43	8.51	9.16	9.55	13.00	9.31
Eugenol	4.12	3.30	3.41	1.97	4.05	2.65	2.81
Methyl eugenol	10.54	4.35	6.08	14.5	14.96	4.07	10.86
Elemicin	1.19	0.57	0.63	0.60	2.05	0.69	1.19
Spathulenol	0.44	0.86	1.76	0.26	0.94	0.49	0.78

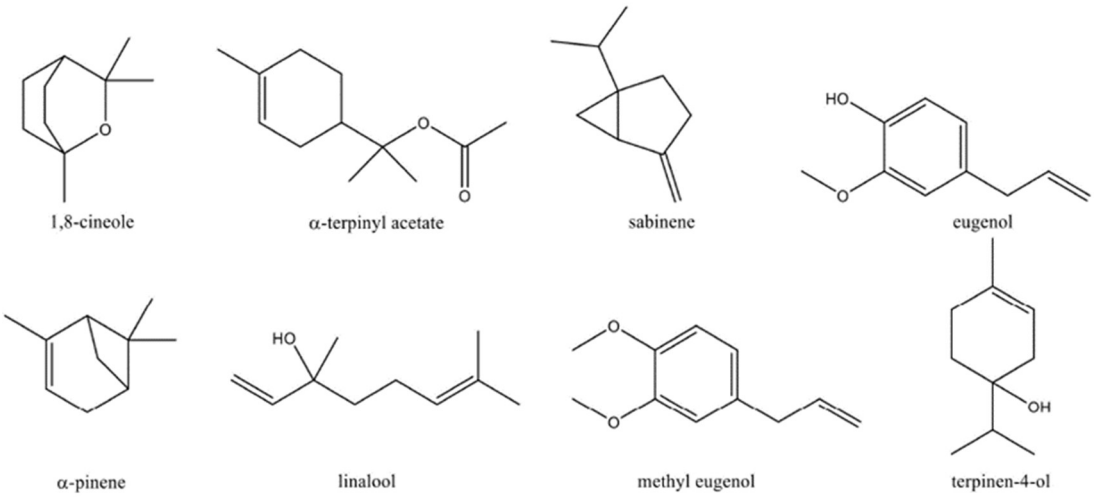


Figure 3.1 - Major volatile constituents (≥1.0%) identified in the essential oils of *Laurus nobilis* from different geographical origins.

3.3.1.1 Multivariate Analysis

Multivariate statistical analysis of the GC–MS data, performed using Principal Component Analysis (PCA) and Partial Least Squares–Discriminant Analysis (PLS-DA), revealed marked chemical variability among the analyzed essential oils. The PLS-DA score plot (*Figure 3.2*) shows a clear separation of the seven accessions, indicating the presence of distinct metabolic profiles related to their different origins.

The model allowed the identification of the metabolites most responsible for sample discrimination through Variable Importance in Projection (VIP) analysis. Compounds with VIP values greater than 1 were identified as the main contributors to the observed separation, as reported in the corresponding published paper.

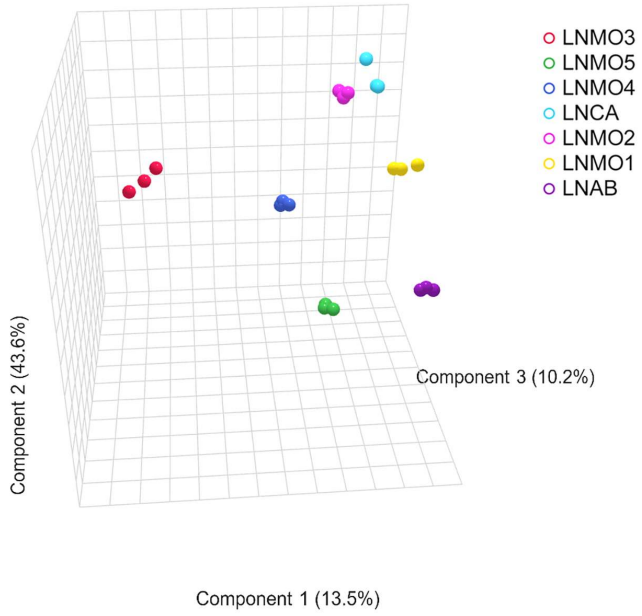


Figure 3.2. 3D Score Plot of the PLS-DA of essential oil of *L. nobilis.*

The model enabled the identification of the metabolites most responsible for sample separation, as determined by Variable Importance in Projection (VIP) analysis.

Compounds with VIP values greater than 1 were identified as the main contributors to sample discrimination, as reported in the published paper.

3.3.2 Antioxidant activity

Antioxidant activity and total phenolic content of the essential oils were evaluated using the Folin–Ciocalteu method, DPPH, and FRAP assays. The results highlight variability among the different accessions and are summarized in *Table 3.2*.

Table 3.2. Total phenolic content (TPC) and antioxidant capacity of *Laurus nobilis* essential oils (EOs) from leaves, evaluated by DPPH and FRAP assays. Ascorbic acid was used as a positive control. IC_{50} represents the concentration required to inhibit 50% of radical activity. Results are expressed as mean ± SD of triplicate analyses. GAE: gallic acid equivalents; TE: Trolox equivalents.

SAMPLE	TPC *(mg GAE/g EO)*	DPPH *IC_{50} (µg/mL)*	FRAP *(mmol TE/g EO)*
LNMO1	11.36 ± 0.23	465.58 ± 2.80	0.51 ± 0.01
LNMO2	10.55 ± 0.24	411.55 ± 13.11	0.32 ± 0.01
LNMO3	10.13 ± 0.14	423.04 ± 26.24	0.30 ± 0.01
LNMO4	6.60 ± 0.29	535.90 ± 28.02	0.27 ± 0.01
LNMO5	10.24 ± 0.30	505.91 ± 10.57	0.40 ± 0.01
LNCA	7.97 ± 0.24	519.90 ± 17.12	0.27 ± 0.01
LNAB	8.28 ± 0.17	423.76 ± 10.86	0.30 ± 0.01
Ascorbic acid	-	3.75 ± 0.01	11.10 ± 0.2

3.3.3 sEH Inhibitory Activity

The inhibition of soluble epoxide hydrolase (sEH) was evaluated for all essential oil samples using an *in vitro* fluorimetric assay.

The IC_{50} values obtained are reported in *Table 3.3*, together with a comparison with the synthetic inhibitor AUDA [12-(3-(adamantan-1-yl)ureido) dodecanoic acid], used as a reference compound. AUDA is a potent soluble epoxide hydrolase (sEH) inhibitor for the mouse and human she, respectively [120].

The corresponding concentration–response curves are shown in *Figure 3.3*.

As expected, AUDA exhibited markedly higher inhibitory potency than the essential oils.

Table 3.3 Inhibitory activity of *Laurus nobilis* essential oils against soluble epoxide hydrolase (sEH), expressed as IC_{50} values. Data are reported as mean ± SD of three independent experiments. AUDA was used as a reference synthetic inhibitor.

Tested oils	IC_{50} ± SD (mg/mL)
LNMO5	16.5 ± 4.3
LNAB	48.8 ± 2.6
LNMO3	71.2 ± 3.4
LNCA	125.7 ± 9.4
LNMO4	421.2 ± 14.6
LNMO1	4513.0 ± 695.1
LNMO2	8062.3 ± 580.9
AUDA (known inhibitor)	0.068 ± 0.003

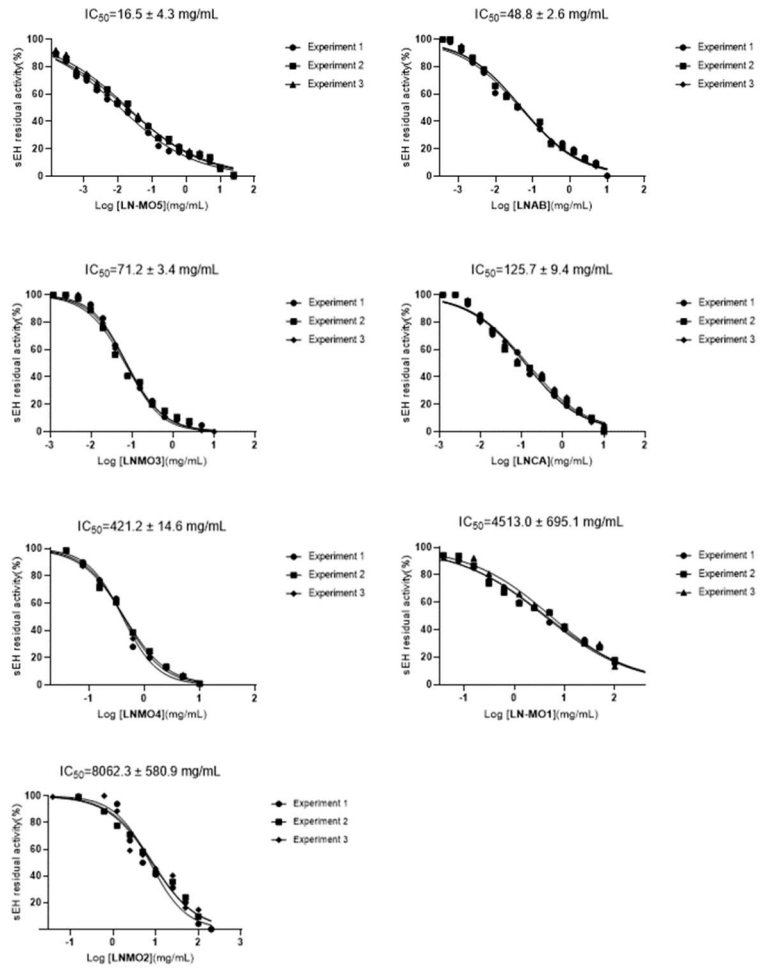


Figure 3.3 - Concentration–response curves describing the inhibitory effects of the seven *Laurus nobilis* essential oils on isolated sEH enzyme activity. Data are expressed as percentage of control (100%) and reported as mean ± SD (n = 3).

3.3.4 Molecular Docking Studies

Molecular docking simulations were performed on the main constituents identified in the essential oils in order to evaluate their potential affinity for the active site of soluble epoxide hydrolase (sEH). Docking results were used to assess the binding modes of selected metabolites qualitatively and to rationalize the experimental inhibitory activity observed *in vitro*.

A comparative visualization of the interactions between selected essential oil constituents and the sEH active site is shown in *Figure 3.4*, highlighting the different orientations within the binding pocket and the key non-covalent interactions involved in enzyme binding.

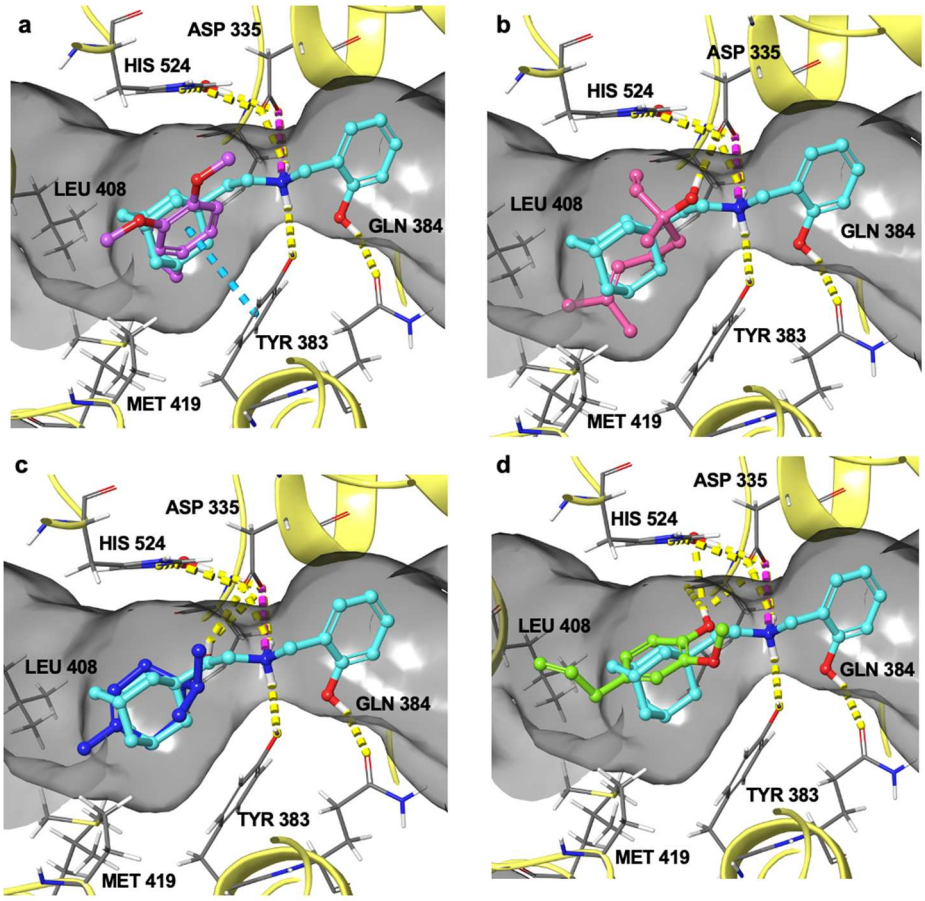


Figure 3.4 - Superposition of the binding modes of the known sEH inhibitor 4A0 and selected constituents of *Laurus nobilis* essential oil within the active site of sEH. The inhibitor 4A0 is shown in cyan, while (a) methyl eugenol, (b) linalool, (c) terpinen-4-ol, and (d) eugenol are shown in different colors. Hydrogen bonds are indicated by yellow dotted lines, π–π stacking interactions by blue dotted lines, and salt bridges by purple dotted lines.

3.4 Discussion

The comprehensive analysis of the collected data underscores the presence of substantial compositional and functional variability in *Laurus nobilis* essential oils, primarily influenced by the geographic origin of the accessions and the environmental factors associated with the harvesting regions. The chemical differences observed among the oils are consistent with the literature on this aromatic species, which is known for its variable composition depending on soil and climate conditions and extraction parameters [121–123]. In particular, the predominance of 1,8-cineole in all samples confirms the presence of an eucalyptol-dominant chemotype, typical of Mediterranean populations of *L. nobilis* [107,124,125]. However, the concentrations of other compounds, such as methyleugenol, α-terpinyl acetate, and linalool, are more variable and potentially responsible for the observed functional differences.

Multivariate analysis plays a pivotal role in the interpretation of data, as it enables the clear differentiation of accessions according to their metabolic fingerprint. The employment of PLS-DA, a recognized effective tool for analysing complex datasets in metabolomics [126], enabled the identification of the compounds most likely to influence sample clustering. This statistical approach thus provides objective support for the observation that the composition of *L. nobilis* oils is not only qualitatively similar between accessions, but also differs sufficiently to generate unique metabolic profiles.

The correlation between chemical profile and antioxidant activity is particularly evident. Oils with higher oxygenated monoterpenoid and phenylpropanoid content demonstrated superior scavenging capacity and higher reducing power in DPPH and FRAP assays. It is acknowledged that these classes of compounds exhibit a notable reactivity towards radical species, attributable to the presence of functional groups that are capable of donating electrons or hydrogen atoms [127]. This phenomenon contributes to the explanation of the observed differences between the samples.

Whilst total phenolic content (TPC) does not serve as a direct indicator of antioxidant activity, it does offer a supplementary interpretative element which helps to elucidate some of the variability observed among the samples. This suggests that the nature and quantity of secondary metabolites act in concert to modulate the overall antioxidant response.

The inhibitory activity against sEH represents an additional level of interpretation. Despite the markedly higher IC_{50} values of essential oils when compared to the synthetic inhibitor AUDA, which is characterized by affinities in the nanogram range, the obtained data suggest the capacity of the oils to interact significantly with the enzyme. This finding indicates a model of action that cannot be attributed to a single ligand but rather suggests the simultaneous intervention of multiple components. This behaviour is consistent with recent evidence demonstrating that natural compounds, such as terpenes and phenylpropanoids, can modulate sEH through hydrophobic interactions or weak polar bonds [5,120,128–132].

In this context, oils from accessions collected at higher altitudes - such as LNMO5 - present a chemical profile particularly rich in oxygenated metabolites, which could favour enzymatic interaction, thereby promoting the hypothesis that specific ecological factors selectively influence the biosynthesis of functional metabolites.

This interpretation of the data is further corroborated by molecular docking studies, which provide a structural rationale for the observed experimental activity. The ability of the main essential oil constituents to stably locate in the active site of the sEH, delineating significant hydrophobic interactions, reinforces the idea that the *L. nobilis* phytocomplex possesses structural elements that can be used as scaffolds for the design of new drug candidates [120,129]. From this standpoint, the absence of a urea or amide moiety - characteristic of high-affinity sEH inhibitors - does not preclude the possibility of enhancing the activity of these natural scaffolds through rationally guided chemical modification.

3.5 Remarks

The experimental evidence emerging from this study, combined with a critical and comparative analysis of the data, allowed the delineation of a comprehensive picture of the chemical and functional characteristics of *Laurus nobilis* L. essential oils from different geographical areas.

Analytical characterization revealed high compositional variability among samples, while confirming the consistent presence of several key metabolites, particularly 1,8-cineole, α-terpinyl acetate, linalool, and phenylpropanoid compounds, which contribute to the species' chemotype. Multivariate analysis further corroborated this finding, demonstrating a clear

separation between the metabolic profiles of the oils and suggesting a significant influence of environmental factors on their composition.

The essential oils under scrutiny in this study exhibited substantial antioxidant properties, manifesting in the capacity to scavenge free radicals and reduce oxidising agents. Despite the inhibitory activity against sEH being lower than that of the reference synthetic inhibitor, the values obtained indicate a biologically relevant interaction with the enzyme, suggesting that specific combinations of metabolites can selectively modulate this target. The results of the *in silico* study lend support to this hypothesis, offering a rational structural interpretation of the experimental activity and identifying potential pharmacophoric fragments in the lipophilic components of the essential oils.

The data obtained from this study, when considered as a whole, lends further support to the hypothesis that *L. nobilis* essential oils represent a natural source of bioactive compounds capable of exerting antioxidant effects and modulating the sEH enzyme.

These findings provide concrete prospects for further investigations focused on the isolation and characterization of the most representative individual metabolites, as well as on the rational design of new inhibitors inspired by natural scaffolds.

CHAPTER 4 *Cannabis sativa* L. var. *Kompolti*

4.1 Background and rationale

Cannabis sativa L. is an annual herb belonging to the Cannabaceae family. It has been extensively studied for the diversity of its secondary metabolites, including phytocannabinoids, which exhibit various biological activities of pharmacological interest [6]. Recently, varieties with low Δ9-tetrahydrocannabinol (Δ9-THC) content have attracted growing interest, such as the *Kompolti cultivar*. This cultivar is characterized by the prevalent presence of non-psychoactive compounds, making it particularly suitable for nutraceutical and therapeutic uses [133–138].

Cannabidiol (CBD), one of the main phytocannabinoids, is the non-psychoactive cannabinoid of greatest pharmacological interest thanks to its antioxidant, anti-inflammatory, and neuroprotective properties, which are supported by numerous experimental studies [19,20,139].

Unlike Δ9-THC, CBD does not act as a potent direct agonist on CB1 and CB2 receptors, but rather interacts with multiple intracellular pathways involved in the response to oxidative stress and the modulation of neuroinflammation — central pathogenetic elements in neurodegenerative diseases [140–142].

Alongside the growing interest in phytocannabinoids, increasing attention has been given to phenolic and flavonoid compounds that are naturally present in plant matrices and recognized for their role in modulating oxidative stress [143,144]. This is complemented by the evaluation of antioxidant activity using specific assays, such as DPPH and FRAP, which aim to measure the sample's ability to neutralise radical species or reduce transition metals that are responsible for propagating oxidative processes [112,145]. However, the correlation between polyphenolic content and antioxidant activity is not always linear, suggesting a synergistic and structural role for the different components of the plant phytocomplex [112,145].

In this study, two preparations obtained from the inflorescences of *C. sativa* var. *Kompolti* were analyzed:

- the aqueous infusion (WI), which is representative of the plant's traditional use;

- the hydroalcoholic extract (HEC), which is aimed at enriching metabolites with greater lipophilicity [146,147].

Although the HEC exhibited higher TPC and TFC values, the WI infusion demonstrated superior scavenging capacity in the DPPH assay, indicating that the relationship between phenolic composition and antioxidant activity is not straightforward and depends on the specific composition and availability of the compounds.

Furthermore, the presence of macro- and micronutrients contributes to the overall nutraceutical value of the extracts [148].

In light of the Keap1/Nrf2 system's involvement in neurodegenerative processes, molecular docking studies were conducted to investigate CBD's interaction with potential protein targets. This provides a structural rationale for the data obtained in the cellular model [114,115].

In light of these findings, the aim of this chapter is to:

i. characterize the chemical profile of the infusion and hydroalcoholic extract of *Cannabis sativa* var. *Kompolti*, paying particular attention to phytocannabinoids and phenolic compounds.
ii. evaluate the antioxidant potential of the two extracts by determining their total phenol and flavonoid content using specific assays based on scavenging and reducing properties;
iii. analyze the mineral composition to define its nutraceutical contribution;
iv. examine the effect of CBD in a cellular model of rotenone-induced dopaminergic neurotoxicity, paying particular attention to the response to oxidative stress.
v. interpret the experimental results through molecular docking studies to assess the interaction of CBD with key components of the Keap1/Nrf2 pathway.

4.2 Experimental design

This section outlines the experimental design adopted for the study of *Cannabis sativa* L.

Detailed experimental procedures are reported in Chapter 10.

4.2.1 Plant material and sample preparation

The inflorescences of *Cannabis sativa* L. var. *Kompolti* were collected, dried, ground and stored away from light until they were used in the experiment.

Two extracts were obtained from the same plant material: an aqueous infusion (WI), which represents the traditional use of the plant, and a hydroalcoholic extract (HEC), which was obtained using ultrasound-assisted extraction.

To prepare the infusion, the plant material (7 g in 100 mL of water) was treated with boiling water for 15 minutes, after which it was filtered and concentrated [146]. The extract was then fractionated using the Kupchan method to obtain four fractions of increasing polarity: n-hexane, chloroform, n-butanol and an aqueous residue. These fractions were subsequently used for phytochemical characterization.

The hydroalcoholic extract (HEC) was obtained by extracting 0.45 g of inflorescences with 9 ml of 80% (v/v) methanol using sonication [146,147]. The extracts were stored at 4 °C and protected from light until analysis.

