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PhD Thesis in

A multidisciplinary approach for the study of Origanum *genus*

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Abstract

This study investigates the phytochemical profile and biological potential of selected species of the genus *Origanum*, with a focus on *O. dictamnus*, *O. majorana*, *O. vulgare*, and *O. heracleoticum*. The work was structured into three main objectives. First, the volatile and non-volatile fractions of *O. dictamnus* were evaluated. The essential oil was characterised through GC–MS and evaluated for its inhibitory effects on key enzymes involved in neurodegenerative and metabolic disorders. Kinetic analyses were performed to clarify the mode of inhibition and by *in silico* analysis, molecular docking supported the *in vitro* results by clarifying the interactions of the main constituents, in particular carvacrol and *p*-cymene, with the active sites of the enzyme. For the non-volatile fraction, three extraction methods (maceration with 70% ethanol, maceration with 20% ethanol, and pressurized liquid extraction (PLE) with 20% ethanol) were compared in terms of extraction efficiency and biological activity (antioxidant and enzymatic assays). The extract obtained via PLE, which showed the best overall performance, was further analyzed by LC-HRESIMS/MS to characterize its metabolic profile. The second objective consisted of an interspecific comparison of essential oils from *O. majorana*, *O. vulgare*, and *O. heracleoticum*. Their chemical composition was determined through GC–MS, and their antioxidant properties and enzyme inhibitory activities were assessed on targets related to neurodegenerative and metabolic diseases. Enzyme kinetic studies were performed to investigate potential differences in their mechanisms of action. The third objective focused on an intraspecific comparison of *O. heracleoticum* collected from different Italian regions. Eight samples were analysed for the volatile fraction, allowing the identification of chemical variability through GC–MS and multivariate statistical analysis. Selected samples were tested for activities related to the central nervous system and metabolic disorders. For the non-volatile fraction, six samples were extracted using the optimized PLE method and evaluated for their chemical features and biological properties, particularly regarding enzymes involved in metabolic dysfunction. Overall, the findings demonstrate chemical diversity among *Origanum* species and highlight their potential as sources of natural inhibitors targeting enzymes implicated in neurodegenerative and metabolic disorders. The study supports the relevance of *Origanum* spp. as promising candidates for nutraceutical and phytotherapeutic applications.

1. INTRODUCTION

1.1 *Origanum* genus

The name *Origanum* derives from the Greek words "oros" (meaning mountain) and "ganos" (meaning joy), referring to the "joy of the mountain," which reflects the natural habitat of these plants (Fonnegra & Jiménez, 2007). The *Origanum* genus, belonging to the Lamiaceae family, includes a group of aromatic and medicinal plants characterized by the distinctive scent and flavor of their leaves and flowers (Bakha et al., 2019; Cid-Pérez, et al., 2016; Kosakowska et al., 2019). Based on various morphological characteristics, such as stem length, number of sessile glands on the leaves, arrangement of whorls, stem and leaf indument, and branching pattern, the genus *Origanum* was divided into three groups, ten sections, thirty-eight species, six subspecies, and seventeen hybrids (Ietswaart & Ietswaart, 1980). The distribution of *Origanum* species follows the general phytogeographical pattern of the Aegean region and Turkey (Arabaci et al., 2020; Kontopanou & Panitsa, 2020). In the Mediterranean region, and particularly across Italy, Greece, and Crete, the most widely distributed *Origanum* species include *O. vulgare* L., *O. majorana* L., *O. dictamnus* L., and *O. heracleoticum* L. Since ancient times, plants of the genus *Origanum* played an important role in the daily lives of many cultures. In fact, they were especially popular in southern Europe, mainly in Greece, Italy, Spain, and France, and later in the Americas, particularly in Mexico. Oregano is a staple of Mediterranean cuisine and is used to flavor pizza, salads, sauces, soups, meats, cheeses, and preserved foods. In Germany, it is commonly called "sausage herb," while in Spain it is often added to egg and vegetable dishes. The leaves are also used to flavor baked goods, beverages, wines, vermouth, and beers, contributing both aroma and preservative properties (Salvo et. al, 2019). Furthermore, the flowers can produce a red-orange dye

for wool, while the leaves can produce a black dye for cotton when combined with iron sulfate.

Since ancient times, *Origanum* species attracts great interest for their medicinal applications. Indeed, *Origanum* species exhibit a wide range of pharmacological and biological properties, including antioxidant, antimicrobial, antitumor, antidiabetic, antinociceptive, insecticidal, hepatoprotective, cytotoxic, and anti-lipase effects (Teixeira et al., 2013). The *Origanum* essential oils (EOs) exhibit anthelmintic, herbicidal, insecticidal, larvicidal, anti-inflammatory, and antioxidant properties (Giatropoulos et al., 2018; López et al., 2018). The volatile fraction is predominantly rich in terpenes, mainly carvacrol and thymol (figure 1), followed by γ-terpinene, α-terpinene, and *p*-cymene (Orhan et al., 2011; Marrelli et al., 2018).

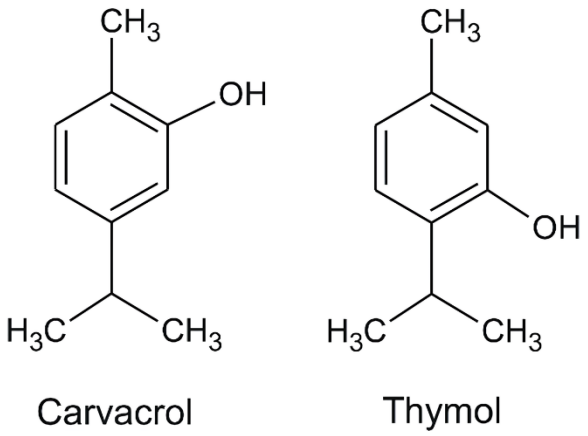


Figure 1. *Chemical structure of main compound of Origanum EOs*

The non-volatile fraction is mainly composed of phenolic acids (figure 2), such as rosmarinic acid and caffeic acid (Hadjadj & Hazzit, 2020; Mahomoodally et al., 2019), and flavonoids such as apigenin and luteolin (Cavero et al., 2006; Hadjadj & Hazzit, 2020).

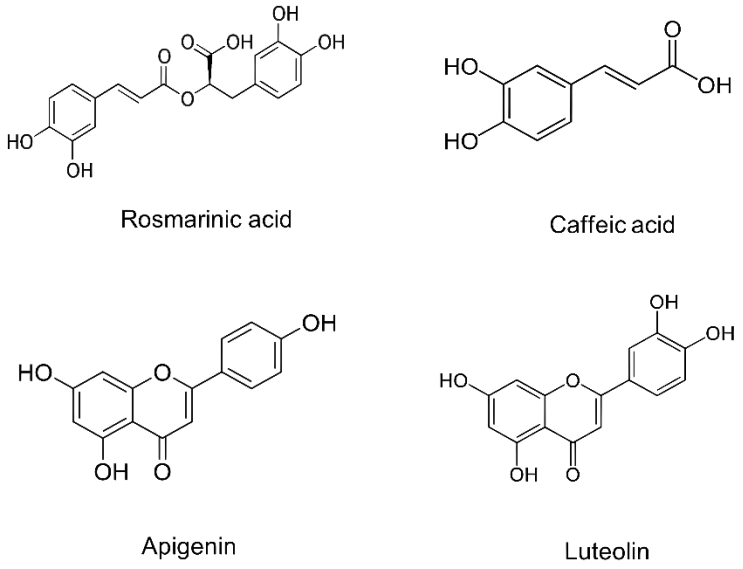


Figure 2. *Chemical structure of main compound of Origanum extract*

1.1.1 Origanum vulgare L.

Origanum vulgare L. commonly known as oregano, is a perennial, aromatic, herbaceous plant native to the Mediterranean region and western Eurasia (Pignatti, 1982). It reaches a height of 20–80 cm and has a bushy habit with a woody base and herbaceous stems. The opposite oval-lanceolate leaves have entire or slightly toothed margins, with a variable apex ranging from rounded to pointed. The small flowers (figure 3) are gathered in terminal and lateral paniculate inflorescences, featuring a white or purple corolla composed of five fused petals and a fused calyx. The androecium comprises four stamens and the gynoecium is formed by two united carpels (Şahin et al., 2004).