Both extracts were stored in the dark at 4 °C until analysis.

4.2.2 Phytochemical characterization

The WI and HEC extracts were subjected to phytochemical characterization using the following methods:

- LC-ESI-HR-MS/MS for identifying cannabinoids and main phenolic metabolites [133–138];
- NMR spectroscopy for confirming the structure of the detected compounds [149,150].

These methods were applied to both the WI and the HEC extracts to highlight compositional differences due to the different polarities of the solvents used.

4.2.3 Determination of total phenolic content (TPC)

Total phenolic content (TPC) was determined using the Folin–Ciocalteu method, which is based on the reduction of the phosphomolybdic reagent in a basic environment. Spectrophotometric readings were taken and the results were expressed as gallic acid equivalents [143].

The assay was performed in triplicate for both WI and HEC extracts.

4.2.4 Determination of total flavonoid content (TFC)

TFC was quantified using the Zhishen method, which is based on complexation with aluminium chloride and spectrophotometric analysis [144]. The results were expressed as catechin equivalents.

The assay was conducted in triplicate for WI and HEC.

4.2.5 Antioxidant activity assays

The antioxidant activity of the extracts was evaluated using two complementary assays:

The DPPH assay, which measures the ability to neutralize the 2,2-diphenyl-1-picrylhydrazyl radical as a function of the decrease in absorbance at 517 nm [145], and the FRAP assay, which is based on the reduction of the ferric–TPTZ complex and measures absorbance at 593 nm [112].

Both assays were conducted in triplicate for the WI and HEC extracts.

4.2.6 Mineral composition (ICP–OES)

The determination of macro- and micronutrients was carried out using ICP-OES, a highly sensitive technique for quantifying minerals [148]. These results enabled us to evaluate the nutraceutical contribution of the infusion in terms of micronutrients.

4.2.7 Cellular model and induction of neurotoxicity

The biological effects of the extracts and CBD were evaluated using the human neuronal cell line SH-SY5Y. The cells were grown in DMEM medium supplemented with 10% FBS and maintained at 37 °C and 5% CO_2 [19]. Neurotoxicity was induced using rotenone, an inhibitor of mitochondrial complex I which is used as a model agent for Parkinson's disease due to its ability to generate oxidative stress and mitochondrial impairment [19].

4.2.8 Cell-based biological assays

To evaluate the neuroprotective effect of the extracts and CBD on the cellular model, the following were performed:

- a cell viability assay to verify the ability of the extracts and CBD to counteract rotenone-induced cytotoxicity;
- an intracellular ROS determination to quantify the impact on oxidative stress;
- an analysis of the Keap1/Nrf2 pathway to investigate the antioxidant effects at a molecular level [140–142].

4.2.9 *In silico* studies (molecular docking)

In order to evaluate the interactions between CBD and the binding sites of the Keap1 protein, a molecular docking study was performed using the three-dimensional structure of the protein target and software dedicated to the simulation of ligand-receptor interactions [114,115]. This methodological approach facilitated the correlation of the experimental findings with potential molecular mechanisms.

4.3 Results

Extended datasets, tables, and supplementary figures supporting the results discussed in this chapter are available in the Supplementary Materials of the corresponding peer-reviewed publication (see Appendix).

4.3.1 Phytochemical profile of WI and HEC extracts

Phytochemical analysis, conducted by means of LC-ESI-HR-MS/MS and confirmed by NMR spectroscopy, has highlighted the presence of various classes of bioactive metabolites in the inflorescences of *Cannabis sativa* var. *Kompolti*, including phytocannabinoids, phenolic compounds, flavonoids, and organic acids [133–138,149,150].

A representative LC-ESI-HR-MS/MS profile of the WI extract is reported in *Figure 4.1*.

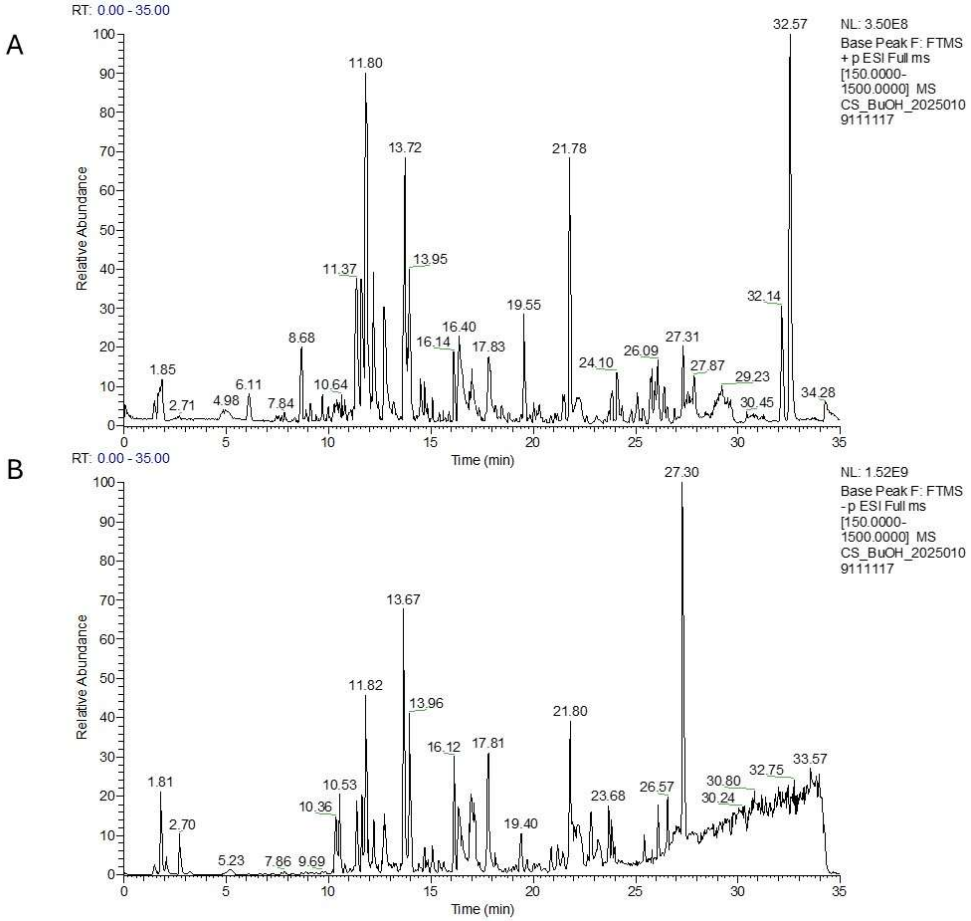


Figure 4.1 - Representative LC-ESI-HR-MS/MS chromatographic profiles of the aqueous infusion (WI) obtained from *Cannabis sativa* L. var. *Kompolti* inflorescences, acquired in positive (A) and negative (B) ion modes. The profiles highlight the chemical complexity of the WI extract and support the identification of phytocannabinoids, phenolic compounds, and other polar metabolites by high-resolution mass spectrometry.

A comparison of the two extracts revealed significant differences attributable to the different polarity of the solvents used in the extraction procedures. In particular, the HEC hydroalcoholic extract demonstrated a greater relative abundance of lipophilic metabolites, including phenolic compounds with a higher affinity for organic solvents, in comparison to the WI infusion, which is more representative of the hydrophilic fraction.

Among the identified phytocannabinoids, cannabidiol (CBD) and its precursor cannabidiol acid (CBDA) were present in both extracts, but with different signal intensities, confirming the influence of the extraction method on metabolite recovery.

The main bioactive compounds identified in the aqueous infusion are summarized in *Table 4.1*.

Table 4.1 Representative bioactive metabolites identified in the aqueous infusion (WI) of *Cannabis sativa* L. var. *Kompolti* by LC-ESI-HR-MS/MS and NMR analysis. The table summarizes the main classes of compounds detected in the WI extract and their distribution among the different fractions obtained by Kupchan partitioning.

Compound	Chemical class	WI fraction	Identification method
Cannabidiol (CBD)	Phytocannabinoid	n-hexane / $CHCl_3$	LC-MS/MS, NMR
Cannabidiolic acid (CBDA)	Phytocannabinoid	n-hexane / $CHCl_3$	LC-MS/MS, NMR
Apigenin derivatives	Flavonoids	n-BuOH	LC-MS/MS
Luteolin derivatives	Flavonoids	n-BuOH	LC-MS/MS
Kaempferol glycosides	Flavonoids	n-BuOH / H_2O	LC-MS/MS
Protocatechuic acid hexoside	Phenolic acid	H_2O	LC-MS/MS
Sinapoyl glucose	Phenolic acid derivative	H_2O	LC-MS/MS
Organic acids (e.g., citric acid)	Organic acids	H_2O	LC-MS/MS

The data obtained provide evidence that:

- HEC presents a richer and more diverse phytocomplex, with a greater abundance of compounds that have been effectively extracted in a hydroalcoholic solvent;
- WI, in contrast, is characterized by a less complex composition, yet it contains water-soluble bioactive molecules that are compatible with aqueous extraction. These molecules were subsequently investigated.

4.3.2 Total phenolic content, flavonoid content, and antioxidant activity

The hydroalcoholic extract (HEC) exhibited a higher total phenol (TPC) and flavonoid (TFC) content in comparison to the aqueous infusion (WI).

The quantitative results of total phenolic content, flavonoid content, and antioxidant activity are reported in *Table 4.2*.

Table 4.2 Total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity of the aqueous infusion (WI) and the hydroalcoholic extract (HEC) of *Cannabis sativa* L. var. *Kompolti*. Antioxidant capacity was evaluated by DPPH radical scavenging and ferric reducing antioxidant power (FRAP) assays. Data are expressed as mean ± SD of three independent determinations.

SAMPLE	Total Phenolic *(mg g^{-1} GAE d.w.)*	Flavonoids *(mg g^{-1} QUE d.w.)*	DPPH *(mg Kg^{-1} TE d.w.)*	FRAP *(mmol kg^{-1} TE d.w.)*
HEC	3.29 ± 0.22	1.39 ± 0.3	58.87 ± 0.54	2.76 ± 0.03
WI	1.00 ± 0.13	0.48 ± 0.1	11.28 ± 0.04	1.49 ± 0.01

GAE: Gallic acid equivalents. QUE: Quercetin equivalents. TE: Trolox equivalents. Each value is a mean ± SD of triplicate analysis

However, analysis of antioxidant activity revealed a behaviour contrary to that of phenolic distribution: the WI infusion exhibited greater scavenger capacity in the DPPH assay, despite the lower overall phenolic content, while the HEC extract demonstrated higher activity in the FRAP assay, consistent with the higher concentration of reducing compounds.

A comparative graphical representation of phenolic content and antioxidant activity of WI and HEC is shown in *Figure 4.2*.

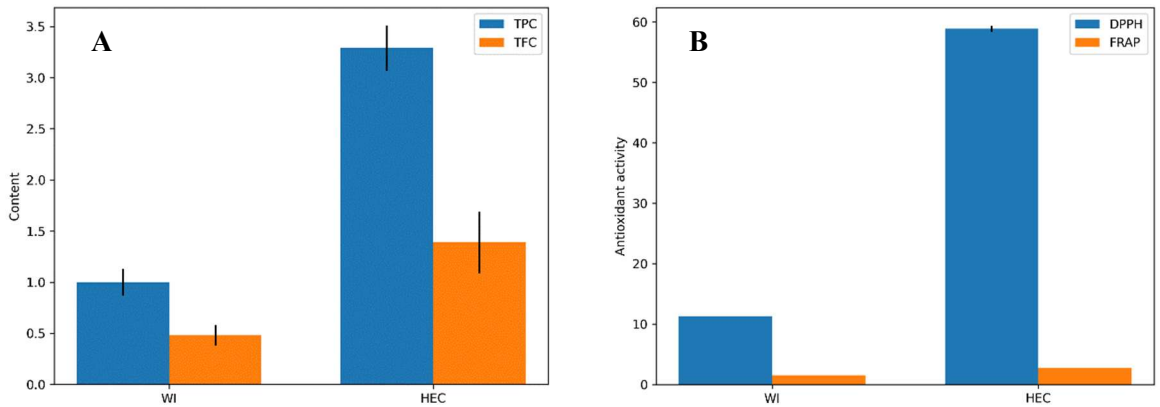


Figure 4.2 – Panel (A) Total phenolic and flavonoid content of WI and HEC. Panel (B) Antioxidant activity of WI and HEC evaluated by DPPH and FRAP assays.

These results indicate that antioxidant activity is not solely contingent on the quantity of phenolic compounds, but is also influenced by their structural characteristics, solubility, and potential synergistic interactions among the molecules comprising the phytocomplex.

Therefore, WI exerts a more pronounced effect in the neutralization of free radicals and HEC exhibits a heightened reducing capacity in the presence of oxidized ferric species.

It is evident that both properties hold significance in the context of oxidative damage associated with Parkinson's disease.

4.3.3 Mineral composition

A mineral analysis was conducted by ICP-OES, which identified a series of macro- and microelements. The concentrations of macro- and microelements detected in mineralized *Cannabis* inflorescences and in the aqueous infusion are reported in *Table 4.3*.

Table 4.3 Microelements and macroelements composition determined by ICP-OES in mineralized *Cannabis sativa* L. var. *Kompolti* inflorescences (MCI) and in the corresponding aqueous infusion (WI). Element concentrations are expressed as mean ± SD of three independent determinations. Values below the detection limit are reported as " < ".

		Inflorescence MCI	**Infusion WI**
Microelements ***(mg Kg^{-1})***	**Wavelength** ***(nm)***	***Mean ± SD***	
Cu	327.395	7.99 ± 0.59	3.43 ± 0.21
Fe	238.204	365.39 ± 37.03	2.86 ± 0.14
Ni	231.604	1.12 ± 0.29	0.57 ± 0.04
Ba	455.403	33.94 ± 1.12	1.00 ± 0.07
Co	230.786	0.24 ± 0.07	< 0.07
Cr	267.716	0.79 ± 0.11	0.02 ± 0.01
Pb	220.353	1.43 ± 0.26	< 0.14
V	292.401	0.61 ± 0.12	< 0.14
Zn	213.857	58.41 ± 0.82	19.00 ± 0.11
B	249.772	66.91 ± 1.96	8.57 ± 0.08
Macroelements ***(g Kg^{-1})***			
Ca	393.366	50.40 ± 0.24	5.34 ± 0.09
K	766.491	12.65 ± 0.38	11.16 ± 0.17
Mg	279.553	2.92 ± 0.12	1.17 ± 0.08
P	214.914	2.77 ± 0.16	0.53 ± 0.07
S	181.972	1.71 ± 0.09	0.72 ± 0.01
Si	251.611	2.20 ± 0.11	0.27 ± 0.02

These elements were found to be involved in essential physiological processes in the two extracts.

The presence of these elements contributes to the nutraceutical profile of the samples. It suggests that their contribution could complement that of phytochemical components in modulating the observed antioxidant activity, thereby supporting the hypothesis that the extracts' activity is not solely attributable to phytocannabinoids or phenolic compounds.

4.3.4 Biological responses in the cellular model

In the SH-SY5Y cell model exposed to rotenone, both extracts and CBD demonstrated protective effects, as evidenced by the following observations:

- an increase in cell viability in comparison to the control treated with the toxin alone;
- a reduction in intracellular ROS levels;
- modulation of the Keap1/Nrf2 pathway, indicative of the activation of endogenous antioxidant mechanisms.

The effects of CBD on cell viability, oxidative stress, and modulation of antioxidant-related signaling pathways in rotenone-treated SH-SY5Y cells are summarized in *Figure 4.3*.

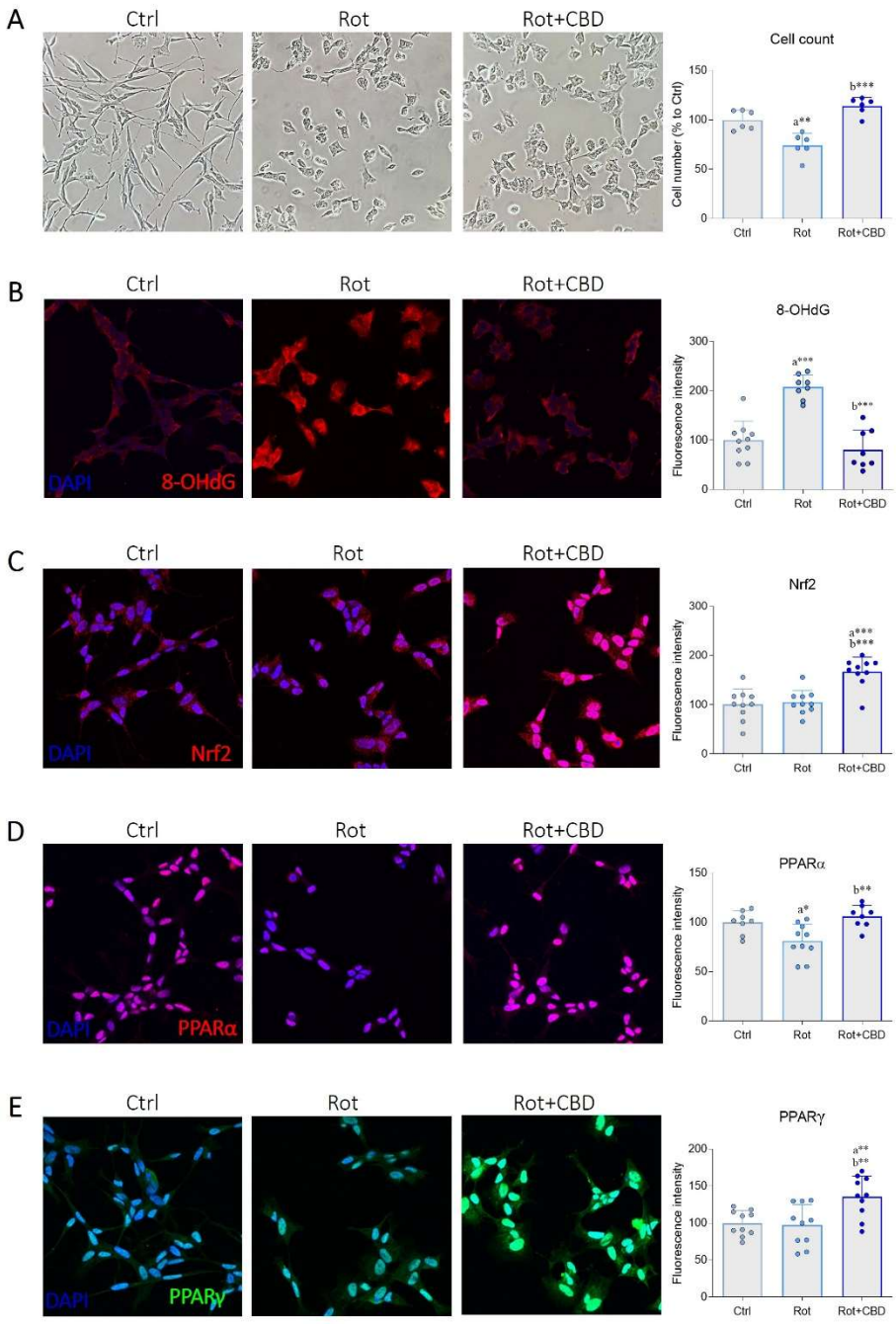


Figure 4.3 - Protective effects of cannabidiol (CBD) in SH-SY5Y cells exposed to rotenone. Panel (A) shows representative phase-contrast images and cell count analysis. Panel (B) reports immunofluorescence analysis of the oxidative stress marker 8-OHdG. Panels (C–E)

These results provide confirmation of the potential neuroprotective role of CBD, and suggest a synergistic contribution of the phenolic and mineral components present in the extracts.

4.3.5 Molecular docking insights

Molecular docking studies have highlighted possible interactions between CBD and specific regions of the Keap1 protein, suggesting a potential mechanism through which CBD may modulate the Keap1/Nrf2 axis.

These observations provide a structural rationale for the observed biological effects and support the hypothesis that CBD may interfere with Nrf2 degradation, promoting its nuclear translocation and activation of the antioxidant response.

The predicted interaction between CBD and the Keap1 protein is illustrated in *Figure 4.4*.

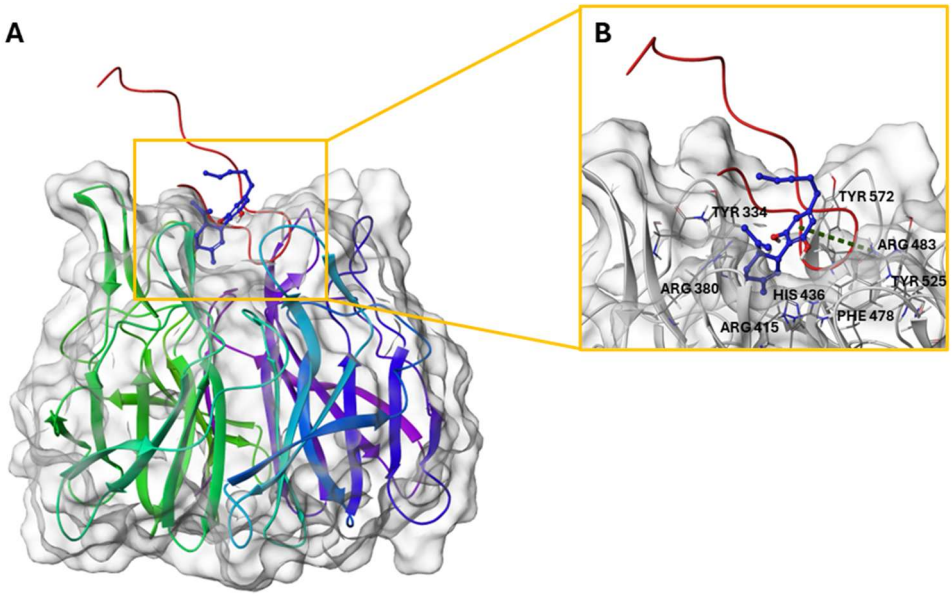


Figure 4.4 - Molecular docking analysis of cannabidiol (CBD) within the Keap1 protein binding site. Panel (A) shows the three-dimensional structure of the Keap1–Nrf2 peptide complex in the presence of CBD, while panel (B) illustrates the predicted binding mode of CBD within the Keap1 Kelch domain.

4.4 Discussion

The results obtained in this study confirm the phytochemical complexity of *Cannabis sativa* var. *Kompolti* inflorescences demonstrate that different extraction strategies lead to distinct metabolic and functional profiles. In particular, the hydroalcoholic extract (HEC) exhibited a higher abundance of phenolic compounds and flavonoids compared to the aqueous infusion (WI), in agreement with previous reports indicating that solvents of intermediate polarity enhance the extraction of lipophilic bioactive metabolites from plant matrices.