Figure 3. *Origanum vulgare* L.

From an ethnobotanical perspective, oregano is renowned and valued worldwide for its aromatic and medicinal properties. The earliest evidence of its use dates to the 7th century BC, when it was used to flavour fish, meat, vegetables and wine (Pignatti, 1982). In addition to its culinary uses, the plant was traditionally employed in traditional medicine to treat respiratory, digestive, rheumatic, menstrual and urinary disorders (Ličina et al., 2013; Rat et al., 2016; Bennaoum et al., 2017). In Iran, for instance, *O. vulgare* was employed as a tonic, expectorant, carminative, stimulant and antibacterial agent (Bahmani et al., 2018). Traditional forms of administration include infusions, decoctions, and tinctures, which are used to relieve colds, coughs, gastric pain, and respiratory disorders (Pieroni et al., 2002). The aerial parts of the plant represent the primary source of bioactive metabolites, including phenolic glycosides, flavonoids, tannins, sterols, and terpenoids. The presence of these compounds varies depending on the chemotype, geographical area, and harvest period (Pezzani et al., 2017; Martins et al., 2014). The EO of *O. vulgare* is the most extensively studied fraction due to

its composition variability and biological importance. EO, mainly extracted by steam distillation of the flowering tops, is a rich source of monoterpenes (including thymol, γ-terpinene, carvacrol, and *p*-cymene), acyclic monoterpenes (e.g. geraniol and linalool), and sesquiterpenes (e.g. β-caryophyllene, spathulenol, and germacrene-D) (Leyva-López et al., 2017). Variations in the qualitative and quantitative compositions of the volatile fraction can be attributed to genetic and ecological factors (D'Antuono et al., 2000). The non-volatile fraction of *O. vulgare* is primarily constituted by phenolic acids, including rosmarinic, caffeic, vanillic, o-coumaric, and protocatechuic acids, as well as flavonoids, such as luteolin-7-*O*-glucoside and luteolin-*O*-glucuronide (Skendi et al., 2017). These compounds significantly contribute to the plant antioxidant and anti-inflammatory activities. Several studies demonstrated the free radical scavenging capacity of its extracts, although the activity of the EO is generally lower than that of methanolic or ethanolic extracts (Moshayedi et al., 2013; Tusevski et al., 2014). Methanolic extracts showed to possess high reducing capacity and strong antioxidant activity *in vitro* assays (Tusevski et al., 2014; Skendi et al., 2017). In addition to its antioxidant activity, *O. vulgare* demonstrated antimicrobial and anti-inflammatory properties. The EO exhibited efficacy against Gram-positive and Gram-negative bacteria, thanks to its hydrophobic nature, which facilitates its penetration into bacterial cell membrane, causing the loss of cytoplasmic components and cell death (Teixeira et al., 2013). Both *in vitro* and *in vivo* studies also highlighted notable anti-inflammatory activity, with inhibition of nitric oxide (NO) and Tumor Necrosis Factor-α (TNF-α) production in cellular models of microglia and macrophages (Gunawardena et al., 2014), and reduction of the release of proinflammatory cytokines (Ocana-Fuentes et al., 2010).

1.1.2 Origanum majorana L.

Origanum majorana L., commonly known as sweet marjoram or marjoram, is a perennial herbaceous species. It is native to the eastern Mediterranean and widespread in Europe, western Asia, and North Africa (Prerna &

Vasudeva, 2015). This semi-shrub grows to a height of 30–60 cm, featuring erect, branched stems, and opposite, elliptical leaves (figure 4), that are finely downy on both surfaces. Small white or purple flowers are arranged in terminal spikelets surrounded by overlapping bracts (Ietswaart, 1980). The glandular trichomes, abundant on the leaves and inflorescences, are the main sites of EO biosynthesis, responsible for the characteristic aroma and pharmacological properties of the species (Vagi et al., 2004; Erenler et al., 2016).

Figure 4. *Origanum majorana* L.

Marjoram has been used, since ancient times, both as an aromatic plant and as a medicinal remedy. In Mediterranean and Middle Eastern folk medicine, it is used to relieve respiratory, digestive, and nervous disorders, such as coughs, asthma, indigestion, insomnia, and anxiety, as well as to treat muscle, arthritic, and menstrual pain (Benali et al., 2017; Cano & Volpato, 2004). Traditionally, infusions and decoctions of the leaves or flowering tops are used as a tonic, carminative, antispasmodic, sedative, and antiseptic (Zougagh et al., 2019; Bina & Rahimi, 2017).

From a phytochemical point of view, *O. majorana* presents a complex profile of secondary metabolites with high biological potential. The EO, obtained mainly by steam distillation of the aerial parts, is dominated by oxygenated monoterpenes such as terpinen-4-ol, *cis*-sabinene hydrate, α-terpineol, and γ-terpinene, followed by *p*-cymene, carvacrol, and thymol, whose concentrations vary depending on geographic origin, phenology, and environmental conditions (Jelali et al., 2011; Hussain et al., 2011). In addition to the volatile fraction, numerous phenolic and flavonoid compounds have been identified in the polar extracts, including rosmarinic, caffeic, ferulic and *p*-coumaric acids, luteolin, apigenin, quercetin, rutin, catechin; moreover, also triterpenes such as ursolic and oleanolic acids (Dhull et al., 2016; Taamalli et al., 2015) have been detected.

The biological activities of *O. majorana* are well documented. The EO exhibits antimicrobial activity against Gram-positive and Gram-negative bacteria (including *Staphylococcus aureus, Escherichia coli, Salmonella typhimurium*, and *Listeria monocytogenes*) and phytopathogenic *(Aspergillus niger*), mainly attributed to the disruptive action of carvacrol and terpinen-4-ol on the microbial cell membrane (Aligiannis et al., 2001; Ouedrhiri et al., 2016; Ezzeddine et al., 2001). The ethanolic and methanolic extracts exhibit good antioxidant activity, assessed by DPPH, FRAP, and ABTS assays, due to their high content of polyphenols and flavonoids (García-Risco et al., 2017; Arranz et al., 2019). These compounds can neutralize reactive oxygen species (ROS) and enhancing endogenous antioxidant defences, suggesting a protective role against chronic degenerative diseases (Idris & Mohd Nadzir, 2021; Valadez-Vega et al., 2013). Recent studies have also highlighted anti-inflammatory and analgesic properties, with a significant inhibition of nitric oxide (NO), TNF-α, and interleukin-6 (IL-6) production in cellular models of activated microglia and macrophages, as well as a reduction in lipoxygenase and cyclooxygenase activity (Subedi et al., 2021 Ishijima & Nakajima, 2021; Wilms et al., 2010). These effects appear to be due to the presence of phenolic acids and terpenoids. In parallel, *O. majorana* extracts have demonstrated cytotoxic and *in vitro* antitumor activity against various cell lines (HeLa, MCF-7, HepG2), through the induction of apoptosis and cell

cycle arrest (Al Dhaheri et al., 2013; Fakhri et al., 2022). Another study reports several biological effects. These include antidiabetic activity, achieved through the inhibition of α-glucosidase and the improvement of insulin sensitivity (Kawabata et al., 2003), and hepatoprotective properties against toxin-induced or oxidative stress–related damage (Soliman et al., 2016). Moreover, neuroprotective and anxiolytic effects have been observed, attributed to the modulation of the GABAergic system and the reduction of cortisol levels (Amaghnouje et al., 2020).
Finally, the plant also exhibits marked repellent and insecticidal activity against harmful insects such as *Aedes aegypti* and *Sitophilus oryzae* (Sefeer & Elumalai, 2018; Chaves et al., 2019).

1.1.3 Origanum dictamnus L.

Origanum dictamnus L. (figure 5), known as Cretan dittany, is a species endemic to the island of Crete, where it grows spontaneously from coastal areas to the highest mountainous zones. It is a small, woolly plant typical of shaded limestone crevices, flowering from June to October (Ietswaart, 1980; Turland, 1995). Known since ancient times for its healing properties, dittany was considered as a sacred plant linked to the Cretan goddess Diktynna, later assimilated to Artemis (Thanos, 1994; Skoula & Kamenopoulos, 1997). The name “dictamnus” derives from “Dicti”, the Cretan Mountain dedicated to Zeus, and from “thamnos”, meaning shrub in Greek (Skrubis, 1979). Classical and medieval sources describe it as a remedy for wounds and poisons, with numerous attestations in the Greco-Roman world and later in European medical texts (Simó, 2010).

Figure 5. *Origanum dictamnus* L.

Today, *O. dictamnus* maintains an important economic and cultural role in Crete, where it is used as an aromatic, medicinal, and food plant (Skoula & Kamenopoulos, 1997; Hanlidou et al., 2004). Furthermore, its safety as a spice is recognized by the Code of Federal Regulations (CFR) and the Herbal Medicinal Products Committee of the EMA has included the plant among the herbal products of traditional use (Chinou, 2012). The chemical composition of *Origanum dictamnus* L. has been extensively investigated in recent decades, highlighting the presence of numerous primary and secondary compounds of biological relevance. Initial studies conducted on fresh and dried leaves identified fatty acids, lipids, sterols, and EOs among the main non-polar constituents (Azcan et al., 2004; Krigas et al., 2015). Numerous polyphenols, flavonoids, and coumarins have been isolated from the various polar extracts of the plant—particularly the methanolic and aqueous ones (Sharifi-Rad et al., 2021). Among the main phenolic compounds identified are *p*-coumaric and ferulic, as well as catechin and derivatives (Proestos et al., 2008). The biological properties of *Origanum dictamnus*—particularly its antioxidant, antimicrobial, and preservative activities— were widely documented (Lillehoj et al., 2018; Daferera et al., 2003; Chinou et al., 2007; Liolios et al., 2009). Its traditional use against

gastric ulcers has been confirmed by *in vitro* studies, which have demonstrated the activity of 70% hydroalcoholic extracts against clinical strains of *Helicobacter pylori*, with minimum inhibitory concentration values around 2.5 mg/mL (Awaad et al., 2013). Methanolic extracts also showed antibacterial activities against several Gram-negative strains, including *Acinetobacter haemolyticus* and *Pseudomonas aeruginosa* (Chatzopoulou et al., 2010), as well as an antioxidant action comparable to that of α-tocopherol (Couladis et al., 2003). In general, polar extracts—rich in phenolic compounds and flavonoids—showed the greatest antioxidant capacity, supporting the potential use of dittany as a possible natural alternative to synthetic antioxidants (Zheng & Wang, 2001). Nonpolar extracts, such as those in cyclohexane, also show appreciable antioxidant activity and a high content of tocopherols, particularly γ-tocopherol (Capecka et al., 2005).

Comparisons between extracts of different polarity have shown that the most polar (ethanolic) ones possess the greatest DPPH radical scavenging capacity, while nonpolar extracts are more effective in protecting vegetable oils from oxidation, suggesting that the polarity of the compounds influences their bioavailability (Kouri et al., 2007). Among the isolated bioactive compounds, ursolic acid stood out for its *in vitro* cytotoxic activity against leukemia tumor cell lines, murine and human lung carcinoma (Chinou et al., 2007).

The *O. dictamnus* EO is known for its antioxidant, antimicrobial, and antiproliferative activities attributed primarily to the monoterpene phenols carvacrol and thymol, while their biosynthetic precursors, *p*-cymene and γ-terpinene, are inactive (Calo et al., 2015; Elshafie & Camele, 2017). Numerous studies have confirmed the antifungal activity of the EO and its main components against *Penicillium digitatum, Botrytis cinerea, Fusarium* spp., and other plant pathogens (Daferera et al., 2003; Vokou et al., 1993). Finally, the EO demonstrated an insecticidal effect against *Drosophila auraria*, causing a significant reduction in egg hatching and larval malformations, confirming the broad biological spectrum of *O. dictamnus* (Kim et al., 2003).

1.1.4 Origanum heracleoticum L.

Origanum heracleoticum (syn. *Origanum vulgare* subsp. *hirtum* (Link) Ietsw.), commonly known as Greek oregano (figure 6), is an aromatic subspecies native to the eastern Mediterranean regions, particularly Greece, where it grows wild in well-drained rocky and calcareous habitats (Kokkini, 1997). It is a highly aromatic, herbaceous perennial plant, 30–80 cm tall, with erect or semi-woody stems at the base, oval, tomentose leaves, and terminal inflorescences consisting of small white flowers (Ietswaart, 1980). The term “hirtum” refers to the fine pubescence that covers the leaves and bracts, a distinctive feature compared to other subspecies of *O. vulgare*.

Figure 6. *Origanum heracleoticum* L.

Historically, Greek oregano has been known since ancient times for its aromatic and medicinal properties: Hippocrates and Dioscorides described it as a "hot and dry" plant, useful for treating digestive and respiratory disorders

as well as a natural antiseptic (Alekseeva et al., 2020). In Greek folk tradition, oregano was considered as a symbol of joy and purity and was also used as a herbal remedy to relieve pain, illnesses and intestinal disorders (Skoufogianni et al., 2019).

Phytochemically, Greek oregano is characterized by a high EO content, primarly composed of phenolic monoterpenes—mainly carvacrol and thymol—along with their precursors *p*-cymene and γ-terpinene (Wogiatzi et al., 2009; Knez et al., 2020). Geographic variability and environmental factors significantly influence the proportion of these constituents, generating different carvacrol/thymol chemotypes (Kokkini et al., 1997; Bakkali et al., 2020). Other relevant secondary metabolites include phenolic acids (rosmarinic, caffeic, ferulic), flavonoids (apigenin, luteolin, quercetin), and tannins (Michalaki et al., 2023; Exarchou et al., 2002).

The EO and ethanolic extracts showed a significant antioxidant activity, comparable to that of standard compounds such as butylated hydroxytoluene (BHT) or α-tocopherol (Paraskevakis, 2015; Tsimogiannis et al., 2006). The antimicrobial activity is mainly attributed to carvacrol and thymol, which are effective against Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus, Escherichia coli, Listeria monocytogenes*, and *Salmonella typhimurium* (Sakkas & Papadopoulou, 2017, Stefanakis et al., 2013). Recent studies have also highlighted an antibiofilm and antifungal effect, especially against *Candida albicans* and *Aspergillus niger* (Kosakowska et al., 2024; Manohar et al., 2001). In addition to its known antimicrobial properties, Greek oregano EO has shown anti-inflammatory and neuroprotective effects: in zebrafish models, the extract attenuated oxidative stress and improved short-term memory (Georgantopoulos et al., 2023, Stojanović et al., 2024). A recent study also demonstrated antitumor activity in murine colorectal cancer, associated with increased apoptosis and modulation of inflammatory cytokines (Aindelis et al., 2025).

1.2 Application of natural products

The therapeutic use of plant-based products dates back thousands of years and remains highly relevant in modern healthcare (Brondani et al., 2018). Many plants, whose leaves, seeds, or flowers are used for flavoring, perfuming, or healing, such as *Rosmarinus officinalis* L. (rosemary) and *Origanum vulgare* (oregano), have long played a central role in traditional medicine (Meo et al., 2017). According to the World Health Organization, approximately 60% of the global population relies on herbal remedies for the treatment of various diseases (Khan and Ahmad, 2019). Beyond their traditional applications, natural compounds and their derivatives have been pivotal in the development of modern pharmacotherapy, particularly in the management of infectious diseases (Atanasov et al., 2021). Many plant-derived molecules have also served as templates for the synthesis or semi-synthesis of new drugs (Balandrin et al., 1993; Bahar et al., 2008), demonstrating the lasting impact of botanical sources on pharmaceutical innovation. In recent decades, growing concerns about antimicrobial resistance have renewed interest in discovering new therapeutic agents from natural sources (Boakye et al., 2019). Typically, the process of drug discovery from medicinal plants involves five main steps: species collection, extraction, chemical separation, structural identification, and biological evaluation (Kinghorn et al., 2003). This systematic approach has its roots in ancient phytotherapeutic practices, when tinctures, teas, infusions, decoctions, and macerations represented the earliest medicinal preparations (Orch et al., 2021; Benkhnigue et al., 2022). Over centuries, such empirical knowledge was transmitted orally and later codified in traditional pharmacopoeias (Avery & Hains, 2017; d'Avigdor et al., 2014). The formal scientific study of medicinal plants began to flourish in the 19th century, marked by the isolation of morphine from *Papaver somniferum* L., which laid the foundations of modern pharmacognosy (Brook et al., 2017; Yuan et al., 2016). Subsequent discoveries, including digitoxin, cocaine, pilocarpine, codeine, and quinine, further revealed the immense pharmaceutical potential of plant-derived compounds (Kong et al., 2003). Plant-derived products continue to play a central role in drug discovery and development. Their

remarkable structural diversity and broad pharmacological potential not only yield effective therapeutic agents but also inspire the creation of innovative compounds, ensuring that natural products remain a vital source of inspiration in modern medicine (Ernest et al., 2010).