Similar evidence was reported by Dudonné et al. [151], who highlighted the strong dependence of extraction yield and antioxidant potential on solvent composition.

Despite its lower total phenolic and flavonoid content, the WI infusion displayed a higher radical scavenging activity in the DPPH assay. This apparently counterintuitive result is consistent with observations reported in the literature, where aqueous extracts frequently exhibit high antioxidant efficacy despite reduced polyphenolic concentrations [151]. These findings suggest that antioxidant activity is not exclusively related to the total amount of phenolic compounds, but rather depends on their qualitative composition, chemical reactivity, and bioavailability within the phytocomplex.

Conversely, the higher reducing power observed for the HEC extract in the FRAP assay aligns with previous studies showing that hydroalcoholic extracts are more effective in electron-transfer–based antioxidant mechanisms [152]. Taken together, these results indicate that WI and HEC exert complementary antioxidant effects, reflecting the selective enrichment of different classes of bioactive compounds according to the extraction method.

The mineral composition of the extracts introduces an additional level of interpretation. Several trace elements play a critical role in maintaining redox homeostasis, acting as cofactors for antioxidant enzymes, and their dysregulation has been associated with neurodegenerative processes. As highlighted by Doroszkiewicz et al. [153], elements such as zinc, copper, iron, and manganese are closely involved in oxidative stress regulation. The presence of both macro- and microelements in *C. sativa* extracts suggests that inorganic components may contribute synergistically with organic metabolites to the overall antioxidant and cytoprotective activity of the phytocomplex.

The biological effects observed in the SH-SY5Y cellular model further support this integrative interpretation. The increase in cell viability, the reduction of intracellular oxidative damage, and the modulation of key transcription factors involved in antioxidant defense confirm the neuroprotective potential of the *Cannabis* phytocomplex. In particular, the activation of the Keap1/Nrf2 pathway is consistent with previous reports describing the involvement of cannabidiol (CBD) in antioxidant and cytoprotective responses [154].

The role of CBD in modulating the Keap1/Nrf2 axis is further supported by the work of Talebi et al. [21], who demonstrated that CBD can interfere with the Keap1–Nrf2 interaction, thereby promoting endogenous antioxidant responses. In this context, the molecular docking analysis performed in this study provides a structural rationale for the observed biological effects, suggesting that CBD may interact with functional regions of the Keap1 protein and potentially influence Nrf2 degradation and activation.

Overall, the data indicate that the neuroprotective potential of *Cannabis sativa* var. *Kompolti* cannot be attributed exclusively to CBD, but rather arises from the combined and synergistic action of multiple molecular classes - including phytocannabinoids, polyphenols, flavonoids, and mineral components - integrated within a complex phytochemical system capable of modulating oxidative stress and molecular pathways involved in neuronal survival.

4.5 Remarks

In this study, the phytochemical profile, antioxidant activity, and potential neuroprotective effects of the aqueous infusion (WI) and a hydroalcoholic extract (HEC) obtained from *Cannabis sativa* L. var. *Kompolti* inflorescences were characterized, with the aim of evaluating these properties in a cellular model of neurodegeneration. The results showed that the extraction method strongly influences both the chemical composition and the biological activity of the preparations. In particular, the hydroalcoholic extract contained higher levels of phenolic compounds and flavonoids, whereas the aqueous infusion showed a stronger ability to scavenge oxygen radicals.

The presence of macro- and microelements in the extracts suggests that inorganic components also contribute to the overall antioxidant activity. This supports the view of *Cannabis sativa* as a complex phytochemical system, in which biological effects result from the interaction of different molecular classes rather than from a single compound.

Experiments performed on rotenone-treated SH-SY5Y cells confirmed that both the extracts and pure cannabidiol (CBD) can counteract oxidative stress, improve cell viability, and modulate the Keap1/Nrf2 signaling pathway. These findings indicate a protective role of CBD and suggest a possible synergistic action with other constituents present in the plant matrix.

Molecular docking analysis provided a structural explanation for these effects, showing a potential interaction between CBD and regulatory regions of the Keap1 protein. This interaction may contribute to the activation of endogenous antioxidant defense mechanisms.

Overall, the results indicate that *Cannabis sativa* var. *Kompolti* should not be considered only as a source of CBD, but as a phytocomplex with antioxidant and cytoprotective properties arising from the combined action of cannabinoids, phenolic compounds, and minerals. This supports the idea that whole-plant–based nutraceutical or therapeutic approaches may be beneficial in the context of neurodegenerative diseases associated with oxidative stress and mitochondrial dysfunction.

Further studies are needed to evaluate the bioavailability of the active compounds, clarify the specific contribution of each molecular class, and confirm these findings in in vivo models. Nevertheless, the data presented here suggest that *Cannabis sativa* and its derivatives represent a promising resource for the modulation of neurodegenerative processes.

CHAPTER 5 *Vicia faba* L.

5.1 Background and rationale

Vicia faba L., commonly known as broad bean, is one of the oldest cultivated legumes, native to the Near East and currently widespread in Europe, the Middle East, Asia, Oceania, and South America [22]. Its adaptability to cold climates, ability to grow without irrigation, and high nitrogen fixation efficiency give this species a strategic agronomic role in the transition toward more sustainable agricultural systems [155]. *V. faba* seeds are a valuable nutritional source, as they are rich in carbohydrates, fibre, proteins, and minerals such as iron and zinc [156–158], and contain both saturated and unsaturated fatty acids [159].

Beyond their nutritional value, *V. faba* seeds have attracted scientific interest due to their high content of secondary metabolites, including phenolic compounds, flavonoids, proanthocyanidins, saponins, and bioactive amino acids such as L-DOPA [22,160–164]. These compounds exhibit antioxidant, anti-inflammatory, and antibacterial activities [22,160–162], while concurrently contributing to the maintenance of a low glycemic index, which is conducive to effective blood glucose control [163].

However, the use of *V. faba* seeds may be limited by the presence of antinutritional factors, particularly vicine and convicine [165–167]. These compounds are precursors of the molecules responsible for favism in individuals affected by glucose-6-phosphate dehydrogenase (G6PD) deficiency, a condition common in Mediterranean and Middle Eastern populations [168–170]. To reduce this risk, cultivars with low vicine/convicine content have been developed [171], and technological treatments such as dehulling have been shown to significantly modify the nutritional and bioactive composition of the seeds [156,161].

In recent years, scientific interest in ancient crop varieties has increased. Although these varieties are not used in industrial agriculture, they preserve a unique genetic heritage and often show higher levels of bioactive metabolites than modern cultivars [172]. In this context, the present study focuses on two ancient *Vicia faba* varieties with pigmented seed coats: one with black seeds (VFB) and one with purple seeds (VFP).

The objectives of this study were:

i. to characterise the phenolic composition and proanthocyanidin content of the two varieties;
ii. to evaluate their antioxidant activity using *in vitro* chemical assays;
iii. to characterise their metabolomic profile by LC-ESI-HR-MS analysis;
iv. to develop and apply a targeted LC-ESI-MS method for proanthocyanidin quantification;
v. to investigate the anti-inflammatory activity of the extracts using COX and sEH enzymatic assays;
vi. to determine the mineral profile of seed coats and cotyledons in relation to their nutritional value.

Therefore, this chapter provides a chemical, biological, and nutritional characterization of the two *Vicia faba* varieties, using an integrated approach based on antioxidant assays, metabolomic profiling, and mineral analysis, with particular attention to the role of pigmented seed coats.

5.2 Experimental design

This section outlines the experimental design adopted for the study of *Vicia faba* L.

Detailed experimental procedures are reported in Chapter 10.

5.2.1 Plant material and sample preparation

The two ancient *Vicia faba* varieties analyzed in this study were collected from local farms managed by Fulget Vita SRL, Braccia Gerardo Carmine farm, and Oasis srl - Centro Di Ricerche, all located in Avellino (Italy). These farms have not been subjected to modern genetic selection in order to preserve the phytochemical variability associated with traditional genotypes [172].

Seeds were selected based on seed coat pigmentation, identifying a black-seeded variety (VFB) and a purple-seeded variety (VFP). Seed coat colour is considered a phenotypic marker associated with the content of phenolic compounds and proanthocyanidins[173–176].

To evaluate the distribution of bioactive compounds, seeds were manually separated into two anatomical compartments, obtaining four samples: black cotyledons (VFBC), black seed coats (VFBS), purple cotyledons (VFPC), and purple seed coats (VFPS). This distinction is relevant because, in legumes, polyphenol biosynthesis mainly occurs in the external seed tissues [173,174].

All samples were ground into a homogeneous powder and stored away from light and humidity to preserve thermo- and photosensitive compounds.

5.2.2 Metabolite extraction

Phenolic compounds and proanthocyanidins were extracted using 80% hydroalcoholic solvents (methanol or ethanol), selected for their effectiveness in extracting polar and semi-polar compounds, according to established protocols for legume matrices [23,177].

A constant sample-to-solvent ratio was maintained for both seed coats and cotyledons to ensure reproducible extraction conditions.

The use of both methanol and ethanol allowed selective extraction of different chemical classes: methanol was more efficient for flavan-3-ols and their oligomers, while ethanol was considered more suitable for food-grade applications [177].

5.2.3 Determination of total phenolic content and flavonoids

Total phenolic content was determined using the Folin-Ciocalteu method, according to Singleton [143], with minor adaptations for legume matrices. Results were expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE g^{-1} DW), in agreement with previous studies on *V. faba* [173–175].

The total flavonoid content was assessed using a colourimetric assay based on the formation of a complex with $AlCl_3$ [145], and the results were expressed as milligrams of catechin equivalent per gram of dry weight (mg CE g^{-1} DW). This approach is widely recognized for legumes and other flavonoid-rich matrices [174,175].

5.2.4 Quantification of proanthocyanidins (PAs)

Proanthocyanidins, defined as oligomers and polymers of flavan-3-ols, were quantified using a selective spectrophotometric method [178–180]. Results were expressed as absorbance units per gram of dry weight (AU g^{-1} DW). Proanthocyanidins are considered key contributors to the antioxidant activity of pigmented legumes and useful markers for varietal discrimination [173–176].

5.2.5. Antioxidant activity assays

Antioxidant activity was evaluated using three complementary assays: DPPH (radical scavenging) [145], ABTS (cation radical scavenging) [181] and FRAP (ferric reducing antioxidant power) [112].

Results were expressed as milligrams of Trolox equivalents per gram of dry weight (mg TE g^{-1} DW). The combined use of these assays provides a more comprehensive assessment of antioxidant capacity and overcomes the limitations of individual methods [177].

5.2.6. Untargeted metabolomic profiling (LC-ESI-HR-MS/MS)

Metabolomic profiling was performed using liquid chromatography coupled with high-resolution mass spectrometry equipped with an electrospray ionization source, following an untargeted approach. This method allowed the identification of major metabolite classes, particularly polar and phenolic compounds, as previously reported for legumes [23].

Metabolite annotation was based on accurate mass measurements, isotopic patterns, MS/MS fragmentation, and database comparison. Detailed instrumental conditions are reported in the reference publication from which this chapter is derived.

5.2.7. Targeted analysis of proanthocyanidins (LC-ESI-MRM)

Targeted quantification of major proanthocyanidins was performed using a Multiple Reaction Monitoring (MRM) approach, which allows selective detection of structurally similar oligomeric species. This method is particularly suitable for matrices rich in flavan-3-ols, such as pigmented legume seed coats.

5.2.8 Mineral profile (ICP-OES)

The determination of macro- and micronutrient content was conducted using ICP-OES, a technique that has been employed extensively for the simultaneous quantification of elements in plant samples [182]. This approach facilitates the evaluation of mineral distribution between seed coats and cotyledons, a crucial parameter for defining the nutritional potential of the varieties analyzed.

5.3 Results

Extended datasets, tables, and supplementary figures supporting the results discussed in this chapter are available in the Supplementary Materials of the corresponding peer-reviewed publication (see Appendix).

5.3.1 Total phenolic content, flavonoids, and proanthocyanidins

The results obtained demonstrate a marked difference between the two varieties of *Vicia faba* examined, both in relation to seed coat pigmentation and the extraction solvent used. In general, higher values were observed in the seed coats compared to the cotyledons,

confirming that the biosynthesis and accumulation of phenolic compounds occurs predominantly in the external tissues of the seed, as previously reported in the literature [173,174].

5.3.1.1 Total Phenolic Content (TPC)

In the seed coats of the purple variety (VFP), methanolic extraction revealed a TPC of 1.96 mg GAE g^{-1} DW. As shown in *Table 5.1*, the TPC in the corresponding ethanolic extract (1.82 mg GAE g^{-1} DW) was slightly lower. In contrast, the black variety (VFB) showed lower TPC values, ranging from 0.88 to 1.07 mg GAE g^{-1} DW.

In the cotyledons, the phenolic content was found to be considerably lower for both varieties, ranging between 0.62 and 0.93 mg GAE g^{-1} DW. These values were found to be significantly lower than those detected in the seed coats. These results confirm the structural and functional role of the external seed tissues as the main polyphenolic reservoir [173–176].

Table 5.1 Total phenolic content (TPC), total flavonoids (TFC) and proanthocyanidins (PAs) in seed coats and cotyledons of black (VFB) and purple (VFP) *Vicia faba*, extracted with 80% methanol (MeOH) and 80% ethanol (EtOH).

	VFB Extract				**VFP Extract**			
	Seed Coats		**Cotyledons**		**Seed Coats**		**Cotyledons**	
	Ext_MeOH	**Ext_EtOH**	**Ext_MeOH**	**Ext_EtOH**	**Ext_MeOH**	**Ext_EtOH**	**Ext_MeOH**	**Ext_EtOH**
TPC ***(mg GAE g^{-1})***	0.88 ± 0.1 ^{b}	1.07 ± 0.3 ^{b}	0.80 ± 0.2 ^{b}	0.62 ± 0.1 ^{b}	1.96 ± 0.1 ^{a}	1.82 ± 0.4 ^{a}	0.72 ± 0.1 ^{b}	0.93 ± 0.1 ^{b}
TFC ***(mg CE g^{-1})***	0.94 ± 0.1 ^{b}	1.00 ± 0.04 ^{b}	0.54 ± 0.1 ^{c}	0.43 ± 0.1 ^{c}	1.75 ± 0.3 ^{a}	1.18 ± 0.1 ^{b}	0.80 ± 0.2 ^{c}	0.58 ± 0.2 ^{c}
PAs ***(AU g^{-1})***	0.0478 ^{bc}	0.0321 ^{bc}	0.0024 ^{c}	0.0008 ^{c}	1.1531 ^{a}	0.1322 ^{b}	0.0982 ^{bc}	0.0012 ^{c}

GAE: Gallic acid equivalent. CE: Catechin equivalent. AU absorbance units. Values are expressed as mean ± SD ($n = 3$). Different superscript letters within the same column indicate statistically significant differences (ANOVA followed by Tukey's test, $p < 0.05$).

5.3.1.2 Total Flavonoids (TFC)

The flavonoid profile demonstrates a comparable trend to that observed in total phenols. The VFP variety exhibited the highest values, with 1.75 mg CE g^{-1} DW in methanolic extracts and 1.18 mg CE g^{-1} DW in ethanolic extracts, while the VFB variety demonstrated lower concentrations (0.94–1.00 mg CE g^{-1} DW).

Flavonoid levels in the cotyledons were markedly lower than in the corresponding seed coats. This finding suggests a close relationship between flavonoid accumulation and seed pigmentation.

5.3.1.3 Proanthocyanidins (PAs)

The most evident discrepancy between the two varieties pertains to the content of proanthocyanidins, which are regarded as one of the primary bioactive markers of pigmented legumes [173–176].

In the seed coats of the purple variety, the methanolic extract has a value of 1.1531 AU g^{-1} DW, which is approximately 24 times higher than that detected in the seed coats of the black variety (0.0478 AU g^{-1} DW).

In the cotyledons, the values are negligible in both varieties, thus confirming the hypothesis that phenolic and proanthocyanidin bioactivity is a characteristic of the seed coat, rather than the internal seed.

5.3.2. *In vitro* antioxidant activity

The antioxidant activity of the extracts obtained from the two *Vicia faba* varieties showed clear differences depending on seed tissue, variety, and extraction solvent.

The scavenging capacity for the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) exhibited marked variations between the two varieties. The methanolic extracts of the purple seed coat (VFP) showed the highest value (14.84 ± 1.0 mg TE g^{-1} DW), while the ethanolic extracts, although slightly less active, still exhibited significant radical scavenging capacity (10.16 ± 0.6 mg TE g^{-1} DW).

The seed coat extracts of the black variety (VFB) demonstrated significantly lower values (3.13 ± 0.1 mg TE g^{-1} DW), while the cotyledons of both varieties exhibited almost negligible activity.

The ABTS assay confirmed the trend observed in the DPPH test. The methanolic extracts of the purple seed coat have been shown to reach 16.88 ± 0.3 mg TE g^{-1} DW, a value more than double that of the black variety (6.30 ± 0.2 mg TE g^{-1} DW). The extraction of ethanol yields

slightly lower values than methanol, thereby confirming the latter's greater efficiency in recovering phenolic antioxidant molecules [177].

The Ferric Reducing Antioxidant Power (FRAP) assay provides the most distinct analysis of the differences between the two varieties. The purple seed coat exhibits a value of 388.79 ± 20.4 mg TE g^{-1} DW, which is more than four times higher than the value of the corresponding black matrix (90.36 ± 2.9 mg TE g^{-1} DW). This result suggests that the purple-pigmented seed coat has a greater capacity to donate electrons, a mechanism closely associated with the presence of phenolic compounds that exhibit high redox reactivity.

A comprehensive comparison of antioxidant activities across varieties, seed tissues, and extraction solvents is reported in *Table 5.2*.

Table 5.2 - In vitro antioxidant activity (DPPH, ABTS, and FRAP assays) of seed coats and cotyledons from black (VFB) and purple (VFP) *Vicia faba*, extracted with 80% methanol and 80% ethanol.

	VFB				**VFP**			
	Seed Coats		**Cotyledons**		**Seed Coats**		**Cotyledons**	
	Ext_MeOH	**Ext_EtOH**	**Ext_MeOH**	**Ext_EtOH**	**Ext_MeOH**	**Ext_EtOH**	**Ext_MeOH**	**Ext_EtOH**
DPPH ***(mg TE g^{-1})***	3.13 ± 0.1 ^{c}	2.78 ± 0.1 ^{c}	1.58 ± 0.1 ^{a}	1.67 ± 0.1 ^{b}	14.84 ± 1.0 ^{d}	10.16 ± 0.6 ^{d}	0.96 ± 0.1 ^{d}	0.99 ± 0.1 ^{d}
ABTS ***(mg TE g^{-1})***	6.30 ± 0.2 ^{c}	5.82 ± 0.2 ^{cd}	4.26 ± 0.3 ^{f}	3.81 ± 0.2 ^{f}	16.88 ± 0.3 ^{a}	9.50 ± 0.1 ^{b}	5.59 ± 0.1 ^{d}	4.93 ± 0.1 ^{e}
FRAP ***(mg TE g^{-1})***	90.36 ± 2.9 ^{c}	85.20 ± 0.8 ^{c}	62.06 ± 2.1 ^{d}	56.21 ± 0.6 ^{d}	388.79 ± 20.4 ^{a}	226.22 ± 17.7 ^{b}	39.10 ± 2.1 ^{d}	37.74 ± 1.3 ^{d}

TE: Trolox equivalent. Each value is a mean ± SD of triplicate analysis. Values are expressed as mean ± SD (n = 3). Different superscript letters within the same column indicate statistically significant differences (ANOVA followed by Tukey's test, $p < 0.05$).

5.3.2.1 Correlation between phenols and antioxidant activity

The positive correlations observed between total phenolic content (TPC), total flavonoid content (TFC), and antioxidant assay values indicate that the phenolic fraction, and particularly proanthocyanidins, plays a major role in the scavenging and reducing capacity of the extracts. These results are consistent with previous studies on pigmented legumes [173–176].

Pearson's correlation coefficients (R) between phenolic contents and antioxidant activities in *Vicia faba* are reported in *Table 5.3*.

Table 5.3 Pearson's correlation coefficients (R) between phenolic contents and antioxidant activities in *Vicia faba* extracts

Correlation Coefficient (R)	Total Phenolic	Flavonoids	Proanthocyanidins	DPPH	ABTS	FRAP
TPC	1					
TFC	0.889 **	1				
PAs	0.740 *	0.854 **	1			
DPPH	0.958 **	0.911 **	0.856 **	1		
ABTS	0.899 **	0.952 **	0.951 **	0.959 **	1	
FRAP	0.932 **	0.920 **	0.906 **	0.993 **	0.977 **	1

Correlation is significant at the 0.01 level (**); at the 0.05 level (*).

5.3.3. Metabolomic profiling by LC-ESI-HR-MS/MS

LC-ESI-HR-MS/MS analysis enabled the identification of over one hundred metabolites belonging to different chemical classes. These included flavonoids such as quercetin, kaempferol, and myricetin, as well as oligomeric proanthocyanidins, particularly those derived from flavan-3-ols. The analysis also revealed phenolic acids and their esters, free amino acids and derivatives, and polar lipids, including oxidative intermediates.

The detection of polar lipids and oxylipins in pigmented seed coats represents a relevant metabolic feature that has been rarely reported in *V. faba* and may be associated with varietal adaptation.

The data obtained demonstrate a clear distinction between the two anatomical components of the seed. Specifically, the cotyledons are dominated by primary metabolites involved in energy and structural metabolism, while a high concentration of phenolic compounds and PAs characterizes the seed coats. These findings are in accordance with those of TPC, TFC, and antioxidant assays.

This differential distribution is consistent with the physiological role of the seed coat, which protects the seed from oxidation and external stresses [173,174].

The VFP variety (purple seed coat) displays a significantly richer and more complex metabolic profile than the VFB variety (black seed coat). The discrepancy is especially pronounced in the aggregation of phenolic metabolites and proanthocyanidins, which manifest in greater abundance, more pronounced expression, and are distinguished by distinctive fragmentation patterns.

This enrichment is consistent with the greater antioxidant activity and higher phenolic values previously observed in analyses.

The combination of chemical diversity, MS/MS fragmentation pattern, intensity, and selective presence of metabolites allowed clear distinction between the two varieties, highlighting that metabolomics not only confirms spectrophotometric data, but also extends them by identifying molecules that cannot be detected using conventional colorimetric methods.