In recent years, scientific research has increasingly focused on exploring the pharmacological activities of traditionally used plants, to identify new nutraceutical ingredients or potential adjuvants to conventional synthetic drugs used in the management of neurodegenerative diseases, metabolic disorders and biofilm-induced infections (Chávez-Delgado & Jacobo-Velázquez, 2023; Fernando et al., 2020; Hyun et al., 2014).

1.2.1 Enzymes involved in neurodegenerative disease

Among the several mechanisms of action exhibited by natural compounds, enzyme inhibition has emerged as a particularly relevant strategy. Enzyme inhibitors represent a major class of therapeutic agents, and consequently, several enzymes have become key targets in modern drug discovery.

Neurodegenerative diseases are a group of disorders characterized by the progressive loss of specific neuronal populations, with serious consequences for cognitive and motor functions. Among these, Alzheimer's disease (AD) and Parkinson's disease (PD) are the most common and extensively studied. In Alzheimer's disease, the cholinergic theory suggests that cognitive decline is largely due to a reduction in the activity of cholinergic neurons projecting to the hippocampus and cerebral cortex, resulting in decreased levels of the neurotransmitter acetylcholine (ACh) (Perry, 1986; Bartus et al., 1982). Under physiological conditions, acetylcholinesterase (AChE) is the main enzyme responsible for the hydrolysis of acetylcholine, while butyrylcholinesterase (BChE) plays a secondary role (Bartolucci et al., 2001). However, in individuals with AD, AChE activity decreases (figure 7), while BChE activity increases as a compensatory response, resulting in an enzymatic imbalance that contributes to the loss of cholinergic function (Greig et al., 2002). This

represents the therapeutic strategy of most symptomatic drugs available today: cholinesterase inhibitors. Molecules such as donepezil, rivastigmine and neostigmine act by preventing the degradation of acetylcholine, temporarily improving cognitive abilities (López et al., 2002; Lahiri et al., 2002). In recent years, however, there has been growing interest in naturally occurring inhibitors that can selectively modulate AChE and BChE, potentially offering a better safety profile.

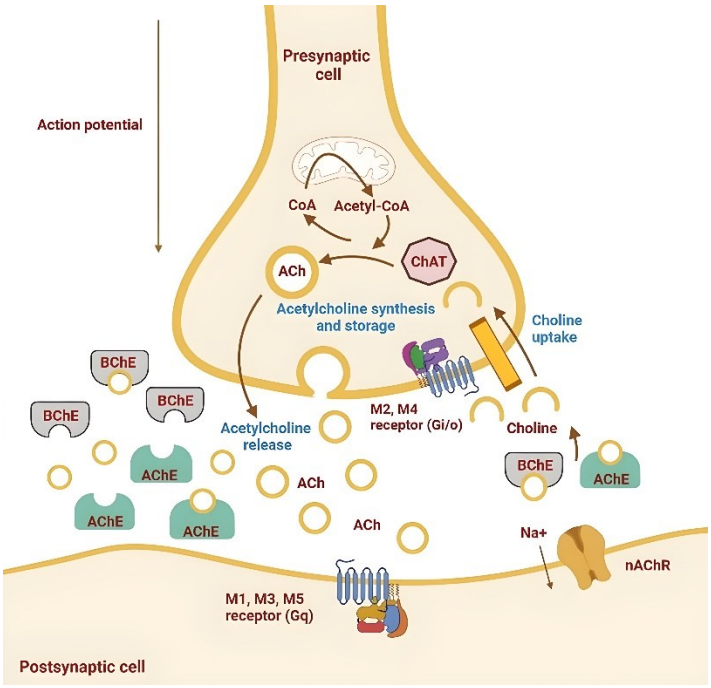


Figure 7. *Schematic representation of a cholinergic neuron (Žnidaršič et al., 2023)*

Parkinson's disease, on the other hand, is associated with selective degeneration of dopaminergic neurons in the substantia nigra, resulting in reduced dopamine (DA) levels in the striatum (Sayre et al., 2001). The main pathogenic mechanisms include oxidative stress, mitochondrial dysfunction and the abnormal accumulation of α-synuclein, a protein that forms toxic intracellular aggregates (Kikuchi et al., 2002; Paris et al., 2007). The enzyme tyrosinase (figure 8) a copper-containing metalloenzyme involved in the synthesis of melanin and neuromelanin, plays an important role in these processes (Hasegawa, 2010). Tyrosinase catalyses the hydroxylation of L-

tyrosine to L-DOPA and its subsequent oxidation to dopaquinone, a highly reactive intermediate that may contribute to the formation of neuromelanin and neurotoxic oxidative products (Baber et al., 2023; Pillaiyar et al., 2017). Althought the presence of tyrosinase in the central nervous system has been debated, recent studies have confirmed its gene and protein expression in dopaminergic neurons (Li et al., 2021; Zecca et al., 2001).

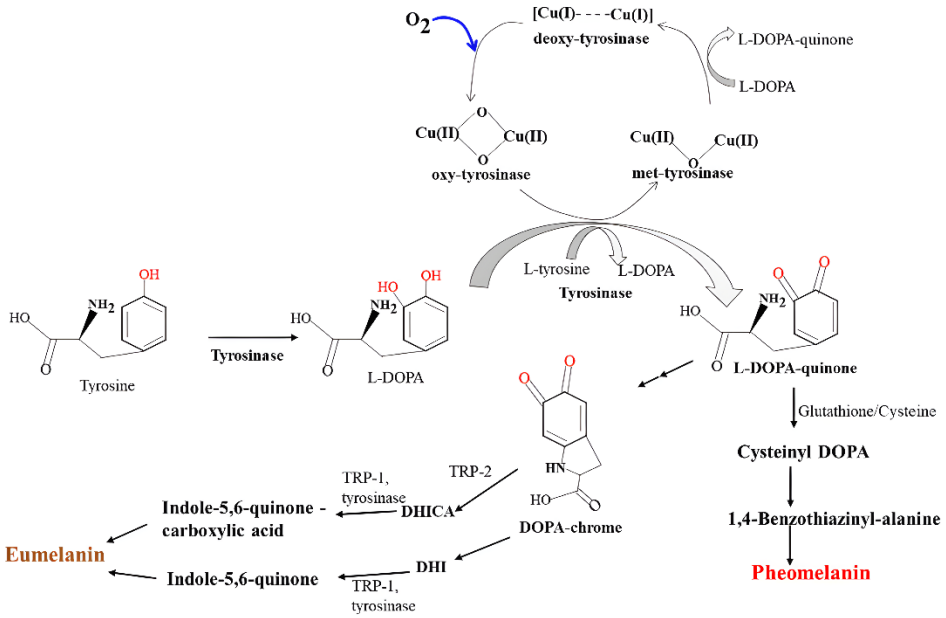


Figure 8. *Tyrosinase uses L-tyrosine and L-DOPA as catalysis substrates (Kim et al., 2023)*

For this reason, the enzyme is considered an emerging drug target in Parkinson's disease. Several natural tyrosinase inhibitors, such as arbutin, azelaic acid, and kojic acid, show interesting therapeutic properties, although some of them present safety concerns and potential toxicity (Hassan et al., 2023; Ogiwara et al., 2015). Currently, research is focused on developing new tyrosinase inhibitors with greater efficacy and tolerability, that can modulate neuromelanin synthesis without inducing systemic side effects.