Only representative and discriminant secondary metabolites are reported in *Table 5.4*, while the complete list of annotated compounds and detailed MS/MS data are available in the reference publication.

Table 5.4 Selected and discriminant secondary metabolites identified by LC-ESI-HR-MS/MS in seed coats and cotyledons of black (VFB) and purple (VFP) *Vicia faba*.

Compound	Chemical class	VFBC	VFBS	VFPC	VFPS
Myricetin hexose deoxyhexose	Flavonoid	–	+	–	+
Myricitrin	Flavonoid	+	+	+	+
Myricetin	Flavonoid	–	+	–	+
Quercetin 3-robinobioside	Flavonoid	+	+	+	+
Rutin	Flavonoid	+	+	+	+
Astragalin	Flavonoid	–	–	+	+
Catechin*	Proanthocyanidin	+	+	+	+
Epicatechin*	Proanthocyanidin	+	+	+	+
Procyanidin B*	Proanthocyanidin	+	+	+	+
Procyanidin A*	Proanthocyanidin	–	+	–	+
(Epi)gallocatechin	Flavan-3-ol	–	+	–	+
Gallic acid	Phenolic acid	–	+	–	+
Piscidic acid	Phenolic acid	+	+	+	+
12(13)-DiHOME	Oxylipin	+	+	+	+
13-HOTrE	Oxylipin	–	+	–	+

An overview of the major discriminant metabolite classes emerging from the LC-ESI-HR-MS/MS analysis is provided in *Table 5.4a*.

Table 5.4a Summary of major discriminant metabolite classes identified in seed coats of black (VFB) and purple (VFP) *Vicia faba* by LC-ESI-HR-MS/MS

Class	Representative compounds	Significant occurrence
Flavonoids	Rutin, Hyperin, Myricetin	VFP seed coats
Proanthocyanidins	Procyanidin A/B, epigallocatechin dimers	VFP ≫ VFB
Polar lipids	Oxylipins (9,12,13-TriOH-ODE), LysoPE	Rarely reported in *V. faba*
Bioactive amino acids	L-DOPA, N-acetyl-tyrosine	Both seed coats

5.3.4. Targeted quantification of proanthocyanidins (LC-ESI-MRM)

The results obtained demonstrate a significant enrichment of proanthocyanidins in the seed coats in comparison to the cotyledons for both varieties, with a considerably greater contribution from the purple variety (VFP).

Targeted LC-ESI-MRM analysis enabled the quantitative determination of major proanthocyanidins in seed coats and cotyledons, as shown in *Table 5.5*.

For the VFP variety, analyses revealed:

- Procyanidin B: 260.3 ± 23.8 μg g^{-1} DW;
- Epigallocatechin-(epi)gallocatechin dimers: high concentrations, particularly in methanolic extracts;
- Procyanidin A (ethanolic extraction): 51.1 ± 1.6 μg g^{-1} DW.

The VFB variety, despite containing the same molecular classes, exhibits lower values:

- Procyanidin B: 175.3 ± 12.2 μg g^{-1} DW;
- Flavan-3-ol dimers were detected, but at significantly lower concentrations;
- Procyanidin A: not significantly detected.

In the cotyledons of both varieties, levels of procyanidins were found to be almost negligible, consistent with their primary storage and metabolic role and the limited presence of phenolic compounds, as highlighted by TPC, TFC, and FRAP results.

The presence of procyanidin A and B, flavan-3-ol dimers, and epigallocatechins derived exclusively or predominantly from the seed coats is a distinguishing feature between the two varieties. These molecules have been demonstrated to possess antioxidant and anti-inflammatory properties, as evidenced in other Fabaceae [173–176].

The MRM approach plays a complementary role to untargeted metabolomics for the following reasons:

- confirms the molecules suggested by LC-ESI-HR-MS/MS profiling;
- quantifies the proanthocyanidin species with the greatest nutraceutical relevance;
- clearly differentiates the two varieties based on their PAs content.

Table 5.5 Targeted quantification of major proanthocyanidins in seed coats and cotyledons of black (VFB) and purple (VFP) *Vicia faba* by LC-ESI-MRM.

Metabolite	VFBS-M	VFBS-E	VFBC-M	VFBC-E	VFPS-M	VFPS-E	VFPC-M	VFPC-E
Catechin *	195.6 ± 2.09	260.5 ± 4.8	136.6 ± 0.63	134.1 ± 0.85	322.9 ± 67.2	393.2 ± 0.1	134.7 ± 0.89	136.9 ± 0.63
Epicatechin *	187.2 ± 2.7	253.1 ± 2.1	128.7 ± 0.27	126.4 ± 0.10	238.7 ± 31.5	292.6 ± 16.7	126.0 ± 0.1	127.2 ± 0.4
Epigallocatechin	152.1 ± 2.7	169.0 ± 1.04	nd	nd	148.5 ± 10.3	150.8 ± 1.04	nd	nd
Epigallocatechin catechin I	70.3 ± 1.3	64.7 ± 1.3	nd	nd	130.4 ± 22.7	108.8 ± 5.5	nd	nd
(Epi)gallocatechin-(epi)gallocatechin I	515.8 ± 0.01	355.6 ± 36.7	nd	nd	475.8 ± 50.0	371.6 ± 83.2	nd	nd
2 × [(Epi)gallocatechin]-(epi)catechin I	35.5 ± 0.01	35.5 ± 0.09	nd	nd	36.7 ± 0.4	35.6 ± 0.1	nd	nd
Procyanidin A *	39.7 ± 0.13	47.9 ± 1.3	nd	nd	41.4 ± 1.6	51.1 ± 1.6	nd	nd
Procyanidin B *	175.3 ± 12.2	98.2 ± 27.9	36.0 ± 0.35	35.2 ± 0.02	260.3 ± 23.8	240.8 ± 34.4	36.8 ± 0.36	36.1 ± 0.76
Total proanthocyanidin	1371.5	1284.5	301.3	295.7	1654.7	1644.5	297.5	300.2

nd: not determined; Total proanthocyanidins were calculated as the sum of all individual proanthocyanidins quantified by LC–MS analysis.

5.3.5. Anti-inflammatory activity (COX and sEH)

The anti-inflammatory activity of the extracts was evaluated by assessing their ability to inhibit two enzymatic targets involved in inflammatory processes: cyclooxygenases (COX-1 and COX-2) and soluble epoxide hydrolase (sEH), a key enzyme involved in the degradation of lipid epoxides with anti-inflammatory activity [183–186]. The combined use

of these assays allows verification of the biological activity observed in antioxidant tests and provides insight into the potential modulation of inflammatory pathways.

5.3.5.1 Inhibition of sEH

Extracts from the seed coats of the purple variety (VFP) showed the strongest inhibitory activity against sEH, with an IC_{50} value of 31.51 ± 1.16 μg μL^{-1}, indicating a dose-dependent effect and a marked interference with lipid epoxide metabolism (*Figure 5.1*).

In contrast, extracts from the black variety (VFB) exhibited a lower inhibitory capacity, with higher IC_{50} values. This result supports the key role of proanthocyanidins and flavonoids present in the pigmented seed coats of the purple variety as major contributors to the observed enzymatic inhibition [183–186].

5.3.5.2 Inhibition of cyclooxygenases (COX-1 and COX-2)

Both varieties exhibited moderate inhibitory activity against cyclooxygenases, with a higher selectivity toward COX-2 compared to COX-1 (*Figure 5.1*). Consistent with previous results, extracts from the purple seed coats showed greater inhibitory activity than those from the black variety, in agreement with their higher proanthocyanidin content and the presence of flavonoid glycosides with reported anti-inflammatory properties in other Fabaceae species [187,188].

In contrast, cotyledon extracts showed negligible or no inhibitory activity, confirming their limited content of bioactive compounds.

Although the anti-inflammatory activity cannot be attributed to a single compound, the presence of procyanidin B, flavan-3-ol oligomers, and quercetin and myricetin derivatives - known modulators of NF-κB/COX-2 and TAK1 signalling pathways [178–181] - supports the hypothesis of a synergistic molecular effect underlying the greater efficacy of the purple variety compared to the black one.

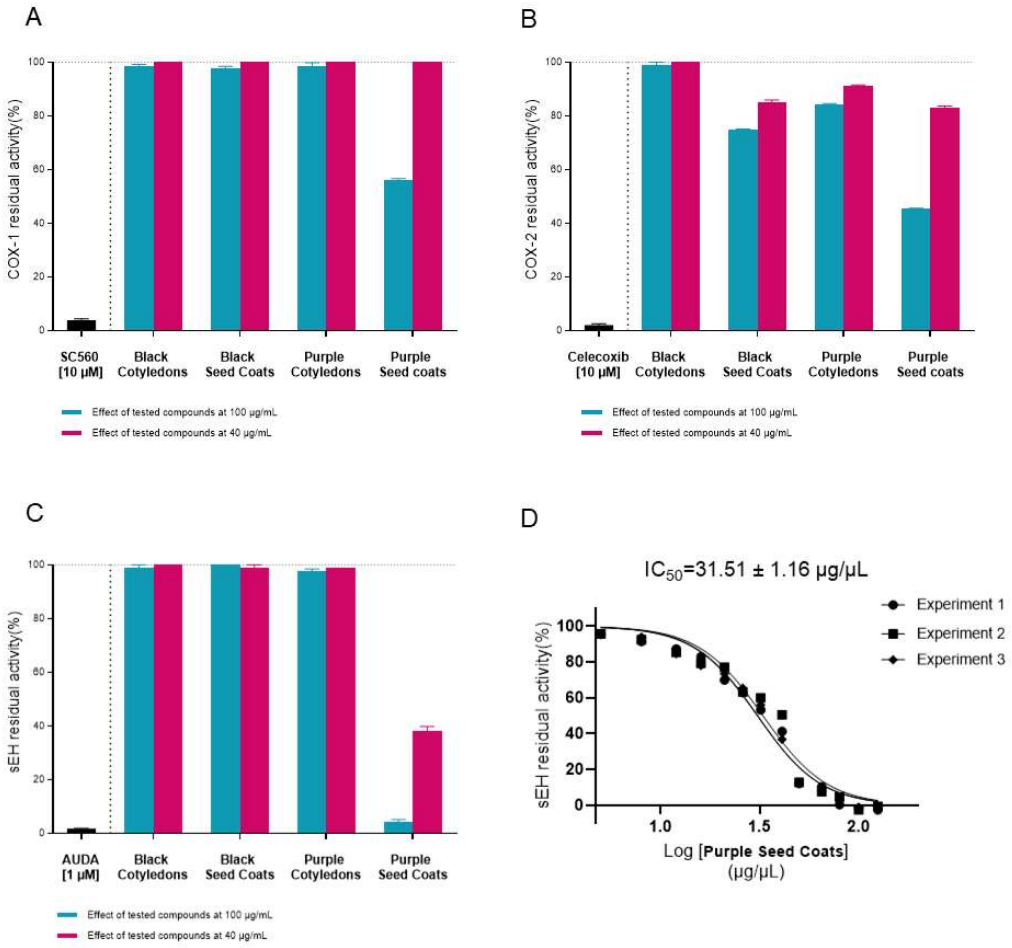


Figure 5.1 - In vitro evaluation of *Vicia faba* extracts (80% MeOH) against COX-1 (A), COX-2 (B), and sEH (C) at 100 and 40 µg mL^{-1}. Dose–response curve (D) of purple seed coat extracts (VFPS) against sEH. Data are expressed as mean ± SD (n = 3).

5.3.6. Mineral Profile

Cotyledons represent the primary reserve of mineral nutrients for embryonic development and exhibited elevated levels of the most abundant macronutrients. In particular, potassium (K) concentrations ranged from 12,615 to 13,548 mg kg^{-1} DW, while phosphorus (P) levels ranged from 5,081 to 6,160 mg kg^{-1} DW.

The predominance of potassium reflects the role of the seed as a metabolically active tissue with high osmotic demands. In contrast, phosphorus, largely stored in the form of phospholipids and phytates, plays a central role in energy metabolism.

In addition to macronutrients, the mineral profile highlighted differences in the distribution of essential microelements between seed fractions. Seed coats showed higher concentrations of elements involved in redox processes, particularly zinc (Zn) and manganese (Mn).

Zinc content reached up to 88.04 mg kg^{-1} DW in the VFB variety, while manganese (Mn) levels were up to 39 mg kg^{-1} DW.

This finding suggests that the outer seed fraction may contribute to the overall bioactivity of the extracts not only through phenolic compounds, but also through the presence of transition metals involved in redox mechanisms.

Overall, the mineral patterns observed are consistent with those reported for other legume species [182]. Although the VFB variety showed a slightly higher zinc content compared to the VFP variety, this difference does not appear sufficient to compensate for the lower phenolic content of the black seed coat, which remains less relevant from a nutraceutical perspective. The elemental distribution is reported in *Table 5.6*.

Table 5.6 Mineral composition (mg kg−1 DW) of seed coats and cotyledons from black (VFB) and purple (VFP) *Vicia faba* L. varieties.

Element (λ, nm)	VFB Seed coats	VFB Cotyledons	VFP Seed coats	VFP Cotyledons
Cu (327.395)	4.80 ± 0.07	16.55 ± 0.40	1.12 ± 0.02	14.55 ± 0.04
Fe (259.940)	4.89 ± 0.09	65.09 ± 1.58	25.36 ± 0.65	44.21 ± 1.05
K (766.491)	7675.95 ± 2.30	12,615.34 ± 5.88	6242.13 ± 2.28	13,548.84 ± 4.65
B (249.772)	19.61 ± 0.11	6.11 ± 0.02	20.16 ± 0.23	8.99 ± 0.06
Mg (280.270)	2539.25 ± 1.04	953.41 ± 1.87	1977.69 ± 1.03	927.30 ± 1.52
Mn (257.610)	39.96 ± 0.22	16.35 ± 0.11	7.46 ± 0.03	13.00 ± 0.02
Ni (216.555)	0.65 ± 0.02	2.00 ± 0.06	0.07 ± 0.01	2.74 ± 0.03
Zn (206.200)	88.04 ± 0.94	85.15 ± 1.32	23.08 ± 0.11	47.50 ± 0.09
P (213.618)	387.35 ± 1.02	5 080.98 ± 1.16	366.55 ± 0.09	6159.66 ± 1.64
S (181.972)	329.35 ± 4.18	1934.99 ± 1.61	297. 27 ± 2.23	1721.12 ± 1.45

5.3.7. Computational study

To provide a molecular rationale for the anti-inflammatory activity observed in the enzymatic assays, an *in silico* molecular docking study was performed on cyclooxygenases (COX-1 and COX-2) and soluble epoxide hydrolase (sEH), in agreement with the biological targets investigated experimentally.

Based on the metabolomic profiling and targeted LC-ESI-MRM analyses, representative proanthocyanidins were selected as ligands, with particular attention to procyanidin A and procyanidin B, which were among the most abundant compounds detected in the methanolic extracts of the purple seed coats and showed a strong correlation with the observed inhibitory effects.

Docking simulations indicated that proanthocyanidins are able to bind within the catalytic pockets of both COX isoforms. Although COX-1 and COX-2 share a high degree of structural similarity, the larger and more flexible active site of COX-2 favours the accommodation of bulky polyphenolic ligands. In this context, both procyanidin A and procyanidin B established multiple stabilising interactions within the COX-2 binding site, including hydrogen bonds and π–π stacking interactions with aromatic residues lining the catalytic channel.

The overall interaction pattern resulted in a more favourable binding affinity toward COX-2 compared to COX-1, in agreement with the preferential COX-2 inhibition observed experimentally.

In parallel, docking simulations performed on soluble epoxide hydrolase revealed that proanthocyanidins can be accommodated within the enzyme catalytic tunnel, where they interact with residues involved in substrate binding and catalysis. Procyanidin A, in particular, exhibited a binding mode characterised by an extensive hydrogen-bonding network and steric interactions capable of partially occluding the catalytic channel.

This binding behaviour is consistent with an effective interference with lipid epoxide metabolism and provides a molecular explanation for the strong sEH inhibition observed for the purple seed coat extracts. The bulky molecular structure of proanthocyanidins appears to play a crucial role in limiting substrate accessibility, thereby contributing to the reduction of enzymatic activity.

Overall, the computational results support and complement the experimental findings obtained from enzymatic inhibition assays. The ability of proanthocyanidins to interact with key functional regions of COX-2 and sEH provides a molecular basis for the enhanced anti-inflammatory activity observed in the methanolic extracts of the purple seed coats.

When integrated with chemical profiling and *in vitro* data, these *in silico* results highlight the contribution of polyphenolic compounds to the modulation of inflammatory pathways and further strengthen the nutraceutical potential of pigmented *Vicia faba* varieties. Representative docking poses of selected proanthocyanidins within the active sites of COX-2 and sEH are shown in *Figure 5.2*.

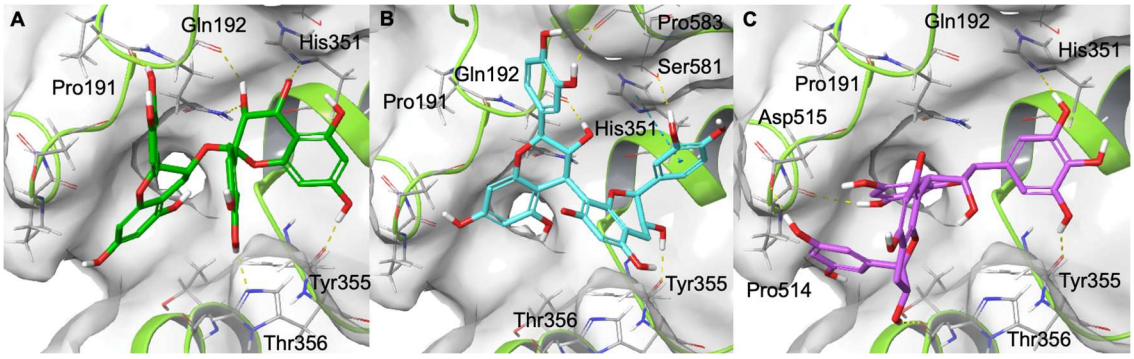


Figure 5.2 - Representative molecular docking poses of selected proanthocyanidins within the active sites of inflammatory enzymes. (A) Procyanidin A bound to the COX-2 catalytic pocket; (B) Procyanidin B bound to the COX-2 catalytic pocket; (C) Procyanidin A docked within the catalytic tunnel of soluble epoxide hydrolase (sEH).

5.4 Discussion

The findings of this study underscore the substantial disparities between the two ancient varieties of *Vicia faba* analyzed with respect to phytochemistry and functionality. These results further support the concept that seed coat pigmentation represents a major phenotypic and metabolic indicator within the species. This element has been previously reported in studies conducted on legumes and other Fabaceae, and is associated with a greater presence of phenolic and flavonoid compounds, which are responsible for antioxidant and anti-inflammatory properties [173–176].

The higher phenolic and flavonoid content observed in the VFP variety compared to the VFB is reflected in the structural characteristics of seed coat pigments, whose biosynthesis is linked to the phenylpropanoid and flavonoid pathways. In other legumes, the presence of anthocyanins and proanthocyanidins in seed coats has been correlated with protective functions against oxidative and microbial stress [173–176], suggesting that the more intense colouration is not simply an aesthetic trait, but a functional index of the tissue's metabolic density.

In this context, the proanthocyanidin content found in the seed coats of the purple variety not only confirms previous observations on the tissue distribution of phenolic compounds in *Vicia faba* [173,174], but also provides a distinctive element that allows us to hypothesise a higher nutraceutical potential of the pigmented varieties.

The elevated antioxidant activities observed in the DPPH, ABTS, and FRAP assays are consistent with the total phenolic content and, most notably, with the presence of proanthocyanidins, which are regarded as among the most efficacious natural free radical scavengers due to their highly redox-reactive oligomeric structure [173–176]. The observation that these activities are more pronounced in methanolic extracts than in ethanolic ones suggests that the choice of solvent is not neutral, but rather guides the preferential recovery of metabolites responsible for bioactivity, with implications for food and nutraceutical applications.

This finding is of particular relevance from an application perspective, as it suggests that appropriately optimized extraction processes could modulate the functional value of *V. faba* derived ingredients.

LC-ESI-HR-MS/MS characterization confirmed the greater metabolic complexity of the purple seed coat, highlighting the presence of flavonoids, proanthocyanidins, and polar lipids not commonly reported in the species. Although *Vicia faba* has traditionally been the focus of research due to its nutritional and antinutritional properties [156–158,167–170], the identification of molecular classes rarely reported in the species serves to broaden the scope of this legume's potential applications.

In particular, the presence of oxylipins and lipid derivatives in the pigmented seed coats suggests the existence of additional biological functions related to resistance to oxidative stress, a finding that is consistent with recent observations in the Fabaceae [189,190].

The LC-ESI-MRM approach was successful in identifying type A and B procyanidins, as well as dimers and epigallocatechins characteristic of the purple variety. The concomitant presence of these molecules, in conjunction with the elevated quantitative levels detected, enables the consideration of VFP as a food matrix of considerable functional value.

The results of the enzymatic assays serve to provide a more comprehensive biological perspective, in conjunction with the findings derived from chemical analyses. The inhibition

of sEH, more pronounced in the purple variety, is consistent with the role attributed to flavonoids and proanthocyanidins in modulating inflammatory pathways through interference with enzymes and lipid mediators [183–186]. While the activity against COX-1 and COX-2 was more moderate, it serves to reinforce the hypothesis of a synergistic effect of phenolic metabolites in the regulation of inflammation.

Finally, the higher concentration of redox-active microelements in the seed coats – including zinc and manganese – serves to complement the functional picture that has emerged from the phytochemical analyses. These elements, which have already been associated with intracellular antioxidant enzymatic systems, may contribute to the stability of phenolic metabolites and explain part of the greater oxidative resilience observed in pigmented varieties [182].

5.5 Remarks

This study demonstrated that the two ancient varieties of *Vicia faba* analyzed exhibit markedly different phytochemical, antioxidant, and anti-inflammatory profiles, thereby confirming the key role of seed coat pigmentation as an indicator of the nutritional and functional potential of the species.

The purple variety (VFP) was characterized by higher levels of phenolic compounds, flavonoids, and proanthocyanidins, together with enhanced antioxidant activity and more pronounced enzyme inhibition. These results suggest that the purple variety may exhibit a higher overall biological potential compared to the black variety (VFB).

The integration of spectrophotometric, metabolomic, and targeted analytical approaches clarified the nature and distribution of the metabolites responsible for these activities, highlighting the seed coats as the main bioactive compartment, while the cotyledons predominantly fulfil a storage function. The presence of redox-active microelements in the seed coats, combined with a rich and diverse phenolic profile, further reinforces the functional relevance of pigmented varieties.

Overall, the results indicate that traditional *V. faba* varieties, particularly those with dark-coloured seed coats, represent promising plant matrices for nutraceutical, food, and cosmetic applications. The significant content of proanthocyanidins and other bioactive metabolites

supports the potential exploitation of these varieties as natural sources of functional ingredients, including those derived from currently undervalued by-products such as seed coats.

Finally, this study contributes to the valorization of local biodiversity and supports the transition towards more sustainable agri-food supply chains, confirming *Vicia faba* as a strategic resource for the development of high-value foods and bio-based products.

CHAPTER 6 REVIEW: Bioactive secondary metabolites in edible plants

6.1 Background and rationale

In recent decades, interest in edible plants as sources of bioactive secondary metabolites has markedly increased. This growing attention is mainly driven by two factors. First, there is a need to identify natural alternatives to synthetic compounds. Second, increasing scientific evidence shows that many phytoconstituents play an important role in the prevention and modulation of major chronic degenerative diseases [3]. Although these molecules are not essential for plant survival, they are involved in defence mechanisms, attraction of reproductive vectors, and ecological adaptation. In humans, these properties are often associated with antioxidant, anti-inflammatory, cardioprotective, and antimicrobial activities [9,191].

At the same time, the concept of nutraceutical—introduced to describe food products with health benefits beyond basic nutrition—has progressively evolved from a focus on single active ingredients to the entire phytocomplex. This perspective highlights that biological efficacy does not depend on a dominant metabolite alone, but on synergistic interactions among different phytochemicals. Epidemiological evidence further supports this view, showing that diets rich in plant-based foods can reduce oxidative stress and chronic disease risk [192,193].

The Mediterranean area, especially the Apennine region, represents a biogeographical hotspot of plant biodiversity, characterized by species adapted to variable soil and climate conditions and therefore rich in secondary metabolites with high functional potential. However, this heritage is increasingly threatened by the progressive loss of traditional ethnobotanical knowledge. For this reason, the collection, organization, and valorization of available data should be considered a priority [194,195].

In addition, modern analytical methodologies—particularly LC–MS and GC–MS—play a key role in phytochemical research. These techniques allow the identification of metabolites even at very low concentrations, as well as their structural and functional characterization. The integration of targeted and untargeted approaches with dedicated bioinformatics tools has shifted research from a purely chemical description of phytocomplexes to a more systemic interpretation of the plant metabolome. This progress has opened new perspectives for the nutraceutical and therapeutic use of edible plants [10].

Based on these premises, the present review aims to critically analyse secondary metabolites in edible plants, with particular attention to phytochemical composition, documented biological activities, and potential applications in nutraceutical and functional fields. The objective is to provide an updated and integrated overview of current evidence, highlighting the contribution of the plant species investigated in the previous chapters and defining a scientific basis for future industrial and biotechnological applications.

For clarity, *Figure 6.1* summarises the plant species investigated in this thesis.



Figure 6.1 - Overview of the ten plant species investigated in this thesis

6.2 Review objectives

The present review aims to organise and critically discuss current knowledge on secondary metabolites in edible plants, with particular reference to species traditionally consumed in the Mediterranean and Apennine regions. The overall objective is to define an integrated framework that links phytochemical composition, biological activity, and potential nutraceutical applications.

Specifically, this review aims to:

i. analyze the phytochemical diversity of secondary metabolites, with particular attention to the main molecular classes - polyphenols, flavonoids, terpenes, carotenoids, alkaloids, and volatile compounds - responsible for the ecological functions and biological effects in edible plants;
ii. examine available evidence on documented biological activities in relation to key pathophysiological pathways involved in modern chronic diseases, including oxidative stress, systemic inflammation, cardiometabolic dysfunction, neurodegeneration, and microbial resistance;
iii. evaluate the synergistic contribution of the phytocomplex, moving beyond a reductionist single-compound approach and highlighting interactions among secondary metabolites that may underlie the overall functional efficacy of plant extracts;
iv. contextualize the nutraceutical potential of edible plants by considering their role in the traditional Mediterranean diet, their integration into contemporary dietary patterns, and the growing interest in unconventional plant matrices as sustainable sources of bioactive compounds;
v. identify critical issues, knowledge gaps, and research priorities related to the stability, bioavailability, safety of use, and phytochemical variability of the plant matrices under study.

The primary objective of this review is to provide an advanced interpretative framework that supports understanding of the role of secondary metabolites in edible plants and helps guide strategies for the sustainable and innovative valorization of plant resources.

6.3 Review Methodology

The present review was conducted using a narrative approach to integrate available evidence on secondary metabolites in edible plants and their associated biological activities. Relevant sources were collected from major international scientific databases, including Scopus, Web of Science, PubMed, ScienceDirect, and Google Scholar, using combinations of keywords such as *wild edible plants*, *secondary metabolites*, *polyphenols*, *nutraceuticals*, *antioxidant activity*, *anti-inflammatory*, *cardiometabolic disorders*, and *bioactive compounds*.

The literature search included original articles, reviews, book chapters, and scientific contributions published mainly over the last twenty years, with particular attention to studies reporting phytochemical analyses, biological assays, and functional evaluations of edible plant species of the Mediterranean context. Priority was given to studies employing well-established analytical methodologies, notably high-resolution liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), and *in vitro* antioxidant assays, in order to improve the robustness and comparability of the selected data.

This approach enabled the identification of the main classes of secondary metabolites, the most frequently reported biological activities, and current application trends. Overall, it provided an interpretative framework that considers both plant biodiversity and the potential functional value of the species examined in this thesis.

6.4 Nutritional composition and biological activity of selected species

6.4.1 Plants with antioxidant activity

Antioxidant activity is one of the most widely documented functional properties of edible plants. It is closely associated with the presence of secondary metabolites such as polyphenols, flavonoids, carotenoids, phenolic acids, and terpenoids. These compounds play a key role in counteracting oxidative stress, a pathophysiological condition linked to the excessive production of reactive oxygen species (ROS). Oxidative stress is recognized as a major contributing factor in the development of several chronic degenerative diseases, including atherosclerosis, diabetes, neurodegeneration, and cellular ageing.

From a mechanistic perspective, phenolic metabolites mainly act through redox reactions, donating hydrogen atoms or electrons to free radicals, thereby stabilising them and interrupting oxidative chain reactions.

In addition to flavonoids, other phytochemical classes significantly contribute to antioxidant activity. These include phenolic acids (such as caffeic, ferulic, and gallic acids), which show high radical-scavenging capacity; carotenoids, which can quench reactive species through energy transfer mechanisms; and terpenoids and volatile compounds, which may indirectly modulate cellular redox balance through specific signalling pathways.

The edible plant species examined in this study exhibit marked antioxidant profiles, generally characterized not by the presence of a single dominant compound, but by the synergistic action of multiple classes of metabolites. This feature is particularly evident in *Lavandula angustifolia*, *Origanum vulgare*, *Laurus nobilis*, *Cannabis sativa* var. *Kompolti*, and pigmented varieties of *Vicia faba*, where the combined presence of phenols, flavonoids, and minor metabolites results in enhanced antioxidant activity[196,197].

From a methodological point of view, antioxidant activity is mainly evaluated through *in vitro* assays based on two complementary approaches. Electron transfer (ET) assays, such as FRAP and CUPRAC, measure the reducing capacity of plant extracts, while hydrogen atom transfer (HAT) assays, including DPPH, ABTS, and ORAC, are commonly used to assess radical-scavenging activity [198].

The combined use of these methods allows a more comprehensive evaluation of antioxidant potential, reducing the limitations associated with individual assays, which are often sensitive to specific compounds or reaction mechanisms.

An important observation emerging from the analyzed studies is that antioxidant efficacy does not depend exclusively on the total polyphenol content, but rather on the chemical structure and complexity of the phytocomplex. In *Cannabis sativa* var. *Kompolti* (Chapter 5), for example, the aqueous extract exhibited higher scavenging activity than the hydroalcoholic extract, despite a lower total phenolic content. This result suggests a relevant role for highly water-soluble metabolites and possible synergistic interactions.

Similarly, in pigmented varieties of *Vicia faba* (Chapter 6), the presence of proanthocyanidins in the seed coat appears to be directly linked to the high antioxidant capacity observed, confirming the key role of flavan-3-ols in radical-scavenging processes.

Overall, antioxidant activity should not be considered a simple chemical parameter, but rather a fundamental biological determinant of the nutraceutical potential of edible plants. The growing evidence linking the consumption of phenolic-rich foods with a reduced risk of chronic diseases highlights the importance of preserving and valorising wild and traditional plant matrices, which represent a valuable natural resource for the prevention and modulation of oxidative processes [199].

6.4.2 Anti-inflammatory Activity

Inflammation is an essential physiological response involved in host defence; however, when it becomes chronic, it represents a key pathogenic factor in the development of numerous degenerative diseases [3], including rheumatoid arthritis, type 2 diabetes, cardiovascular disorders, neurodegenerative diseases, and certain cancers. In this context, secondary metabolites from edible plants are of increasing pharmacological interest, as they can modulate inflammatory pathways through both direct and indirect mechanisms [200].

Representative anti-inflammatory compounds include flavonoids, phenolic acids, terpenoids, and proanthocyanidins. Their activity is related to the ability to neutralise reactive oxygen species (ROS), reduce nitric oxide (NO) production, and modulate the activity of enzymes such as cyclooxygenase (COX) and lipoxygenase (LOX), which are involved in the biosynthesis of pro-inflammatory eicosanoids [200]. These effects are rarely attributable to a single metabolite, but rather reflect the synergistic action of the phytocomplex, which confers multifunctional properties to plant extracts that are difficult to reproduce with isolated compounds.

Among the species analyzed, *Origanum vulgare* is characterized by a high content of rosmarinic acid and carvacrol, compounds shown to reduce free radical formation and the production of pro-inflammatory mediators in cellular and *in vitro* models. *Cannabis sativa* var. *Kompolti* (Chapter 5), due to the presence of non-psychotropic phytocannabinoids such as cannabidiol (CBD), exhibits anti-inflammatory effects that are indirectly linked to the modulation of oxidative stress and redox signalling pathways.

Similarly, pigmented varieties of *Vicia faba* (Chapter 6) contain elevated levels of proanthocyanidins, which are thought to contribute to inflammation attenuation through the regulation of redox-dependent processes involved in the acute inflammatory response.

A particularly relevant case is *Laurus nobilis* (Chapter 4), in which specific metabolites, including lauroside B [18,201], display targeted activity towards enzymes and proteins involved in the inflammatory cascade, as well as molecular factors related to cell survival regulation. This evidence supports the concept that the anti-inflammatory properties of edible plants result from multiple complementary mechanisms rather than a single molecular pathway.

Overall, anti-inflammatory activity represents a key component of the nutraceutical value of edible plants.

6.4.3 Cardiometabolic Effects

Cardiometabolic disorders, including obesity, insulin resistance, dyslipidaemia, and cardiovascular complications, represent a major global health challenge. Their development is driven by a complex interaction of genetic, environmental, and behavioural factors, among which diets poor in bioactive plant components and unhealthy lifestyles play a significant role. In this context, increasing scientific attention has focused on edible plants containing secondary metabolites capable of modulating metabolic parameters, improving endothelial function, and counteracting oxidative and inflammatory processes involved in cardiometabolic disease progression [3].

The phytochemical classes most commonly associated with these benefits include polyphenols, flavonoids, phenolic acids, terpenes, and phytosterols. These compounds can influence key biological mechanisms such as lipid absorption and metabolism, glucose homeostasis, and vascular protection against oxidative damage. Polyphenols, in particular, are known for their cholesterol-lowering effects and their ability to inhibit low-density lipoprotein (LDL) oxidation, a critical step in atherogenesis.

Several species examined in this thesis, including *Achillea millefolium*, *Urtica dioica*, and *Foeniculum vulgare*, show relevant evidence in the cardiometabolic field. *Achillea millefolium* is rich in flavonoids and phenolic acids, which have been associated with

reductions in serum triglyceride and total cholesterol levels, suggesting a modulatory effect on lipid metabolism [202,203].

Urtica dioica, in addition to its polyphenolic fraction, contains phytosterols and minerals that may influence cholesterol absorption and glycaemic parameters, supporting its potential role in glucose homeostasis. *Foeniculum vulgare*, characterized by the presence of anethole and other aromatic compounds, has demonstrated beneficial effects on lipid digestion and liver function, a central organ in lipid metabolism.

Additional contributions derive from plant matrices rich in flavan-3-ols and proanthocyanidins, such as pigmented varieties of *Vicia faba* (Chapter 6). Their strong antioxidant activity has been associated with reduced oxidative damage to endothelial cells and increased nitric oxide (NO) bioavailability, promoting vasodilation and vascular protection. Likewise, plant matrices containing hypoglycaemic components—such as soluble fibres, polyphenols, and enzyme-modulating compounds—can reduce postprandial glycaemic peaks and improve insulin sensitivity.

Taken together, these findings indicate that the cardiometabolic benefits of edible plants rely on the integrated action of the phytocomplex rather than on individual compounds. This functional multidimensionality makes these plant matrices valuable resources for nutritional prevention and for the development of functional foods and nutraceutical ingredients aimed at supporting cardiometabolic health.

6.4.4 Antimicrobial Activity

Antimicrobial activity is one of the most documented properties associated with the traditional use of edible plants. Their historical use for food preservation, infection treatment, and prevention of microbial spoilage dates back thousands of years. In recent decades, scientific interest in this activity has increased considerably in response to the rise of antimicrobial resistance and the need for safe and effective natural alternatives.

Secondary metabolites such as phenols, flavonoids, terpenoids, aromatic compounds, and saponins can interfere with essential microbial cellular structures and processes. Although the mechanisms of action vary among chemical classes, the most widely documented effects include damage to bacterial cell membranes through lipophilic interactions, increased

membrane permeability with leakage of intracellular components, interference with enzymes involved in energy metabolism, and alteration of microbial communication and virulence.

Among the species analyzed, *Origanum vulgare* (Chapter 3) is characterized by a high content of aromatic compounds, particularly carvacrol and thymol, which show strong antibacterial and antifungal activity against both Gram-positive and Gram-negative microorganisms. These compounds primarily disrupt cell membrane integrity, leading to lipid disorganization and loss of proton motive force, ultimately compromising microbial viability.

Similarly, *Laurus nobilis* (Chapter 4) contains terpene derivatives and phenolic compounds capable of inhibiting microbial growth and reducing bacterial colonization, supporting its traditional use as a preservative aromatic plant. Some extracts also show selective activity against pathogenic strains involved in food contamination and spoilage, suggesting potential applications in functional food formulations.

Plant matrices with high polyphenol content, such as *Urtica dioica* and *Vicia faba*, also contribute to antimicrobial activity. In these species, flavonoids and proanthocyanidins may exert indirect effects by inhibiting microbial adhesion, modulating local oxidative responses, and reducing environmental conditions favourable to pathogen proliferation.

Overall, antimicrobial activity is not attributable to a single dominant compound, but rather results from synergistic interactions among different chemical classes within the phytocomplex. This reinforces the concept that the phytocomplex itself represents the main functional unit underlying the observed biological effects. Consequently, edible plants emerge as promising natural resources for microbial control, with applications in nutraceuticals, food preservation, and the development of functional ingredients with reduced environmental impact. Their antimicrobial activity complements antioxidant, anti-inflammatory, and cardiometabolic properties, contributing to a phytochemical profile of high applicative value.

To facilitate comparison among the selected species and their potential functional applications, the table below summarizes the main nutraceutical features of the edible plant species considered in the broader framework of this thesis, including their distinctive

metabolites, documented biological activities, and prospective uses, encompassing both species extensively discussed in the review and those analyzed in the experimental chapters.

To facilitate comparison among the selected species and their potential functional applications, *Table 6.1* summarises the main nutraceutical features of the edible plant species discussed in this thesis, including their key metabolites, documented biological activities, and potential uses.

Table 6.1 Nutraceutical features of selected wild edible plants from the Apennine area

Species	Plant part used	Key metabolites	Main nutraceutical activities	Potential applications	Distinctive feature
Urtica dioica	Leaves, roots	Caffeoylquinic acids, flavonoids, minerals	Anti-inflammatory, antioxidant, remineralising	Urinary supplements, anaemia support	High mineral content
Silybum marianum	Seeds	Silymarin complex (silibinin), taxifolin	Hepatoprotective, anti-steatotic	Liver supplements, detox products	Unique phytocomplex
Laurus nobilis	Leaves	1,8-cineole, eugenol, tannins	Digestive, antimicrobial, anti-inflammatory	Digestive infusions, respiratory support	Broad-spectrum essential oil
Foeniculum vulgare	Fruits, leaves	Anethole, estragole, fenchone	Carminative, antioxidant, spasmolytic	Digestive products, herbal teas	Anise-like aromatic profile
Satureja montana	Flowering tops	Carvacrol, thymol	Antimicrobial, antioxidant	Food preservation, phytoprotective products	Strong antimicrobial activity
Juniperus communis	Berries	α-pinene, myrcene, diterpenes	Diuretic, anti-inflammatory, hepatoprotective	Liqueurs, urinary supplements	Long-standing officinal use
Gentiana lutea	Roots	Amarogentin, gentiopicroside	Digestive stimulant	Functional bitters, tonics	Extremely high bitter potency
Achillea millefolium	Aerial parts	Azulenes, flavonoids	Anti-inflammatory, wound-healing	Herbal teas, cosmetics	Mucosal support
Malva sylvestris	Leaves, flowers	Mucilages, anthocyanins	Soothing, gastroprotective	Herbal teas, mucosal nutraceuticals	High mucilage content
Borago officinalis	Leaves, seeds	GLA, flavonoids	Anti-inflammatory, lipid-modulating	Functional oils, joint support	Plant source of GLA

6.5 Nutraceutical Perspectives and Future Applications

Interest in edible plants as nutraceutical resources has grown substantially in recent years, supported by increasing awareness of the role of secondary metabolites in health maintenance and chronic disease prevention. Unlike conventional foods, nutraceuticals derived from plant matrices not only provide essential nutrients, but also contain bioactive compounds capable of modulating specific physiological functions. Their beneficial effects on oxidative stress, inflammation, lipid metabolism, and microbial balance are now widely documented.

The edible plants examined in this thesis are characterized by the richness and diversity of their phytocomplex, which includes polyphenols, flavonoids, terpenes, aromatic compounds, and minor metabolites acting in a synergistic manner. This synergistic behaviour, which is difficult to reproduce using isolated active ingredients, confers a clear functional advantage to plant extracts and supports their suitability for the development of functional foods, dietary supplements, and innovative nutraceutical ingredients.

An important aspect of nutraceutical valorization is the growing demand for natural and sustainable solutions. The use of traditional and native plant species, often overlooked by modern agri-food systems, contributes to the preservation of biodiversity and the recovery of ethnobotanical knowledge, while also creating new opportunities for local and rural economies. Despite the recognized potential of edible plants as sources of bioactive compounds, the translation of this value into marketable nutraceutical products is still limited by several critical issues. These include phytochemical variability related to environmental, genetic, and agronomic factors, as well as concerns regarding metabolite stability during extraction, processing, and storage. In addition, the bioavailability and bioaccessibility of many compounds in the gastrointestinal tract are often lower than those observed under *in vitro* conditions. Finally, further well-designed clinical studies are required to confirm efficacy and safety in human populations.

The integration of targeted agronomic strategies, advanced extraction technologies, and comprehensive metabolomic approaches represents a promising pathway to address these challenges. High-performance analytical platforms, such as LC–MS, GC–MS, untargeted metabolomics, and multivariate data analysis, enable the identification of novel bioactive metabolites, the investigation of interactions within the phytocomplex, and the optimization

of agro-industrial valorization processes. At the same time, technological innovation aimed at improving environmental sustainability - including the use of green solvents and energy-efficient processes - offers new opportunities for nutraceutical production in line with European policies on ecological transition.

Overall, the nutraceutical potential of edible plants represents a rapidly evolving field, characterized by the convergence of expertise from agronomy, food technology, biochemistry, and clinical research. The main future challenge lies not only in identifying new metabolites or confirming known biological activities, but also in translating this knowledge into reliable, safe, and effective products that can be integrated into contemporary dietary patterns and contribute to the improvement of public health.

6.6 Remarks

Edible plants represent a phytochemical heritage of high scientific and nutraceutical relevance. The evidence discussed in this review confirms that their richness in secondary metabolites - such as polyphenols, flavonoids, terpenoids, carotenoids, and aromatic compounds - forms the basis of a wide range of biological activities, including antioxidant, anti-inflammatory, cardiometabolic, and antimicrobial effects.

These activities do not depend on individual compounds, but rather arise from the complexity and synergistic interactions of the phytocomplex, a feature that clearly distinguishes plant matrices from synthetic products. This perspective emphasises the importance of considering the whole metabolite profile when evaluating the nutraceutical potential of edible plants.

At the same time, the available evidence highlights the need for a critical and forward-looking approach. Despite the growing interest in plant-based nutraceuticals, further research is required to address phytochemical variability, processing and transformation effects, bioavailability, and, most importantly, clinical validation of efficacy in humans.

In this context, the integration of technological innovation, advanced metabolomic characterization, and the preservation of ethnobotanical knowledge represents a promising strategy for translating the potential of edible plants into reliable and effective applications for human health.

CHAPTER 7 REVIEW: Valorization of plant waste by LC–MS for the identification of bioactive metabolites for cosmetic applications

7.1 Background and rationale

The increasing focus on environmental sustainability and the adoption of circular production models have led to a significant change in the perception of agro-industrial waste. Rather than being considered a material to be discarded, this waste is now widely recognized as a valuable resource for biotechnology and the development of functional ingredients [24,25]. Its valorization offers an effective strategy to reduce the environmental impact of agricultural and industrial activities, in line with the objectives of the European Green Deal and the circular bioeconomy framework [204,205].

In particular, plant residues generated during food processing - such as peels, leaves, seeds, fibres, and lignocellulosic fractions - represent an important reservoir of secondary metabolites. In many cases, these compounds are present at higher concentrations in waste materials than in the edible portions of plants [206,207]. The high phytochemical content of these residues, together with their antioxidant, anti-inflammatory, antimicrobial, and, in some cases, anti-ageing properties, has stimulated growing interest in their application in emerging sectors, especially the cosmetics industry [208].