1.2.2 Enzymes involved in metabolic disorders

Metabolic syndrome is a cluster of related conditions — including visceral obesity, hypertension, dyslipidaemia, and insulin resistance — that significantly increase the risk of developing cardiovascular disease and type 2 diabetes (Costa et al., 2004; Kaur, 2014). The management of this syndrome is mainly based on lifestyle interventions and drugs targeting individual metabolic parameters (Rask Larsen et al., 2018). However, a promising biochemical approach consists of modulating the activity of specific digestive enzymes, such as α-amylase, α-glucosidase and lipase, which are responsible for the digestion of carbohydrates and fats.

During digestion, α-amylases (salivary and pancreatic) hydrolyze the glycosidic bonds of starch (figure 9) producing oligosaccharides and maltose, while α-glucosidases complete the degradation into glucose, which is rapidly absorbed and causes a rise in postprandial blood sugar levels (Yoon & Robyt, 2002; Warren et al., 2015). The inhibition of these enzymes reduces the rate of carbohydrate absorption, contributing to glycemic control and to the prevention of type 2 diabetes (Obiro et al., 2008).

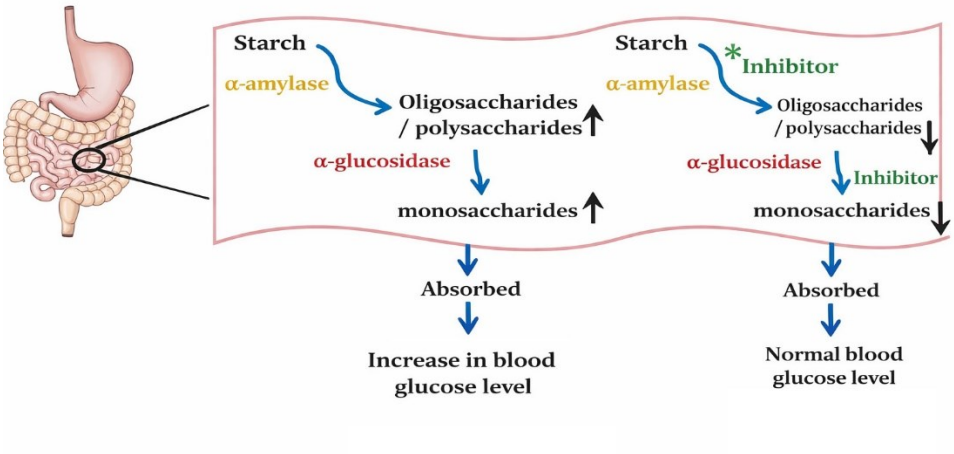


Figure 9. *Role of α-amylase, α-glucosidase in starch digestion and metabolism (Naveen & Baskaran, 2018)*

At the same time, pancreatic lipases (figure 10) catalyse the hydrolysis of dietary triglycerides into free fatty acids and monoglycerides, promoting their intestinal absorption (Lowe, 2002). Inhibiting this enzyme is a useful strategy for reducing fat absorption and, consequently, combating obesity (Basque & Ménard, 2000; Aloulou & Carrière, 2008). Furthermore, in October 2025, Italy took a historic step in public health by officially recognizing obesity as a chronic, progressive, and relapsing disease: it was the first nation in the world to do so (Bifulco et al., 2025; D.L 149/2025)

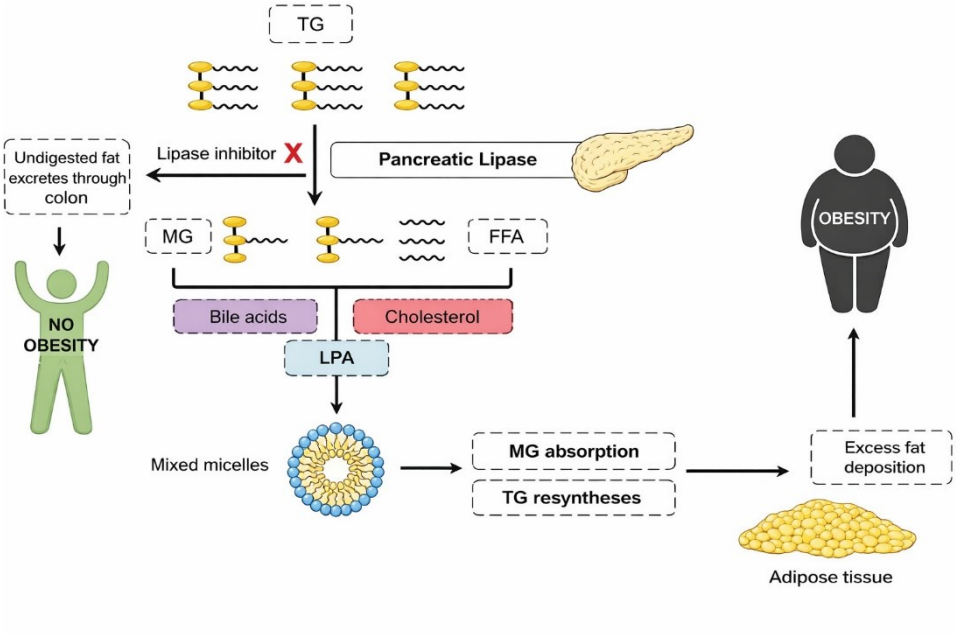


Figure 10. *Role pancreatic lipase in digestion and metabolism (Rocha et al., 2024)*

Numerous natural compounds from plant and marine origin show inhibitory activity against α-amylase, α-glucosidase and lipase, providing safer alternatives to synthetic drugs (Guo, 2017; Williamson et al., 2020). Clinical and preclinical studies have shown that plant extracts and phytochemicals can improve insulin sensitivity, reduce blood sugar and lipid levels, and promote weight loss (Jang et al., 2016). The growing interest in these approaches reflects the global trend towards natural and preventive

therapeutic strategies based on bioactive molecules capable of finely regulating energy metabolism.

1.2.3 Biofilms and antimicrobial resistance

Microbial biofilms are highly organised communities of microorganisms adhering to biotic or abiotic surfaces, immersed in a self-produced matrix of extracellular polymeric substances (EPS). The ability of microorganisms to form biofilms plays an important role in many scientific fields, including medicine, food safety, biotechnology and environmental microbiology. Biofilm development (figure 11) follows a well-defined sequence of stages (Costerton et al., 1999; Donlan & Costerton, 2002):

1) **Initial attachment**: The phase begins when planktonic bacterial cells adhere to a specific surface reaching a critical cell density. In this phase, the bacteria aggregate to form microcolonies that anchor themselves through physical-chemical interactions, mainly van der Waals forces and hydrophobic interactions (O'Toole et al., 2000). Since the cells are still weakly attached, it is relatively easy to remove them by gentle mechanical or chemical means.
2) **Transition and matrix formation**: Subsequently, the transition from planktonic to sessile form involves a profound metabolic reorganization. The cells start to secrete exopolysaccharides, proteins and nucleic material, which form a protective viscoelastic dome-shaped matrix. At this stage, intercellular communication is established through quorum sensing (QS), a molecular signalling system that regulates collective gene expression and phenotypic diversification within the microbial consortium (Parsek & Greenberg, 2005). QS allows cells to coordinate their metabolic activity, optimising nutrient utilization and improving survival under adverse environmental conditions (Fazeli-Nasab et al., 2022). Interfering with QS mechanisms is therefore a promising strategy for reducing biofilm cohesion and resistance,

increasing the sensitivity of cells to antimicrobial agents (Kaplan, 2010).

3) **Maturation and dispersion**: During maturation, the biofilm takes on a complex three-dimensional structure, crossed by aqueous channels that facilitate the transport of nutrients and waste metabolites (Zhao et al., 2023). Some sessile cells may subsequently detach, returning to a planktonic state to colonize new surfaces. The extracellular matrix consists of a variety of macromolecules: polysaccharides, responsible for the cohesion and adhesion of the biofilm (Wang et al., 2024); proteins and enzymes, which stabilize its structure and increase its resistance; extracellular DNA (eDNA), which contributes to both mechanical stability and genetic exchange (Das et al., 2013); and lipids, which improve adhesion to hydrophobic surfaces. From a structural point of view, the biofilm has a layered arrangement: a surface layer populated by metabolically active cells; an intermediate layer, which is denser and rich in EPS; and a deep layer in which quiescent or dormant cells predominate (Billings et al., 2015).