Modern cosmetic formulations are increasingly oriented towards natural and sustainable products based on bioactive compounds of plant origin, progressively replacing synthetic ingredients that may be poorly compatible with skin physiology [209]. Within this context, plant-derived waste is particularly attractive, as its exploitation enables the recovery of functional molecules while reducing production costs, lowering CO_2 emissions, and promoting new industrial processes consistent with circular economy principles [210–212].

This shift has been strongly supported by the development of advanced analytical techniques capable of identifying, quantifying, and characterising bioactive metabolites in plant waste with high sensitivity and accuracy. Among these, liquid chromatography coupled with mass spectrometry (LC–MS) has become the reference platform for plant metabolomics, owing to its ability to analyse a wide range of chemical classes, often present at trace levels within complex matrices [13].

At the same time, the diffusion of eco-friendly extraction techniques—such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), and the use of biodegradable solvents or supercritical fluids—has further

enhanced the recovery of bioactive compounds from agro-industrial waste. These approaches reduce the use of toxic solvents and energy consumption compared to conventional methods, enabling the production of functional extracts suitable for cosmetic applications while meeting current market demands and sustainability goals [213–215].

7.2 Objectives of the review

The main objective of this chapter is to critically examine the role of liquid chromatography coupled with mass spectrometry (LC–MS) in the valorization of plant waste as a source of bioactive metabolites for cosmetic applications. In the current industrial context, increasing attention is directed towards the use of sustainable raw materials and the reduction of environmental impact. In this framework, the transformation of agri-food residues into functional ingredients represents an innovative strategy aligned with the principles of the circular economy [24,205].

To achieve this objective, the review aims to:

i. Contextualise the issue of plant waste by outlining the environmental, economic, and regulatory motivations for its valorization, emphasising that these matrices represent not only a disposal cost but also a valuable source of bioactive compounds of cosmetic interest [206,208];
ii. examine LC–MS analytical platforms by describing the main approaches used for metabolite characterization in plant by-products, with particular attention to targeted and untargeted analyses, the advantages of high-resolution instruments, and recent technological developments that extend the applicability of LC–MS to complex matrices [13,14];
iii. identify the main classes of bioactive metabolites detected in plant waste by LC–MS, with a focus on polyphenols, carotenoids, terpenoids, alkaloids, and polysaccharides, highlighting their functional properties and relevance for cosmetic applications, especially in antioxidant, anti-ageing, antimicrobial, and soothing formulations [208,210–212];
iv. analyse reported application examples showing how extracts obtained from post-harvest residues and agri-food by-products have been incorporated into experimental or commercial cosmetic formulations,

demonstrating the technical and industrial feasibility of plant waste upcycling [210–212];

v. identify critical issues and future perspectives by addressing challenges related to extraction process standardization, phytochemical variability of raw materials, analytical limitations of LC–MS, and regulatory aspects associated with the marketing of cosmetic ingredients derived from plant waste, with particular emphasis on the growing role of green technologies [213,215].

7.3 LC–MS platforms for the characterization of bioactive metabolites

The chemical complexity of plant waste, characterized by a wide diversity of compounds belonging to different structural classes and present over broad concentration ranges, requires analytical methodologies that combine high sensitivity, selectivity, and advanced data processing capabilities. In this context, liquid chromatography coupled with mass spectrometry (LC–MS) has emerged as the reference platform for metabolomic studies of plant materials and agro-industrial by-products intended for cosmetic applications [13].

The effectiveness of LC–MS relies not only on the combination of chromatographic separation and mass spectrometric detection, but also on the availability of advanced software tools for raw data processing, peak detection, annotation, and metabolite identification. This bioinformatic component, which is now an integral part of LC–MS workflows, enables the management of large datasets and the transformation of complex metabolic profiles into functional information useful for extract selection and product development [15,216].

LC–MS is consists of two main functional components:

- a chromatographic stage, responsible for the separation of metabolites according to their physicochemical properties, such as polarity, molecular weight, and affinity for the stationary phase;
- a mass spectrometric stage, responsible for the detection, identification, and quantification of compounds based on their mass-to-charge ratio (m/z).

The synergy between these components allows the simultaneous analysis of structurally related molecules present at low concentrations within complex matrices, which is typical of agro-industrial residues where phenolic compounds, terpenes, carotenoids, alkaloids, and polysaccharide-related components coexist [14].

7.3.1 Targeted and untargeted approaches

In plant waste metabolomics, two complementary LC–MS strategies are commonly applied: targeted and untargeted approaches.

Targeted analysis is designed for the identification and quantification of known metabolites. This strategy is typically implemented using triple quadrupole instruments operated in specific acquisition modes, such as multiple reaction monitoring (MRM) or parallel reaction monitoring (PRM), which provide high sensitivity, selectivity, and reproducibility [217]. Due to these characteristics, targeted methods are particularly suitable for extract standardization, quality control, and verification of bioactive markers, which are essential requirements in cosmetic formulation and industrial production.

In contrast, untargeted approaches aim at comprehensive metabolome profiling and the discovery of new or unexpected bioactive compounds. These analyses are mainly performed using high-resolution mass spectrometry (HRMS) instruments, such as Q-Orbitrap or Q-TOF systems, which allow the detection of hundreds or thousands of metabolites within a single analysis, including low-abundance or previously uncharacterized compounds [218]. The use of MS/MS fragmentation data supports structural elucidation and facilitates the reconstruction of metabolic pathways related to cosmetic-relevant properties, such as antioxidant, anti-ageing, and antimicrobial activities.

A further development, particularly relevant to untargeted approaches, concerns data acquisition strategies. Data-dependent acquisition (DDA) selectively fragments the most intense precursor ions, enabling a direct association between precursor and fragment ions. In contrast, data-independent acquisition (DIA) fragments all ions within predefined m/z windows, increasing metabolome coverage and improving the detection of low-abundance compounds [16].

Overall, targeted approaches are essential for accurate quantification and certification of characteristic metabolites, while untargeted approaches represent the primary tool for

bioactive compound discovery. Together, these strategies make LC–MS an indispensable platform for cosmetic innovation based on the valorization of plant waste.

7.3.2 Instrumental evolution and recent advancements

Technological progress in LC–MS instrumentation has significantly expanded the analytical possibilities for the study of metabolites present in plant waste, improving both sensitivity and structural information content. Modern platforms integrate different instrumental architectures capable of resolving structurally similar compounds and detecting molecules present at very low concentrations, a common feature of agro-industrial by-products [219].

One of the most important advances in this field is the development of high-resolution hybrid systems, such as Q-Orbitrap and Orbitrap Fusion instruments. These platforms combine high mass accuracy, fast scanning speeds, and advanced MS^n capabilities, enabling detailed characterization of metabolite structures and fragmentation patterns. Such features are particularly valuable for elucidating structural relationships among compounds of cosmetic interest, including polyphenols, terpenoids, and carotenoids.

Another relevant innovation is the integration of ion mobility spectrometry (IMS), which introduces an additional gas-phase separation based on ion mobility. IMS allows the discrimination of isomers, conformers, and co-eluting compounds that are difficult to resolve using conventional LC–MS alone. This capability is especially advantageous for complex plant matrices, where overlapping signals often generate ambiguous identifications [220].

In parallel, the use of alternative ionization sources, such as atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI), has expanded the detectable chemical space. These sources are particularly suitable for less polar or thermolabile compounds, which are frequently present in waste derived from lignocellulosic biomass and lipid-rich plant residues [221].

The integration of information obtained from multiple analytical dimensions ultimately enables the construction of a more comprehensive and reliable metabolic profile. As a result, LC–MS has evolved into a multidimensional analytical platform capable of supporting the identification of functional biomarkers and guiding both extraction strategies and formulation decisions in the cosmetics sector.

The overall LC–MS-based workflow applied to the valorization of plant waste for cosmetic applications is schematically summarized in *Figure 7.1*.

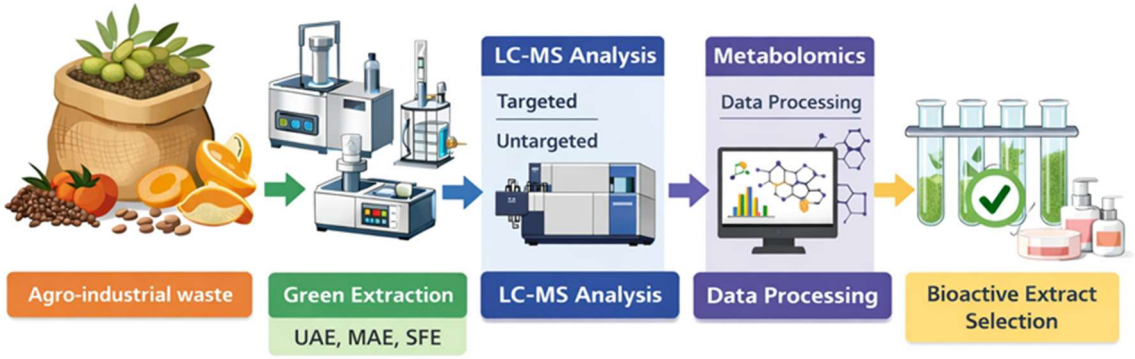


Figure 7.1 - LC–MS-based workflow for the valorization of plant waste in cosmetic applications. The figure illustrates the main steps from agro-industrial residues to the selection of bioactive extracts and cosmetic formulation.

7.4 Main classes of bioactive metabolites obtained from plant waste

The use of plant waste in cosmetic applications is based on the presence of a wide range of secondary metabolites within these matrices. Although phytochemical composition varies depending on plant species, ripening stage, and processing conditions, LC–MS analysis has enabled the identification of recurrent metabolite classes in agro-industrial residues. Each class is characterized by a specific chemical profile, biological activity, and functional relevance for cosmetic formulations [206–208,210–212].

A comparative understanding of these metabolite groups supports more informed decisions regarding extraction strategies and formulation choices in the cosmetics sector.

7.4.1 Polyphenols: the most abundant and functionally versatile class

Polyphenols represent the most extensively studied class of secondary metabolites in plant waste and are the compounds most strongly associated with cosmetic applications. Advances in high-resolution LC–MS have enabled detailed characterization of phenolic acids, flavonoids, tannins, and stilbenes, even in complex matrices where conventional analytical methods are often inadequate [13].

From a cosmetic perspective, polyphenols are mainly valued for their strong antioxidant activity, which plays a key role in counteracting oxidative stress and photoageing processes.

In addition, many phenolic compounds exhibit anti-inflammatory effects by modulating pathways involved in skin ageing and irritation. Their presence also contributes to formulation stability, improving product shelf life.

Plant by-products such as citrus peels, grape seeds, olive leaves, and pruning residues are among the richest sources of polyphenols and often contain higher concentrations than the edible plant parts. This makes them particularly attractive as functional cosmetic ingredients.

7.4.2 Terpenoids: antimicrobial and skin-balancing properties

Terpenoids are widely distributed in aromatic plant residues, especially leaves and flowering tops, and display high structural diversity, including mono-, di-, tri-, and tetraterpenes. LC–MS analysis allows the detection of both polar and lipophilic terpenoids through appropriate selection of ionization conditions.

In cosmetic formulations, terpenoids are primarily appreciated for their natural antimicrobial properties, which contribute to product purification and deodorising effects. Many terpenoids also exhibit soothing and calming activities, making them suitable for formulations designed for sensitive or irritated skin. Furthermore, their antimicrobial action supports the natural preservation of cosmetic products.

Compared with polyphenols, terpenoids are generally more volatile and chemically labile, requiring careful control during extraction and formulation. However, they provide complementary biological activities that enhance the overall functional profile of plant-derived extracts.

7.4.3 Carotenoids: photoprotective and skin uniforming properties

Carotenoids, such as β-carotene, lutein, and lycopene, are commonly detected in residues from tomatoes, carrots, and other pigmented fruits. The use of APCI or APPI ionization sources enables their effective detection by LC–MS, particularly in lipid-rich matrices where electrospray ionization may be less suitable [221].

In cosmetic applications, carotenoids act as lipophilic antioxidants that help protect the skin barrier from oxidative damage. They also contribute to photoprotection against UV-induced

stress and play a role in improving skin tone uniformity by scavenging reactive species in epidermal tissues.

Compared with polyphenols, carotenoids show lower water solubility and higher sensitivity to light and oxygen. As a result, their extraction and stabilization require tailored technological approaches to preserve their functionality.

7.4.4 Alkaloids: an emerging class with high biological specificity

Although less abundant in plant waste than other metabolite classes, alkaloids identified by LC–MS and MS/MS display bioactive profiles of considerable cosmetic interest. Many alkaloids exhibit anti-inflammatory effects through the modulation of pro-inflammatory mediators, while others show vasomodulatory activity that may be beneficial in toning or anti-cellulite formulations.

In addition, certain alkaloids have been proposed as candidates for neurocosmetic applications, owing to their potential interaction with skin receptors and sensory pathways.

Due to their high biological specificity but relatively low abundance, alkaloids are best considered niche functional ingredients, suitable for targeted cosmetic applications rather than bulk formulation components.

7.4.5 Polysaccharides and macromolecules: hydration and film formation

Residual polysaccharides, including pectins, arabinans, and cellulose derivatives, are not always easily detected by LC–MS because of their low ionisability. However, when characterized, these macromolecules exhibit properties of high relevance for cosmetic formulations.

Their strong water-binding capacity confers significant hydrating effects, while their film-forming ability contributes to reinforcement of the skin barrier and improvement of skin texture. In addition, some polysaccharides display prebiotic properties that may support the balance of the skin microbiota.

Unlike other metabolite classes discussed in this section, polysaccharides do not primarily function as antioxidants. Instead, they act as structural and functional components that enhance the sensory and protective properties of cosmetic products.

7.5 Cosmetic applications of extracts from plant waste characterized by LC–MS

The use of plant waste in cosmetic products has attracted increasing attention in recent years, supported by a growing body of evidence demonstrating its efficacy, safety, and technological feasibility. Agro-industrial by-products, when extracted and characterized by LC–MS, represent a sustainable source of bioactive compounds capable of supporting innovative formulation strategies aligned with circular economy principles [206–209].

Within cosmetic analytical research, LC–MS plays a central role by ensuring the chemical characterization and quality control of plant-derived extracts. This technique enables the identification of metabolites responsible for functional activities and supports the standardization of cosmetic formulations, which is essential for industrial scalability and regulatory compliance.

7.5.1 Recovery of antioxidants from pruning residues

A representative example of plant waste valorization is provided by residues derived from the pruning of orange, apricot, and olive trees. LC–MS analysis of these matrices enabled the identification of phenolic compounds with high antioxidant capacity, which were subsequently incorporated into an experimental cosmetic cream [210].

The resulting formulation exhibited good physical stability, with no evidence of phase separation or crystallization, as well as microbiological safety without the need for additional synthetic preservatives. Functional tests further confirmed a high antioxidant activity. This case represents a clear example of successful upcycling, demonstrating how lignocellulosic waste typically destined for disposal can be transformed into functional cosmetic ingredients.

7.5.2 Oils and lipid fractions: anti-aging properties in *Caryocar brasiliense*

The fruit of *Caryocar brasiliense*, widely distributed in tropical ecosystems, generates agroforestry residues from which an oil rich in fatty acids, carotenoids, and other bioactive compounds can be extracted. LC–MS characterization allowed detailed profiling of the lipid fraction and the selection of components most suitable for cosmetic use [211].

Cosmetic formulations developed using this oil demonstrated marked moisturising properties, improvements in skin softness, reduced oxidative processes associated with photoageing, and increased skin elasticity. These results highlight the potential of oils derived from tropical plant waste as natural anti-ageing ingredients.

7.5.3 Supercritical citrus and oleaceous extractsy

Eco-friendly extraction techniques, such as supercritical fluid extraction, enable the production of high-purity extracts characterized by the absence of residual solvents and high compositional reproducibility. When applied to clementine peel and olive leaf residues, this approach - combined with LC–MS profiling - allowed the selection of extracts with elevated phenolic content [212].

Cosmetic creams formulated with these extracts showed excellent physical stability over time, strong antioxidant activity, and good skin compatibility, which is essential for leave-on products. These results confirm the effectiveness of integrating supercritical extraction and LC–MS analysis for the valorization of phenolic-rich plant waste.

7.5.4 Emerging opportunities in the cosmetics supply chain

Beyond consolidated examples, the literature reports an expanding range of plant waste matrices characterized by LC–MS and investigated for cosmetic applications. These include grape pomace and seeds rich in proanthocyanidins, pomegranate peels containing photoprotective tannins, black tea residues with antimicrobial phenols, red cabbage waste as a source of antioxidant anthocyanins, and *Camellia sinensis* leaves rich in photoprotective catechins [222–224].

Overall, these studies demonstrate how the application of LC–MS has significantly broadened the spectrum of cosmetically relevant plant waste, facilitating targeted selection of raw materials for industrial upcycling processes.

Representative examples of cosmetic applications of plant waste characterized by LC–MS are summarized in *Table 7.1*.

Table 7.1 Plant waste analyzed by LC–MS and their documented cosmetic applications. *Detailed bibliographic references for each example are reported in the corresponding review article and are not repeated in this thesis.*

Plant waste	**LC–MS technique**	**Identified bioactives**	**Cosmetic activity**	**Potential applications**
Wine lees	UHPLC–Orbitrap / LC–QTrap	Phenols, hydroxycinnamic acids	Antioxidant	Functional antioxidant creams
Olive leaves	LC–MS (Finnigan AQA)	Polyphenols	Antioxidant, anti-ageing	Protective and anti-age creams
Tomato pomace	LC–Q-Exactive	Polyphenols	Antioxidant, anti-inflammatory	Anti-age skin formulations
Spent black tea	LC–LTQ Orbitrap XL	Phenolic compounds	Antimicrobial, antioxidant	Natural cosmetic preservatives
Aloe vera leaf waste	LC–MS	Anthraquinone derivatives	Soothing, regenerative	Calming ointments
Saffron tepals	LC–LTQ XL	Flavonoids	Anti-ageing	Anti-age creams
Red cabbage waste	LC–Linear Ion Trap	Anthocyanins	Photoprotective, antioxidant	Natural cosmetic colourants
Annatto by-product	UPLC–QTOF	Terpenoids, ceramides	Skin barrier restoration	Dermoprotective formulations
Prunus leaves	LC–MS	Tannins	Antioxidant	Anti-age ingredients
Horse chestnut flowers	LC–MS	Flavanols	ROS scavenging	Antioxidant serums
Camellia sinensis flowers	LC–MS	Catechins, flavonols, caffeine	Anti-ageing, UV-protective	Firming and sunscreen products
Eucalyptus leaves	LC–MS	Polyphenols	Anti-ageing	Anti-age formulations
Harpagophytum procumbens	LC–MS	Verbascoside	Anti-inflammatory	Repairing products
Lycium barbarum seed dreg	LC–MS	Polysaccharides	Moisturising	Hydrating cosmetics
Mentha × piperita	LC–MS	Flavonoids, terpenes	Antioxidant, soothing	Protective creams
Ginseng root	LC–MS	Ginsenosides	Regenerative	Anti-age formulations
Olive by-products	LC–MS	Polyphenols	Antioxidant, anti-inflammatory	Skin barrier support
Tomato waste	LC–MS	Carotenoids, polyphenols	Antioxidant	Skin protection products
Pomegranate peel	LC–MS	Tannins	UV-protective, anti-ageing	Anti-wrinkle creams
Blackcurrant pomace	LC–MS	Polysaccharides	Antioxidant, antimicrobial	Cosmetic emulsifiers

7.6 Remarks

The valorization of plant waste through advanced metabolomic techniques represents a promising direction for innovation in the cosmetics sector. The application of liquid chromatography coupled with mass spectrometry (LC–MS) has shifted the perception of agro-industrial by-products from low-value materials to rich sources of bioactive compounds. Owing to its high sensitivity and resolving power, LC–MS has become an essential tool for the identification and quantification of metabolites in complex residual matrices.

The evidence discussed in this chapter confirms that plant waste constitutes a significant reservoir of polyphenols, terpenoids, carotenoids, alkaloids, and polysaccharides, associated with antioxidant, anti-inflammatory, moisturising, and anti-ageing activities. The integration of targeted and untargeted LC–MS approaches supports comprehensive metabolic profiling and the development of more effective, standardized, and sustainable cosmetic extracts.

The application examples analyzed - ranging from pruning residues to citrus and olive by-products - demonstrate that the conversion of plant waste into cosmetic ingredients is already an established industrial practice within a circular production model. However, challenges remain, including phytochemical variability, extraction standardization, safety assessment for topical use, and regulatory constraints. Despite these limitations, ongoing advances in LC–MS technologies, bioinformatics tools, and eco-friendly extraction processes indicate that these barriers can be progressively addressed.

Overall, LC–MS is not only an analytical technique but also a key enabling tool for sustainable cosmetic innovation. The upcycling of plant waste into high-value cosmetic ingredients represents a strategic opportunity linking scientific research, environmental sustainability, and human well-being.

CHAPTER 8 INTEGRATIVE SCIENTIFIC PRODUCTION

8.1 Summary of contributions to publications

During Ph.D. course (2022-2025), in addition to the experimental research activities described in the preceding chapters, I participated in a number of scientific publications as part of interdisciplinary collaborations. The works address topics related to the chemistry of secondary metabolites, the phytochemical characterization of natural matrices, the evaluation of biological activity, and environmental monitoring. These contributions serve to expand the range of methodological and applicative skills acquired during the three years.

The present chapter has been authored for the purpose of providing a concise overview of the main scientific contributions made by the author during the period 2022–2025. The objective of the chapter is to highlight the objectives of these contributions, the experimental approach adopted, and the most relevant results.

8.1.1 Influence of artificial light spectra on essential oil composition

"*Light spectra of biophilic LED-sourced system modify essential oils composition and plant morphology of Mentha piperita L. and Ocimum basilicum L.*"
P. Beatrice, G. Saviano, M. Reguzzoni, F. Divino, F. Fantasma, D. Chiatante and A. Montagnoli – *Frontiers in Plant Science* (2023) https://doi.org/10.3389/fpls.2023.1093883

The present study evaluated the effect of an innovative biophilic artificial lighting system (CoeLux®) on the growth, leaf morphology, and essential oil composition of *Mentha piperita* and *Ocimum basilicum*. A study was conducted to ascertain the effects of a biophilic light spectrum on the morphology of test subjects. The subjects were exposed to the light spectrum and then subjected to morphometric analysis and chemical characterization of their essential oils using GC-MS. The results of the study indicated that the light spectrum induced morphological changes attributable to shade avoidance phenomena. However, the qualitative composition of the essential oils was only marginally affected compared to the control group. The results suggest that such systems could be used in indoor environments, with potential applications in ornamental and nutraceutical fields.

8.1.2 Environmental monitoring of organic pollutants and heavy metals

"*The Presence of Ultra-Traces of Persistent Organic Pollutants (POPs) and Heavy Metals in Some Areas of Molise*"
I. Notardonato, F. Fantasma, P. Monaco, C. Di Fiore, G. Saviano, C. Giancola, P. Avino and V. De Felice – *Toxics* (2023) https://doi.org/10.3390/toxics11030250

This study investigated the presence of heavy metals and polycyclic aromatic hydrocarbons (PAHs) in areas of the Molise region considered to have low anthropogenic impact. Using standardized analytical protocols, the presence of contaminants at ultra-trace levels was emphasized, thus underlining the necessity of introducing an "environmental blank" as a reference in public health studies. The findings indicate that even regions deemed to be uncontaminated may in fact be subject to pervasive contamination, thus giving rise to substantial ramifications for the purposes of environmental and health risk assessment.

8.1.3 Phenolic content and antioxidant activity in wild edible plants

"*Trend of Antioxidant Activity and Total Phenolic Content in Wild Edible Plants as Part of the Environmental Quality Assessment of Some Areas in Central Italy*"
I. Notardonato, F. Fantasma, P. Monaco, C. Di Fiore, G. Saviano, C. Giancola, P. Avino and V. De Felice – *Separations* (2023) https://doi.org/10.3390/separations10020092

The study analyzed the total polyphenol content, tannins, and antioxidant activity of various edible wild plant species collected in different seasons in areas of central Italy. The results, obtained using standard spectrophotometric assays, demonstrated high seasonal variability and a negative correlation between the presence of heavy metals and the biosynthesis of phenolic metabolites. The present study contributes to our understanding of the role of secondary metabolites as indicators of environmental quality and the nutritional potential of the species analyzed.

8.1.4 Metabolomic and nutraceutical profile of *Phaseolus vulgaris* seeds

The contributions presented in this section concern the study of different Italian commercial varieties of *Phaseolus vulgaris*, addressed using complementary analytical strategies. The common objective of this study was to investigate the chemical characteristics of the seeds and their potential functional value, integrating global and targeted approaches. The adoption

of different methodologies has made it possible to obtain integrated information on the composition of metabolites and associated biological properties.

8.1.4.1

"*NMR Metabolomics and Chemometrics of Commercial Varieties of Phaseolus vulgaris L. Seeds from Italy*"
V. Samukha, F. Fantasma, G. D'Urso, C. Caprari, V. De Felice, G. Saviano, G. Lauro, A. Casapullo, M. G. Chini, G. Bifulco and M. Iorizzi – *Plants* (2024)
https://doi.org/10.3390/plants13020227

The study utilized nuclear magnetic resonance (NMR) spectroscopy in conjunction with multivariate chemometric analyses to compare different varieties of *Phaseolus vulgaris*. The subsequent data processing revealed distinctive metabolic patterns and quantitative variations between samples. These variations were attributable to specific classes of metabolites belonging to both primary and secondary metabolism.
The observed differences were then correlated with the results of *in vitro* biological assays, highlighting associations between chemical profile and antioxidant capacity, as well as the emergence of selective antifungal activity in one of the varieties analyzed. The approach adopted proved effective in terms of varietal discrimination and for identifying characteristics of nutraceutical interest.

8.1.4.2

"Chemical Profiling of Polar Lipids and the Polyphenolic Fraction of Commercial Italian Phaseolus Seeds by UHPLC-HRMS"
V. Samukha, F. Fantasma, G. D'Urso, E. Colarusso, A. Schettino, N. Marigliano, M. G. Chini, G. Saviano, V. De Felice, G. Lauro, F. Maione, G. Bifulco, A. Casapullo and M. Iorizzi – Biomolecules (2024) https://doi.org/10.3390/biom14101336

The present study concentrated on specific classes of bioactive compounds through the use of UHPLC-HRMS. The analysis enabled the detailed characterization of polar lipids and polyphenols present in the seeds, thus highlighting compositional differences between the varieties considered.
The chemical characterization was accompanied by the evaluation of targeted biological activities, with particular reference to the inhibition of the COX-2 enzyme and the modulation of the inflammatory response in a cellular model. The results obtained serve to

provide a more complete picture that emerges from the overall analysis, thereby reinforcing the functional interpretation of the compositional differences that were previously observed.

8.1.5 Anticancer activity of essential oils

"*Lavender Essential Oil and Its Terpenic Components Negatively Affect Tumor Properties in a Cell Model of Glioblastoma*"
M. Russo, N. Martella, D. Gargano, F. Fantasma, C. Marcovecchio, V. Russo, M. A. Oliva, M. Segatto, G. Saviano, S. Di Bartolomeo and A. Arcella – *Molecules* (2024) https://doi.org/10.3390/molecules29246044

The present study evaluated the biological activity of *Lavandula angustifolia* essential oil and its main terpene components in a glioblastoma cell model.
The essential oil, characterized by GC-MS, demonstrated the capacity to curtail cell proliferation, migration, and oxidative stress, with a pivotal function attributed to terpinen-4-ol. The results of the study indicate the potential utilization of natural compounds as adjuvants in anticancer therapeutic strategies.

8.1.6 Native microorganisms for bioremediation applications

"*Natural Hydrocarbon-Contaminated Springs as a Reservoir of Microorganisms Useful for Bioremediation*"
C. Cavone, P. Monaco, F. Fantasma, P. Rizzo, C. Tarracchini, S. Petraro, M. Ventura, C. Milani, F. Celico, G. Naclerio and A. Bucci – *Sustainability* (2025) https://doi.org/10.3390/su17073083

The study investigated naturally hydrocarbon-contaminated sources in Val d'Agri as potential reservoirs of hydrocarbon-degrading bacteria. The selection of bacterial strains capable of using hydrocarbons as their sole carbon source and improving the efficiency of bioremediation processes was achieved through the following methodologies: microbial isolation, genomic analysis, and mesocosm experiments.
The study emphasises the significance of contaminated environments as a biotechnological resource for sustainable environmental applications.

8.2 Remarks

The publications contained within this chapter constitute a selection from the wider research activities undertaken over the course of the three-year doctoral programme. These publications address a range of related topics, albeit from the perspective of different experimental contexts. The works address a range of aspects, including the phytochemical characterization of natural matrices and the evaluation of the biological activity of secondary metabolites. They also consider applications in the environmental and biotechnological fields, reflecting the variety of areas of collaboration activated during the research process. When considered as a whole, these contributions serve to integrate and complement the results presented in the preceding chapters, thereby providing a more comprehensive overview of the methodologies and approaches that have been applied. While not the central focus of the thesis, these studies contribute to outlining a coherent research path based on the use of advanced chemical-analytical and biological techniques and the critical analysis of experimental data.

CHAPTER 9 GENERAL REMARKS

9.1 Integration of chemical and biological results

The work carried out in this doctoral thesis contributes to understanding how plant-derived secondary metabolites can be selected, characterized, and interpreted in relation to their functional potential, highlighting the importance of an integrated approach that consistently links chemical characterization with biological activity.

A key general outcome concerns the role of extraction strategies and the distinction between volatile and non-volatile fractions. Volatile fractions, mainly obtained as essential oils and characterized by GC–MS, are predominantly composed of mono- and sesquiterpenes and are frequently associated with antimicrobial and antifungal activities [225]. In contrast, hydroalcoholic extracts, characterized using LC-based platforms, allow the investigation of polar and semi-polar metabolites such as phenolic acids, flavonoids, and proanthocyanidins, which are commonly related to antioxidant activity and protective effects observed in *in vitro* models [226].

Across the different plant species investigated, the observed biological activities could not be attributed to single predominant compounds, but rather to the overall balance and composition of the phytocomplex.

In studies involving phenolic-rich matrices, particularly those conducted on *Cannabis sativa* and *Vicia faba*, the integration of targeted quantitative analyses with high-resolution metabolomic profiling proved effective in clarifying the relationship between global chemical parameters and specific metabolite classes potentially responsible for the observed biological activities, supporting the formulation and interpretation of biologically plausible hypotheses [225,226].

The two review articles included in this thesis provide a broader interpretative framework for the experimental results. In particular, the review on wild edible plants highlights plant matrices as integrated biochemical systems, in which interactions among different classes of metabolites contribute to the modulation of biological pathways involved in the prevention of chronic-degenerative diseases [226].

At the same time, the review focused on the use of LC–MS techniques for the valorization of plant by-products extends the discussion toward circular economy models, emphasizing

the role of advanced analytical platforms in transforming agro-industrial residues into sources of high-value-added bioactive ingredients, particularly for cosmetic applications [227].

9.2 Positioning within the literature

Numerous studies have documented the antimicrobial and antifungal activities of essential oils from aromatic species belonging to the Lamiaceae family, including *Lavandula angustifolia*, *Origanum vulgare*, and *Laurus nobilis* [227,228], confirming the relevance of monoterpenes and oxygenated monoterpenoids as major contributors to these biological effects [229].

However, compared to literature focusing primarily on single marker compounds, this work reinforces the concept that biological effectiveness derives from synergistic interactions within the phytocomplex, whose relative composition is strongly influenced by geographical, environmental, and technological factors [7]. This aspect is particularly evident in the case of *Lavandula angustifolia*, where geographical variability is reflected in differentiated biological responses [229].

With regard to phenolic-rich matrices from *Laurus nobilis*, *Cannabis sativa*, and *Vicia faba*, the results are consistent with the growing literature supporting their nutraceutical potential [226]. At the same time, the combined use of global quantitative analyses and targeted and untargeted LC–MS-based metabolomic approaches allows a more comprehensive interpretation of the contribution of specific metabolite classes to biological activity, going beyond the sole quantification of total phenolic content [225].

An additional element of originality emerges from the integration of *in silico* approaches within a broader experimental framework, where computational analyses are used to support the interpretation of in vitro results rather than as isolated predictive tools [7].

9.3 Applicative implications

The results of this thesis highlight relevant applicative implications in the pharmaceutical, nutraceutical, cosmetic, agri-food, and bioprotection sectors. Phenolic-rich extracts emerge as promising sources of bioactive compounds potentially useful in the prevention of

oxidative stress and inflammation-related processes associated with chronic-degenerative diseases.

At the same time, the aromatic species investigated confirm their relevance as sources of essential oils with antimicrobial, antifungal, and antioxidant properties, representing potential natural alternatives to synthetic preservatives and biocides. The observed modulation of biological activity as a function of chemical composition underlines the importance of raw material selection and standardization to ensure quality and reproducibility of the final products.

A particularly relevant applicative aspect concerns the use of essential oils in bioprotection and material conservation. The antifungal efficacy observed on wooden substrates under conditions simulating real contexts suggests a possible use of plant-derived products as sustainable alternatives to conventional treatments, although further investigations on stability and compatibility are required.

In the cosmetic field, the valorization of plant by-products through advanced analytical techniques such as LC–MS opens new opportunities for the development of bioactive ingredients within a circular economy framework, contributing to waste reduction and sustainability.

9.4 Future research perspectives

The complexity of the plant matrices investigated and the variety of approaches adopted indicate that the potential of plant-derived secondary metabolites is still only partially explored. Future research should extend biological investigations toward more complex models, including in vivo studies, to validate the physiological relevance of the observed activities and to address issues related to bioavailability, metabolism, and safety.

From an analytical perspective, the integration of metabolomics with other omics disciplines could further clarify the biosynthetic mechanisms underlying chemical variability and support the optimization of plant matrices for specific applicative goals. Overall, the present thesis provides a methodological and conceptual framework that can be further expanded to explore new plant matrices and to support the sustainable valorization of plant-derived secondary metabolites.

CHAPTER 10 MATERIALS AND METHODS

10.1 Scope and rationale of the methodological approach

This chapter describes the materials and experimental methods underlying the experimental designs adopted in Chapters 1–5.

Methodological procedures are presented here in a unified manner to avoid repetition and to ensure coherence across the different case studies.

This thesis investigates plant-derived secondary metabolites through an integrated methodological approach combining chemical characterization and biological activity evaluation. Given the intrinsic complexity of plant extracts, a single analytical or experimental technique is rarely sufficient to fully describe their composition and functional properties.

For this reason, complementary extraction, analytical, and biological strategies were applied. Different extraction procedures were selected to obtain volatile fractions, such as essential oils, and non-volatile fractions enriched in phenolic compounds. Chemical characterization was performed using analytical techniques selected according to the physicochemical properties of the target metabolites. Whenever appropriate, chemical data were supported by *in vitro* assays to relate composition to functional activity, and, in selected cases, *in silico* analyses were applied to support the interpretation of experimental results.

Although the experimental studies presented in this thesis focus on different plant species and matrices, a consistent methodological framework was applied throughout.

The general methodological framework and detailed experimental procedures are therefore reported in this chapter, supporting the experimental designs described in the application chapters.

10.2 Plant Material

The plant material investigated in this thesis includes a selection of aromatic, medicinal, and edible species chosen for their traditional use, phytochemical relevance, and documented biological potential. The selected plants are known to be rich in secondary metabolites of interest, such as terpenoids and phenolic compounds, which are commonly associated with antioxidant, anti-inflammatory, antimicrobial, and other functional properties.

Plant samples were collected from different geographical areas of Italy or obtained from certified suppliers, depending on availability and experimental requirements. When harvested *in situ*, collection was carried out during the appropriate phenological stage, corresponding to the period of maximum accumulation of bioactive constituents.
Botanical identification was performed by qualified personnel, and voucher specimens were deposited at the Herbarium of the Department of Biosciences and Territory, University of Molise (Pesche, Italy), to ensure taxonomic accuracy and traceability.
After collection, the plant material was handled under controlled conditions in order to preserve its chemical integrity. Depending on the nature of the matrix and the subsequent experimental application, samples were air-dried under ventilated conditions or processed fresh, while avoiding direct exposure to light, excessive heat, and moisture. In selected cases, low-temperature storage was adopted prior to extraction to limit enzymatic activity and oxidative degradation.

The plant organs of interest, including flowering tops, leaves, seeds, or fruits, were selected according to the known distribution of secondary metabolites within each species. Dried samples were manually cleaned, ground when required, and stored in airtight containers under protected conditions until further processing. These standardized handling procedures were applied consistently across all experimental studies in order to ensure comparability among the different plant matrices analyzed throughout the thesis.

10.3 Sample preparation and extraction strategies

10.3.1 General principles of extraction

The extraction of secondary metabolites from plant matrices represents a critical step in natural products research, as it directly influences the chemical profile and functional properties of the resulting extracts. In this thesis, extraction strategies were selected according to the chemical nature of the target compounds and the objectives of each experimental study, with the aim of obtaining representative and reproducible phytochemical fractions.
A clear distinction was made between volatile and non-volatile metabolites. Volatile compounds, mainly terpenoids, were isolated through distillation-based techniques suitable for essential oil recovery, while non-volatile metabolites, particularly phenolic compounds, were obtained using solvent-based extraction methods. This separation reflects the different

physicochemical properties of the metabolites and allows a more focused characterization of each fraction.

Extraction conditions were chosen to minimize chemical degradation and to preserve the native composition of the plant material as much as possible. Solvent polarity, extraction time, and temperature were selected in order to balance extraction efficiency with compound stability. When different analytical or biological applications were planned, extraction procedures were adapted accordingly, while maintaining a consistent methodological rationale across studies.

The extraction strategies described in this section were applied throughout the experimental chapters of the thesis. Detailed protocols and specific operating conditions are reported in the corresponding chapters, where each method is described in relation to the plant species and analytical objectives involved.

10.3.2 Extraction of volatile fractions (essential oils)

Volatile compounds were extracted from selected plant species in the form of essential oils. These fractions are mainly composed of terpenoids and other low-molecular-weight compounds that are responsible for the characteristic aroma and many of the biological properties of aromatic plants.

Essential oils were obtained using hydrodistillation, a distillation-based technique widely accepted for the extraction of volatile metabolites from plant matrices. This approach allows the separation of volatile compounds from the aqueous phase without the use of organic solvents and is suitable for preserving the original chemical profile of essential oils.

Hydrodistillation was carried out using a Clevenger-type apparatus, following the procedures outlined in the European Pharmacopoeia.

The main extraction conditions are summarized below:

- Extraction method: hydrodistillation
- Apparatus: Clevenger-type system
- Plant material: 50 – 800 g of fresh or dried plant material
- Distillation principle: steam generated in the distillation chamber facilitates the release and transport of volatile compounds to the condenser
- Essential oil recovery: manual separation from the aqueous phase using a Pasteur pipette
- Drying agent: anhydrous sodium sulfate (Na_2SO_4)

- Storage conditions: amber glass vials at 4 °C

After extraction, essential oils were dried over anhydrous sodium sulfate to remove residual moisture and stored under refrigerated conditions until further analysis. A schematic representation of the steam distillation setup is shown in *Figure 10.1*.

The same extraction strategy was applied to all aromatic species investigated in this thesis, including *Lavandula angustifolia* L., *Origanum vulgare* L., and *Laurus nobilis* L., in order to allow comparison among samples.

Essential oil yield and chemical composition are discussed in detail in Chapters 1–3.



Figura 10.1 – Clevenger-type hydrodistillation apparatus

10.3.3 Extraction of non-volatile fractions

Non-volatile metabolites were extracted from plant matrices using solvent-based procedures designed to recover mainly phenolic compounds and other polar constituents. These compounds cannot be obtained by distillation and therefore require extraction methods based on solvent polarity and diffusion.

Hydroalcoholic mixtures were used as extraction solvents for all plant species investigated in this thesis (*Lavandula angustifolia* L., *Origanum vulgare* L., *Laurus nobilis* L., *Cannabis sativa* L., and *Vicia faba* L.). This approach allowed the recovery of a broad range of non-volatile bioactive metabolites and ensured methodological consistency across different species. The use of comparable solvent systems also facilitated the comparison of chemical and biological data among samples.

Solvent extraction was performed using a methanol/water mixture (80:20, v/v), which is widely employed for the isolation of polyphenolic compounds. Two extraction protocols

were applied depending on the experimental purpose: one optimized for phytochemical profiling by UHPLC–HRMS and one designed for antioxidant activity assessment using spectrophotometric assays.

The main extraction steps are summarized below:

- Extraction solvent: methanol/water (80:20, v/v)
- Matrix: dried and powdered plant material
- Extraction approach: solid–liquid extraction at room temperature
- Separation: centrifugation to remove solid residues
- Post-extraction processing: concentration under reduced pressure or freeze-drying, when required

For phytochemical characterization, extraction was carried out under magnetic stirring at room temperature, followed by centrifugation and concentration of the supernatant.

A schematic overview of the extraction workflow used for UHPLC–HRMS profiling is shown in *Figure 10.2*.

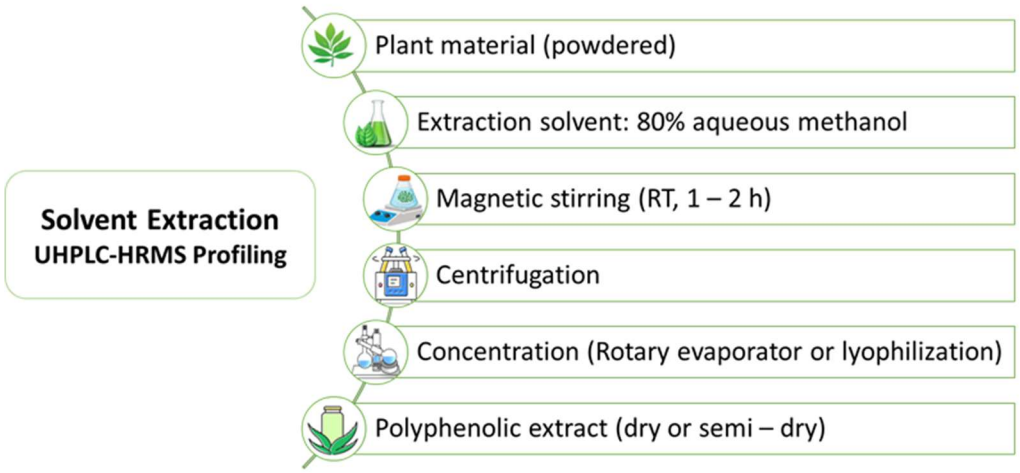


Figure 10.2 – Schematic overview of the solvent extraction workflow used for phytochemical profiling of non-volatile fractions by UHPLC–HRMS.

For antioxidant activity evaluation, extraction conditions were optimized to preserve phenolic integrity and ensure reproducibility of DPPH, ABTS, and FRAP assays.

This protocol involved sonication, short incubation in the dark, and centrifugation, followed by storage of the extracts under refrigerated conditions until analysis.

A schematic representation of the extraction workflow used for antioxidant assays is provided in *Figure 10.3*.

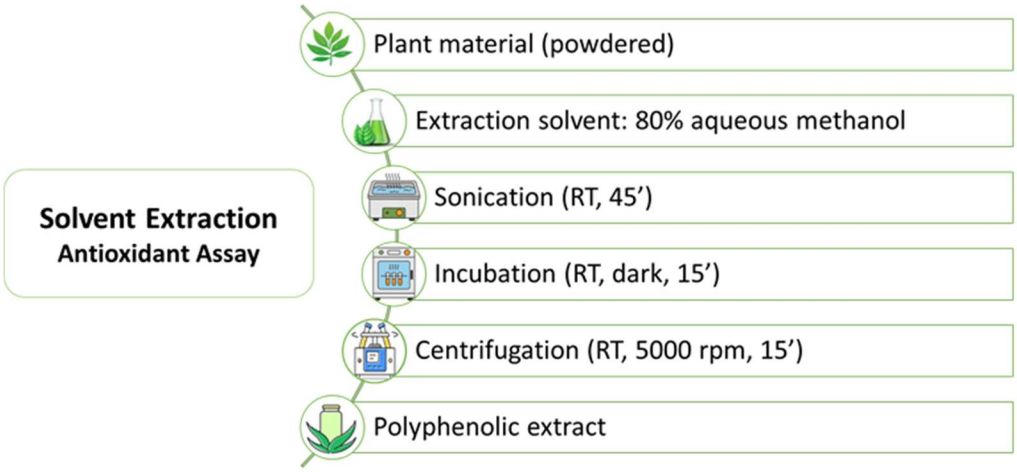


Figure 10.3 – Schematic overview of the solvent extraction workflow applied for the preparation of plant extracts used in antioxidant assays.