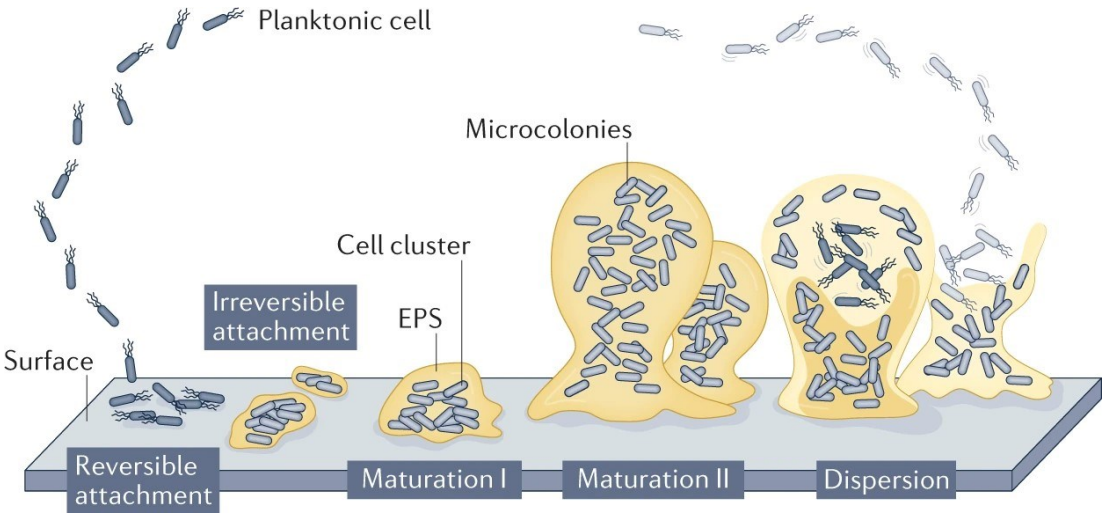


Figure 11. *The cycle of biofilm (Rocha et al., 2024)*

Biofilm protects bacteria from the action of various antibiotics. In fact, the multiple resistance phenotype that characterizes bacteria incorporated into

biofilms is supported by a combination of structural and molecular adaptations. One key mechanism is the increased expression of efflux systems, which actively expel a wide range of antimicrobials and are more active in sessile bacteria than in planktonic bacteria (Mah & O'Toole, 2001; Poole, 2001). The EPS matrix itself acts as a diffusion barrier, limiting antibiotic penetration and creating nutrient and oxygen gradients that induce a metabolic state of quiescence in deeper cells. These conditions reduce the effectiveness of antibiotics that act on actively growing cells (Fernández-Billón et al., 2023). In addition to structural mechanisms, the genetic plasticity of microorganisms within the biofilm allows for rapid changes in gene expression, further strengthening collective resistance. To combat the persistence of biofilms, strategies targeting specific resistance mechanisms must be adopted. Among these, the use of lytic enzymes (DNase, protease or polysaccharidase) to degrade the EPS matrix and the use of quorum sensing inhibitors (quorum-quenching agents) are promising approaches (Stewart, 2002). The integration of these methods into sanitation protocols can significantly reduce the presence of biofilms on food contact surfaces, minimizing the risk of cross-contamination and improving overall health and safety. Furthermore, biofilms are now a strategic target in the development of new antimicrobial drugs (Chadha, 2014). Numerous recent studies have shown that plant-derived secondary metabolites possess anti-biofilm activity by interfering with the microbial structure formation or stability s (Lahiri et al., 2019). These natural compounds are not only effective, but also generally safe and non-damaging to host tissues, making them promising candidates for alternative and complementary treatments against biofilm-related infections.

2. METHODS OF EXTRACTION

Extraction methods are selected according to the chemical nature of the matrix and the type of fraction to be isolated. For the volatile fraction (such as EOs and aromatic compounds), techniques that preserve low-boiling constituents or exploit their volatility are preferred. These methods include steam or hydrodistillation, vacuum distillation, cold pressing (commonly used for citrus peels), headspace analysis, supercritical fluid extraction, and solid phase microextraction (SPME) (Samakradhamrongthai, 2024; Jerkovic, et al., 2012; Mohammadhosseini, 2024). Extraction using highly volatile solvents followed by gentle solvent removal can also be applied.
In contrast, the non-volatile fraction (including lipids, pigments, polyphenols, saponins, and sugars) is better obtained through solvent-based methods applied to solid or liquid matrices, such as maceration, percolation, Soxhlet extraction, ultrasound- or microwave-assisted extraction, supercritical fluid extraction, and pressurized liquid extraction (Braga et al., 2022; Gupta et al., 2012; Azwanida, 2015).
In the present study, attention will be focused on steam distillation for the volatile fraction, and on maceration and pressurized liquid extraction for the non-volatile fraction.

2.1 Steam Distillation

Steam distillation (figure 12) represents the conventional and most extensively applied technique for the extraction of EOs (EOs) from plant materials. It is estimated that approximately 93% of commercial EOs are obtained by this method, while the remaining 7% are produced using alternative extraction techniques (Hanif et al., 2019). This approach is generally preferred because it is environmentally friendly, safe to operate, and easily scalable. Furthermore, steam distillation often results in products of higher purity and with fewer impurities compared to other extraction procedures (Yani et al., 2014). In this method, plant tissues such as leaves or

flowers are placed on a supporting screen, and steam is passed through the material, facilitating the release of volatile compounds. The resulting vapor mixture of water and EOs is subsequently condensed and separated to recover the oil fraction (Machado et al., 2022). The steam temperature must be sufficient to disrupt cellular structures and release aromatic components without promoting their thermal degradation (Tongnuanchan & Benjakul, 2014). Despite these advantages, several limitations are associated with steam distillation. The process is relatively time-consuming and requires extended extraction periods. In addition, a portion of the EO may remain dissolved in the distillation water, leading to odor issues. To maximize recovery, the aqueous phase is frequently redistilled through a procedure known as cohabitation, which can increase energy and operational costs (Masango, 2005).

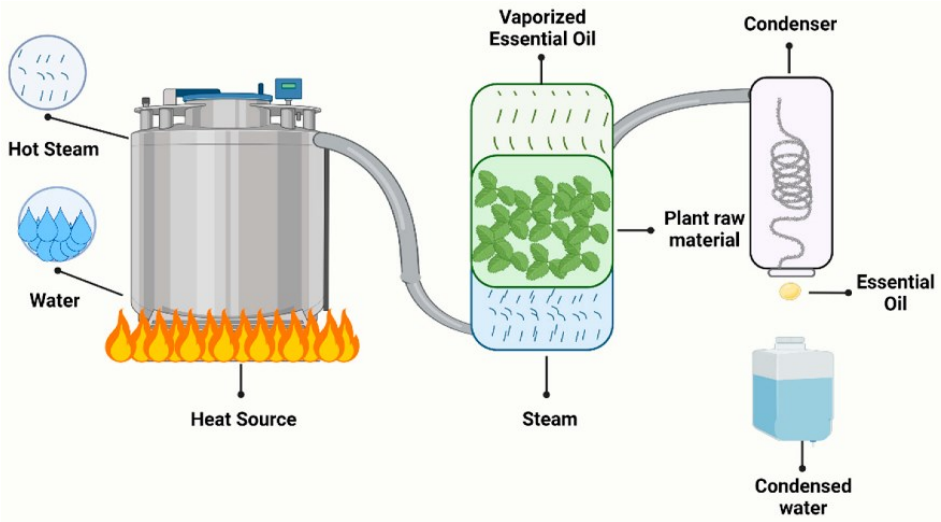


Figure 12. *Steam distillation process*

2.2 Maceration

Maceration is a simple, inexpensive, and widely used extraction technique, particularly suitable for small-scale and laboratory applications. It relies on mass transfer between a solid matrix and a solvent, facilitated by agitation until solid–liquid equilibrium is achieved (figure 13). Although efficient for