In addition to hydroalcoholic extraction, in one specific case (*Cannabis sativa* L., Chapter 4), an aqueous infusion was prepared to simulate a traditional model of use.

This extract was further subjected to liquid–liquid partitioning according to a modified Kupchan scheme, in order to obtain fractions enriched in compounds with different polarity. Briefly, the dried extract was suspended in water and successively partitioned with organic solvents of increasing polarity, allowing the separation of chemically distinct fractions.

This approach facilitated phytochemical profiling and biological evaluation of fractions with reduced chemical complexity.

A schematic representation of the applied partitioning procedure is reported in *Figure 10.4*.

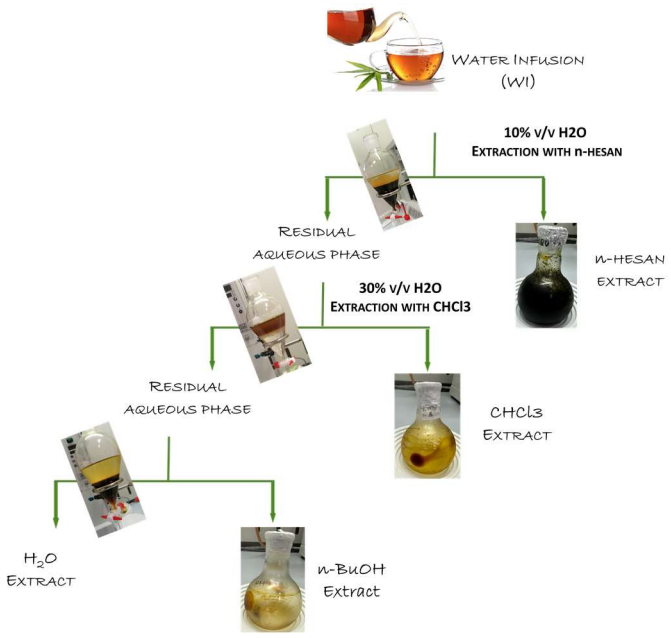


Figure 10.4 - Kupchan partitioning scheme applied to the aqueous extract.

Detailed extraction conditions and processing steps are reported in the relevant experimental chapters (Chapters 4 and 5), while the present section summarizes the general principles adopted for the extraction of non-volatile fractions throughout the thesis.

10.4 Chemical characterization techniques

Chemical characterization of plant extracts was carried out using complementary analytical techniques selected according to the nature of the target compounds. Volatile and non-volatile fractions were analyzed using different platforms in order to obtain reliable and informative chemical profiles.

Analyses were performed at the laboratories of the Department of Biosciences and Territory (University of Molise, Pesche, Italy) and the Department of Pharmacy (University of Salerno, Salerno, Italy), using high-resolution instrumentation and validated protocols commonly reported in the scientific literature. Detailed instrumental parameters and operating conditions are noted in the corresponding experimental chapters.

The chemical characterization of plant-derived extracts followed a stepwise analytical workflow, as illustrated in *Figure 10.5*, involving the sequential use of chromatographic and spectrometric techniques selected according to the nature of the target compounds.

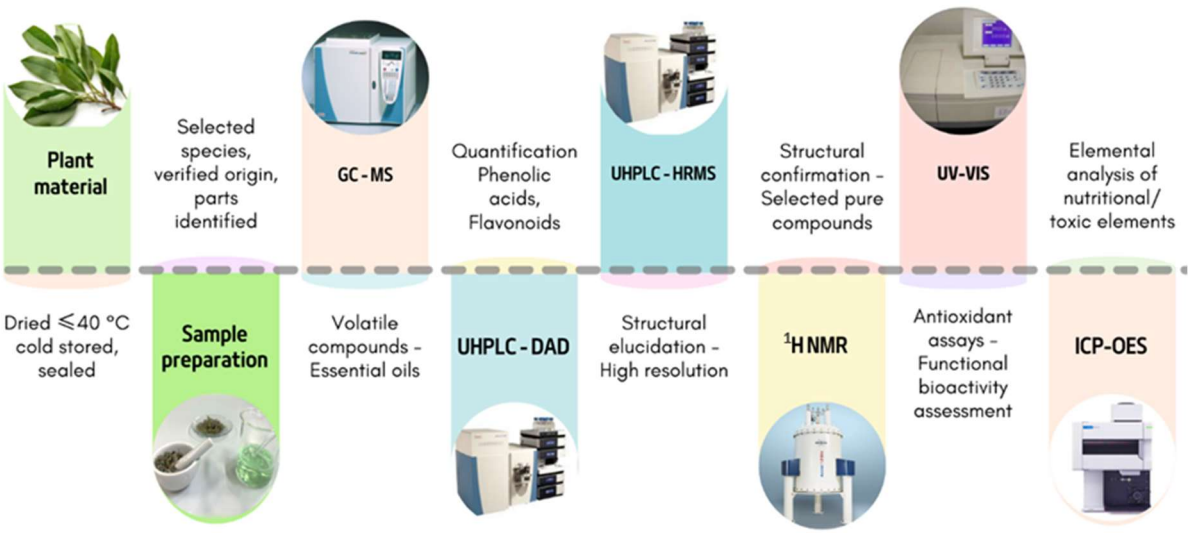


Figure 10.5 - Overview of the analytical workflow employed for the characterization of secondary metabolites in plant extracts

10.4.1 GC–MS analysis of essential oils

Gas chromatography coupled with mass spectrometry (GC–MS) was used for the analysis of essential oils obtained from aromatic plant species in order to determine the composition of volatile compounds. This technique is widely recognized as a reference method for the separation and identification of monoterpenes, sesquiterpenes, and their oxygenated derivatives.

The main GC–MS operating conditions are summarized below:

- Instrumentation: Trace GC Ultra GC coupled with a Polaris Q ion trap MS (Thermo Fisher Scientific, Waltham, MA, USA)
- Column: Rtx-5 Restek capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness)
- Carrier gas: Helium, 1.0 mL/min
- Injector temperature: 250 °C
- Oven temperature program: initial isotherm at 40 °C for 5 min, followed by a temperature increase of 3 °C/min up to 250 °C, with a final isothermal hold of 10 min
- Ionization mode: electron impact (EI), 70 eV
- Ion source temperature: 250 °C
- Mass range: 40–400 m/z
- Software: Xcalibur (Thermo Fisher Scientific)

Compound identification was carried out by comparing mass spectra with those reported in NIST, Wiley, and Adams libraries [48,230,231] and by calculating linear retention indices (RI) relative to a homologous series of *n*-alkanes (C8-C20) analyzed under the same chromatographic conditions. Experimental RI values were further compared with literature data to support compound assignment.

GC–MS analysis was applied to essential oils extracted from *Lavandula angustifolia* L., *Origanum vulgare* L., and *Laurus nobilis* L., allowing qualitative characterization of volatile profiles and comparison among samples obtained from different species or geographical origins. The chemical composition of essential oils and its relationship with biological activity are discussed in detail in Chapters 1–3.

10.4.2 LC-based techniques for phenolic profiling

Liquid chromatography–based techniques were used for the analysis of non-volatile metabolites, mainly phenolic compounds and other polar constituents. These methods are suitable for compounds that cannot be analyzed by gas chromatography due to their low volatility or thermal instability.

Ultra-high-performance liquid chromatography coupled with diode array detection (UHPLC–DAD) and high-resolution mass spectrometry (UHPLC–HRMS) were employed, depending on the analytical objectives of each study.

UHPLC–DAD was applied for the targeted separation and quantification of major phenolic acids and flavonoids, whereas UHPLC–HRMS was employed for untargeted profiling and structural annotation of complex metabolite mixtures.

UHPLC–DAD analysis of phenolic compounds

Targeted phenolic analysis was carried out using a UHPLC system equipped with a diode array detector. Chromatographic separation was achieved on a reversed-phase C18 column using a binary mobile phase consisting of water acidified with formic acid and acetonitrile. Detection was performed at multiple wavelengths in order to monitor different classes of phenolic compounds.

The main UHPLC–DAD operating conditions are summarized below:

- Instrument: Knauer UHPLC system equipped with diode array detector
- Column: C18 reversed-phase column (Phenomenex Synergi Hydro-RP or equivalent)
- Mobile phase:
    - A: water with 0.1% formic acid
    - B: acetonitrile
- Flow rate: 1.0 mL/min
- Detection wavelengths: 260, 280, 320, and 360 nm
- Software: ChemStation / Chromeleon–based data processing

Phenolic compounds were identified by comparison of retention times and UV–Vis spectra with those of authentic reference standards, including caffeic acid, rosmarinic acid, and quercetin. Quantification was performed using external calibration curves.

UHPLC–HRMS analysis

For a deeper metabolomic characterization, UHPLC was coupled with high-resolution mass spectrometry. This approach enabled untargeted profiling and the acquisition of accurate mass data for structural annotation of phenolic and related metabolites.

UHPLC–HRMS analyses were performed using a reversed-phase C18 column and a binary mobile phase composed of water and acetonitrile, both acidified with formic acid. Mass spectrometric detection was carried out using an Orbitrap-based system operating in both positive and negative electrospray ionization modes. Data were acquired in full-scan mode with data-dependent MS/MS fragmentation to obtain structural information on the most intense ions.

The main UHPLC–HRMS operating conditions are summarized below:

- LC–MS system: Ultimate 3000 UHPLC coupled to an Orbitrap Q-Exactive mass spectrometer
- Column: C18 reversed-phase column (Luna Omega or equivalent)
- Ionization mode: electrospray ionization (ESI), positive and negative
- Acquisition mode: full-scan and data-dependent MS/MS
- Software: Compound Discoverer (Thermo Fisher Scientific)

LC-based techniques were applied to different plant species included in this thesis, including *Origanum vulgare* L., *Cannabis sativa* L., and *Vicia faba* L.. In particular, mass spectrometry–based approaches were used for *Cannabis sativa* L. and *Vicia faba* L., where a deeper metabolomic characterization was required.

10.4.3 NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy was used as a complementary technique to support the chemical characterization of selected plant extracts. Compared to chromatographic methods, NMR provides direct information on the structural features of metabolites and allows a global view of the chemical composition of complex mixtures.

In this thesis, proton NMR (^{1}H NMR) spectroscopy was applied in selected cases where additional structural confirmation or an overview of the metabolomic profile was required. In particular, NMR analysis was performed on extracts of *Cannabis sativa* to support the identification of major non-volatile constituents and to complement the information obtained by LC–MS analysis.

^{1}H NMR analyses were carried out using a high-field spectrometer operating at 600 MHz. Samples were dissolved in a deuterated solvent system and analyzed under standardized acquisition conditions. The main NMR operating parameters are summarized below:

- Instrument: Bruker DRX-600 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany)
- Operating frequency: 600 MHz (^{1}H)
- Spectral width: 15.00 ppm
- FID size: 64 K
- Number of scans: 512
- Dummy scans: 4

NMR spectra were evaluated to assess signal distribution and key structural features of major metabolites, providing complementary information to chromatographic analyses. When appropriate, NMR data were further processed using multivariate analysis tools to support metabolomic interpretation.

10.4.4 Elemental analysis – ICP-OES

Elemental analysis was performed to determine the mineral composition of selected plant matrices. The evaluation of macro- and microelements provides complementary information to the phytochemical profile and is relevant for both nutritional and safety considerations.

To determine trace and essential element content, plant samples were subjected to microwave-assisted acid digestion using a pressure-controlled system (ETHOS EASY, Milestone, Sorisole, Italy). This step allowed complete mineralization of the organic matrix prior to instrumental analysis.

Elemental quantification was carried out using inductively coupled plasma optical emission spectrometry (ICP–OES) with a 5800 ICP–OES system (Agilent Technologies, Santa Clara, CA, USA). This technique enables the simultaneous determination of multiple elements with good sensitivity, accuracy, and reproducibility, and is widely applied in food and plant matrix analysis.

Quantification was performed using external calibration with multi-element standard solutions. The elements analyzed included macro- and microelements of nutritional relevance, as well as selected elements of potential toxicological interest. The overall analytical workflow adopted for metal determination is summarized in *Figure 10.6*.

Elemental analysis was applied to *Cannabis sativa* and *Vicia faba*, for which mineral composition is particularly relevant to the interpretation of nutraceutical potential and biological effects.

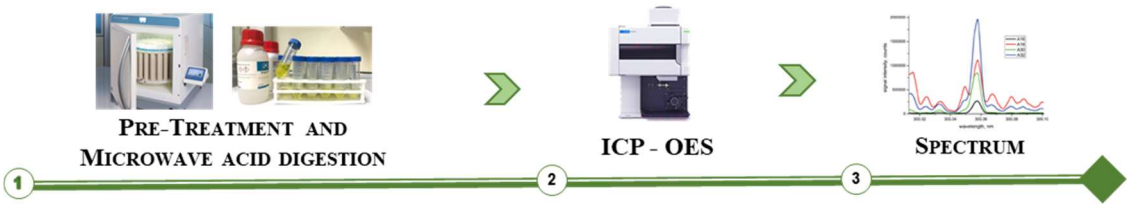


Figure 10.6 - Analytical workflow adopted for elemental determination of plant samples by microwave-assisted acid digestion followed by ICP–OES analysis.

10.5 Quantification of bioactive compounds

The quantification of selected classes of bioactive compounds was carried out to support the chemical characterization of plant extracts and to enable comparative evaluation among different samples. In particular, attention was focused on phenolic compounds, which are widely recognized for their contribution to antioxidant and other biologically relevant activities.

Total phenolic content (TPC), total flavonoid content (TFC), and proanthocyanidins (PAs) were determined using established colorimetric assays. These methods provide an overall estimation of major phenolic classes and are commonly used in studies dealing with plant-derived extracts.

Results were expressed as standard equivalents, allowing comparison with data reported in the literature.

Detailed assay conditions, calibration procedures, and data processing are described in the corresponding experimental chapters, where quantitative results are discussed in relation to antioxidant activity and associated biological effects.

10.6 Assessment of antioxidant activity

The antioxidant activity of plant extracts was evaluated using *in vitro* spectrophotometric assays commonly applied in natural products research.

These methods allow the assessment of the ability of plant-derived compounds to neutralize free radicals or to reduce oxidized species, indicating the overall antioxidant potential of the extracts.

Antioxidant assays are based on different chemical reaction mechanisms and may provide complementary information on the antioxidant behaviour of complex plant extracts. Radical scavenging assays, such as DPPH and ABTS, evaluate the ability of compounds to quench stable free radicals through electron or hydrogen atom transfer reactions, whereas reducing power assays, such as FRAP, assess the capacity of antioxidants to reduce oxidized metal ions. As each method is characterized by specific advantages and limitations, the combined application of multiple assays allows a more comprehensive and reliable assessment of antioxidant potential.

To better illustrate the methodological distinctions between these assays, their core principles and analytical parameters are summarized in *Table 10.1*.

Table 10.1 Comparison of antioxidant assays used in this thesis.

Parameter	**DPPH**	**ABTS**	**FRAP**
Reactive species	DPPH• (stable radical)	$ABTS^{\bullet+}$ (radical cation)	Fe^{3+} (ferric ion)
Reaction mechanism	Radical scavenging	Radical scavenging	Reducing power
Principle	Reduction of DPPH• (violet) to DPPH-H (pale yellow) by	Reduction of $ABTS^{\bullet+}$ (blue-green) to $ABTS^{-}$ (colorless) by antioxidants	Reduction of Fe^{3+}–TPTZ complex to Fe^{2+}–TPTZ
Detection (nm)	517	734	593

DPPH: 2,2-Diphenyl-1-picrylhydrazyl;
ABTS: 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid;
FRAP: Ferric Reducing Antioxidant Power.

Results were expressed using appropriate reference parameters, such as inhibition percentages, IC_{50} values, or Trolox equivalents, depending on the assay and the experimental design.

10.7 *In vitro* biological assays

In vitro biological assays were used to evaluate the functional effects of plant extracts under controlled experimental conditions. These assays allow the investigation of cellular responses to bioactive compounds and provide useful information on potential protective, antioxidant, or modulatory effects.

Cell-based models were applied primarily to non-volatile plant extracts, which were selected based on their chemical composition and relevance to the biological questions addressed in each study. The experimental design included the evaluation of cell viability, oxidative stress, and selected molecular markers associated with cellular response mechanisms. This approach allowed the integration of chemical data with functional outcomes at the cellular level.

Plant extracts were tested at different concentrations in order to evaluate dose-dependent effects and to distinguish between protective and potentially cytotoxic responses. Appropriate controls were included in all experiments to ensure the reliability of the results. The general principles of the *in vitro* assays are outlined in this section, while detailed experimental protocols, cell culture conditions, and analytical procedures are reported in the corresponding experimental chapters.

10.8 *In silico* approaches

In silico approaches were used to support the interpretation of experimental results and to provide additional insight into the potential biological behavior of selected bioactive compounds. Computational tools represent a useful complement to *in vitro* studies, as they allow the exploration of molecular interactions and pharmacokinetic properties in a cost-effective and time-efficient manner.

Molecular docking simulations were performed to investigate the possible interaction between selected plant-derived compounds and biological targets relevant to the observed *in vitro* effects. This approach allowed the evaluation of binding modes and relative interaction strength, supporting hypotheses on potential mechanisms of action.

In addition, *in silico* prediction tools were applied to estimate key pharmacokinetic and safety-related parameters, including absorption, distribution, metabolism, and excretion (ADME) properties. These analyses provided complementary information on the drug-like behavior of selected compounds and helped to contextualize the experimental findings.

The *in silico* analyses presented in this thesis were not intended to replace experimental validation, but rather to support and strengthen the interpretation of chemical and biological data. Detailed computational methods and parameters are described in the corresponding experimental chapters, where *in silico* results are discussed together with *in vitro* findings.

10.9 Statistical analysis

Statistical analysis was performed to support the interpretation of experimental data and to evaluate the significance of the observed results. Quantitative data were analyzed using appropriate statistical methods selected according to the experimental design and data distribution.

Results are generally expressed as mean values with the corresponding measures of variability, such as standard deviation or standard error, as specified in the individual experimental chapters. Statistical comparisons were carried out to identify significant differences between groups or treatments.

The level of statistical significance was set according to commonly accepted criteria. All statistical analyses were performed using validated software tools. Detailed information on the specific tests applied and the number of replicates is provided in the corresponding experimental chapters.

10.10 Integrated methodological workflow

The methodological approach adopted in this thesis is based on a structured workflow that integrates sample preparation, chemical characterization, and biological evaluation. This integrated design allowed the progressive selection and investigation of plant extracts, from initial extraction to functional assessment.

The workflow begins with the collection and preparation of plant material, followed by the extraction of volatile and non-volatile fractions using complementary strategies. Chemical characterization is then performed using appropriate analytical techniques, selected according to the physicochemical properties of the target compounds. Quantitative assays and antioxidant tests were applied to provide additional information on the functional potential of the extracts.

Biological evaluation is carried out through *in vitro* assays, which allow the assessment of cellular responses under controlled conditions. When relevant, *in silico* analyses are used to support the interpretation of experimental data and to explore possible molecular interactions

and pharmacokinetic properties. Statistical analysis is applied throughout the workflow to ensure the robustness and reliability of the results.

This integrated methodological framework ensures coherence among the different experimental phases and supports a comprehensive interpretation of the data generated in the various chapters of the thesis.

A schematic representation of the methodological workflow and a summary table (*Table 10.2*) are provided to illustrate the sequence of the experimental steps.

Table 10.2 Overview of the analytical and experimental techniques applied in this thesis, indicating the corresponding chapters of application and the investigated plant matrices.

Technique	Chapters of application	Investigated matrices
Hydrodistillation	1 – 3	*Lavandula* L., *Origanum* L., *Laurus* L.
GC–MS	1 – 3	Essential oils
Hydroalcoholic	4 – 5	*Cannabis* L., *Vicia faba* L.
LC–MS	4 – 5	Phenolic extracts
^{1}H NMR	4	*Cannabis* L.
Antioxidant assays	1 – 5	Plant extracts
Enzymatic activity	3, 5	*Laurus* L., *Vicia faba* L.
Molecular docking	3, 5	*Laurus* L., *Vicia faba* L.

APPENDIX

Supplementary materials

The supplementary materials associated with the experimental studies included in this Ph.D. thesis are not reproduced in full within this document. Instead, they are available online as Supplementary Materials accompanying the corresponding peer-reviewed publications.

This approach was adopted to ensure clarity and conciseness of the thesis, while maintaining access to extensive datasets, figures, and tables that are available through the official journal platforms. The supplementary materials include detailed experimental data, extended tables, additional figures, and complementary analyses supporting the results discussed in the main chapters of this thesis.

For clarity, the links to the Supplementary Materials are reported below for each corresponding chapter and publication.

Chapter 1 – *Lavandula angustifolia* L.
Supplementary Materials available at:
https://www.mdpi.com/article/10.3390/molecules28010392/s1

Chapter 2 – *Laurus nobilis* L.
Supplementary Materials available at:
https://www.mdpi.com/article/10.3390/foods13142282/s1

Chapter 3 – *Cannabis sativa* L.
Supplementary Materials available at:
https://www.mdpi.com/article/10.3390/plants14101473/s1

Chapter 4 – *Origanum vulgare* L.
Supplementary Materials available at:
https://www.mdpi.com/article/10.3390/plants14162468/s1

Chapter 5 – *Vicia Faba* L.
Supplementary Materials available at:
https://www.mdpi.com/article/10.3390/molecules31010184/s1

Certificate of research activity abroad

This appendix includes the certificate attesting the research activity carried out abroad during the Ph.D. program at the University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca (Romania).



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STATEMENT OF STAY

The hosting facility, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca headquartered in Calea Mănăştur, 3-5, Cluj-Napoca, Romania, hereby declares that PhD Student Francesca Fantasma, born on 04-01-1973, was guested at our premises from 24 June 2024 to 25 July 2024.

During this period, she was involved in research activities regarding the chemistry of natural substances, deepening the basic knowledge and methodologies relating to chromatography and quality control of honey. In particular, she carried out determinations relating to the quality and authenticity of the honey.

Cluj-Napoca, 25 July 2024

If any questions do not hesitate to contact me at:
Email: ddezmirean@usamvcluj.ro
tel.: 0040.726.383.991

Sincerely,

Professor Daniel S. Dezmirean, PhD

Dean of Faculty of Animal Husbandry and Biotechnology,
Head of APHIS-DIA Laboratory

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