preliminary extraction, this method typically requires a subsequent concentration step to obtain the final extract (Azmir et al., 2013). Maceration has long been employed in the preparation of herbal tonics and extracts and generally involves several sequential operations: grinding of the plant material, immersion of the powdered sample in a solvent, removal of the solvent, and pressing of the residue to recover the crude extract (Jha & Sit, 2022). These steps enhance diffusion of target compounds from the plant material to the solvent and facilitate the removal of concentrated solution from the solid surface, thereby improving extraction yield. Despite its simplicity, maceration presents several limitations: it often employs toxic or environmentally unfriendly solvents, requires long extraction times, and may lead to the degradation of thermolabile compounds due to oxidation or prolonged exposure to ambient conditions (More et al., 2022). The technique is poorly selective, frequently necessitating additional purification steps, and the final extracts may contain residual solvent traces, which are undesirable from a health and safety perspective (Gil-Martín et al., 2022). Mechanistically, maceration is a solid–liquid extraction process in which solvent polarity, temperature, and agitation are key parameters affecting solubility and extraction efficiency (Luque de Castro & Priego-Capote, 2010). Compared to both conventional and emerging extraction methods, maceration offers low equipment cost and operational simplicity. Moreover, its versatility allows the recovery of a wide range of bioactive compounds by optimizing solvent composition, temperature, and agitation conditions to promote selective mass transfer (Gallo et al., 2017; Sasidharan et al., 2018). The process is typically carried out by immersing the plant material (coarse or powdered) in a stoppered container with the chosen solvent and leaving it to stand for three to seven days under occasional agitation until the soluble constituents are fully dissolved (Nirmal et al., 2023). This step allows the solvent to penetrate the plant tissues, soften the cell walls, and release intracellular phytochemicals. After maceration, the mixture is filtered or strained, and the solid residue is pressed to recover any remaining liquid.

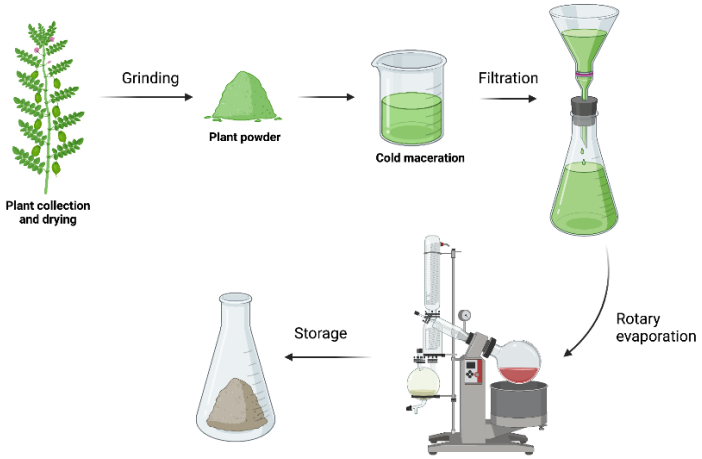


Figure 13. *Maceration process*

2.3 Pressure Liquid Extraction (PLE)

Pressurized Liquid Extraction (PLE) is an advanced extraction technique (figure 14) that utilizes liquid solvents under elevated temperature and pressure to achieve a rapid and efficient recovery of analytes from solid matrices (Carabias-Martínez et al., 2007). This method—also referred to as Accelerated Solvent Extraction (ASE), Pressurized Fluid Extraction (PFE), Pressurized Hot Solvent Extraction (PHSE), or Subcritical Solvent Extraction (SSE)—operates under conditions where the solvent remains in the liquid phase, but its physicochemical properties are significantly altered (Pereira et al., 2024). At high temperature and pressure, solvent viscosity and surface tension decrease, while diffusion and solubility of the target analytes increase, thereby enhancing mass transfer and allowing more effective penetration of the solvent into the solid matrix. As a result, PLE provides higher extraction yields in shorter times and with lower solvent consumption compared to conventional extraction methods (Herrero et al., 2013).

In cyclically pressurized extraction, the solvent pressure varies periodically within a defined range, promoting enhanced sample hydration and improved solute diffusion (Formato et al., 2013). Several parameters influence PLE efficiency, including solvent type and polarity (or solvent mixture), solvent-to-

sample ratio, temperature, pressure, number of extraction cycles, and extraction time. Among these, temperature and solvent type have the greatest influence on extraction efficiency (Lama-Muñoz et al., 2019).

Numerous studies have demonstrated the versatility of PLE for various analytical and industrial applications, such as the extraction of pesticides from food matrices, phenolic compounds from parsley (Luthria, 2008), herbicides from agricultural products (Jouybari et al., 2022), and polychlorinated biphenyls (PCBs) from fatty foods and feeds (Björklund et al., 2006). When compared with traditional extraction techniques such as heat-based or solvent extraction, PLE and other high-pressure approaches offer substantial advantages, including shorter extraction times, higher yields, reduced impurities, and minimal thermal degradation of thermolabile compounds (Huang et al., 2013). PLE is also considered an environmentally friendly extraction technique due to its reduced solvent use and the possibility of employing green and GRAS (Generally Recognized as Safe) solvents, such as ethanol, ethyl acetate, ethyl lactate, and D-limonene (Alvarez-Rivera et al., 2020). The widespread development of automated PLE systems has further improved reproducibility, operational safety, and labor efficiency, reinforcing its adoption in the pharmaceutical, food, and biotechnological industries. These advantages make PLE particularly suitable for the extraction of antioxidants, phenolic compounds, carotenoids, and other bioactive molecules with anti-inflammatory, antimicrobial, and antiviral properties (Kumar et al., 2023).

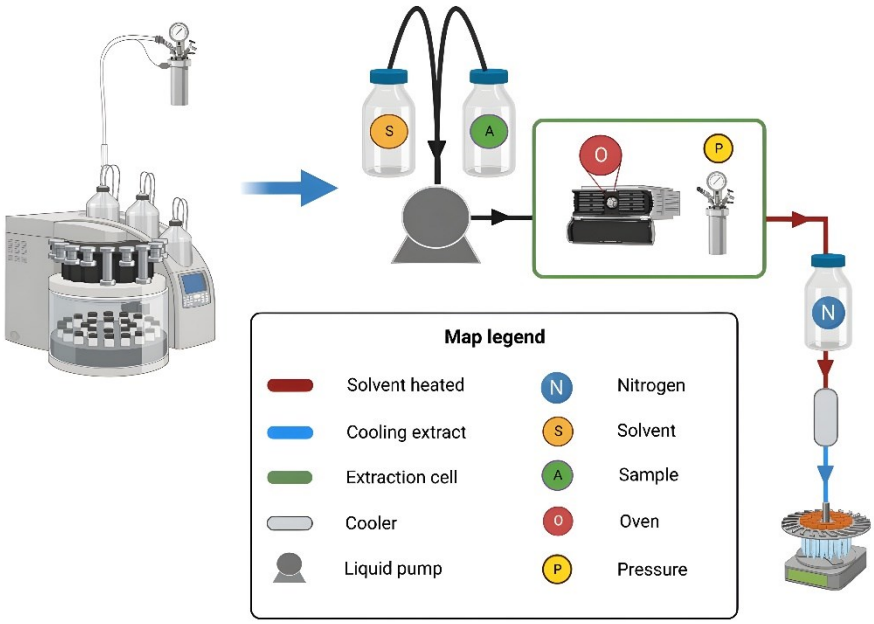


Figure 14. *PLE process* (Perez-Vazquez et al., 2023)

3. AIMS OF THE STUDY

The aims of the following study were to:

1) Investigate the *O. dictamnus* species evaluating both volatile fraction (EO) and non-volatile fraction:

1.1 Volatile fraction

- to characterize its phytochemical profile through GC–MS analysis;
- to assess its nutraceutical potential by examining inhibitory activities against key enzymes related to neurodegenerative and metabolic disorders;
- to elucidate the mechanism of enzyme inhibition through kinetic studies;
- to support and validate *in vitro* findings with *in silico* analyses, focusing on molecular interactions between major EO constituents and the target enzymes.

1.2 Non-volatile fraction

- to evaluate three different extraction conditions: maceration with 70% ethanol, maceration with 20% ethanol, and pressurized liquid extraction (PLE) with 20% ethanol;
- to analyse the biological activities of the extracts, focusing on effects related to neurodegenerative and metabolic disorders;
- to identify the best extraction condition that provides the optimal biological activities and to analyse its phytochemical profile through LC-HRESIMS/MS.

2) Conduct a comparative interspecies evaluation of EOs from Italian *Origanum* species (*O. majorana*, *O. vulgare*, and *O. heracleoticum*):

- to assess chemical profile of these three different species by GC-MS

- to compare their antioxidant capacity and enzyme inhibitory effects on targets implicated in neurodegenerative and metabolic diseases;
- to explore possible differences in their mechanisms of action through enzyme kinetics.

3) Compare *O. heracleoticum* samples (intraspecies comparison) collected from different Italian localities analysing:

3.1 Eight samples for volatile fraction:

- to characterize the chemical composition of each sample using GC–MS**;**
- to perform multivariate statistical analyses to identify similarities, differences, and potential clustering among the samples;
- to select samples for further biological assays based on their chemical profiles: three samples tested for activities related to the central nervous system; five samples evaluated for activities associated with metabolic disorders.

3.2 Six samples for non-volatile fraction:

- to apply the optimized PLE method to all six *O. heracleoticum* samples collected from different Italian areas;
- to characterize the chemical profiles of the extracts and assess qualitative and quantitative differences;
- to evaluate the biological activities of the extracts, focusing on targets related to metabolic disorders.

4. RESULTS AND DISCUSSION

4.1 Investigate on *O. dictamnus* species

4.1.1 *Volatile fraction*

4.1.1.1 Yield and chemical composition

The hydrodistillation of the aerial parts of *O. dictamnus* provided an EO with a yield of 3.01% calculated on the dry weight. This result was like that obtained by Mitropoulou and collaborators (2015) who reported a yield of 2.53%, while Ntalli et al. (2010) and Liolios et al. (2009) obtained yields of 0.94% and 0.46%, respectively, lower compared to our sample. Table 1 reported the chemical composition of the EO *(*peak area normalization was used to get the component relative concentrations); the components are listed according to their elution on an DB-5 capillary column. A total of 70 compounds were identified, accounting for 95.2% of the total oil.

Table 1. *Chemical composition of O. dictamnus EO.*

N.	RT	Compound	%	KI[a]	KI[b]	Identification
1	12.77	α-Thujene	0.40±0.04	927	1110	1,2,3
2	13.10	α-Pinene	0.40±0.03	932	932	1,2,3
3	14.03	Camphene	0.10±0.05	945	945	1,2,3
4	14.94	Benzaldehyde	t	957	956	1,2,3
5	15.98	β-Pinene	0.10±0.04	971	971	1,2,3
6	16.67	1-Octen-3-ol	0.20±0.03	981	982	1,2
7	17.18	3-Octanone	0.10±0.04	988	988	1,2
8	17.43	β-Myrcene	0.30±0.09	991	991	1,2,3
9	18.08	α-Phellandrene	0.10±0.04	1000	1000	1,2,3
10	18.48	3-Carene	t	1005	1005	1,2,3
11	19.00	α-Terpinene	1.20±0.10	1012	1022	1,2,3
12	19.76	*p*-Cymene	20.20±0.20	1023	1023	1,2,3
13	19.90	Limonene	0.40±0.06	1025	1025	1,2,3
14	20.02	1,8-Cineole	0.10±0.04	1026	1026	1,2,3

15	22.13	γ-Terpinene	0.80±0.08	1055	1055	1,2,3
16	22.68	*cis*-Sabinene hydrate	0.50±0.03	1063	1058	1,2,3
17	24.25	Terpinolene	0.10±0.02	1084	1084	1,2,3
18	24.30	*p*-Cymenene	0.10±0.04	1085	1084	1,2,3
19	24.93	*trans*-Sabinene hydrate	0.20±0.06	1094	1094	1,2,3
20	25.34	Linalool	0.80±0.07	1099	1099	1,2,3
21	26.26	β-Thujone	t	1112	1101	1,2,3
22	26.61	*p*-2-Menthen-1-ol	0.10±0.06	1117	1116	1,2
23	27.54	1,3,8-*p*-Menthatriene	t	1130	1118	1,2
24	27.70	Pinocarveol	t	1132	1132	1,2
25	28.09	Camphor	0.10±0.04	1138	1138	1,2,3
26	29.68	*trans*-Borneol	0.30±0.04	1160	1160	1,2,3
27	30.05	*trans*-2-Caren-4-ol	t	1165	1178	1,2
28	30.57	Terpinen-4-ol	1.10±0.2	1172	1172	1,2,3
29	31.09	Thujen-2-one	t	1180	1181	1,2
30	31.25	*p*-Cymen-8-ol	0.10±0.04	1182	1182	1,2
31	31.54	*m*-Cymen-8-ol	0.10±0.03	1186	1182	1,2
32	31.89	α-Terpineol	0.20±0.07	1191	1191	1,2,3
33	32.18	Estragol	t	1195	1195	1,2
34	32.83	Dihydrocarvone	t	1204	1207	1,2
35	33.27	*cis*-Piperitol	t	1211	1211	1,2
36	35.44	Carvacrol methyl ether	t	1242	1244	1,2
37	35.86	Carvone	0.20±0.02	1249	1243	1,2
38	35.96	Thymoquinone	0.30±0.04	1250	1251	1,2
39	38.11	Anethole	0.50±0.03	1282	1284	1,2,3
40	38.95	Thymol	1.00±0.09	1294	1294	1,2,3
41	40.03	Carvacrol	57.70±0.50	1310	1311	1,2,3
42	42.32	α-Cubebene	0.20±0.04	1346	1352	1,2,3
43	42.91	Eugenol	0.10±0.02	1355	1355	1,2,3
44	43.89	Copaene	0.50±0.08	1370	1370	1,2,3
45	44.85	Carvacryl acetate	0.10±0.04	1385	1367	1,2
46	45.74	β-Copaen-4α-ol	t	1399	1565	1,2
47	46.08	Methyleugenol	t	1405	1403	1,2
48	46.53	Caryophyllene	1.40±0.30	1412	1412	1,2,3
49	47.77	α-Bergamotene	t	1432	1432	1,2,3
50	48.18	Thymoquinol	t	1439	1522	1,2
51	48.60	Humulene	0.10±0.02	1446	1446	1,2,3
52	49.22	*cis*-Muurola-4(15),5-diene	0.10±0.04	1456	1468	1,2
53	50.15	γ-Muurolene	t	1471	1471	1,2,3
54	50.32	β-Copaene	t	1474	1443	1,2,3
55	50.67	α-Curcumene	t	1480	1480	1,2
56	51.18	Cubebol	t	1488	1511	1,2,3
57	51.51	α-Elemene	t	1494	1494	1,2,3
58	51.61	α-Muurolene	t	1495	1508	1,2
59	52.23	β-Bisabolene	0.50±0.04	1506	1506	1,
60	52.85	Calamenene	0.30±0.03	1516	1516	1,2
61	52.99	δ-Cadinene	0.30±0.06	1519	1530	1,2,3

62	53.73	α-Cadinene	0.10±0.02	1532	1495	1,2,3
63	54.00	α-Calacorene	0.10±0.04	1536	1536	1,2,3
64	55.18	*p*-Cymene-2,5-diol	2.50±0.50	1557	1522	1,2
65	55.93	Spathulenol	t	1570	1570	1,2,3
66	56.19	Caryophyllene oxide	0.70±0.20	1574	1574	1,2,3
67	57.69	α-Humulene epoxide	t	1600	1614	1,2
68	58.10	Epicubenol	0.20±0.04	1608	1606	1,2,3
69	59.54	τ-Cadinol acetate	0.10±0.02	1634	1479	1,2
70	60.31	α-Cadinol	0.10±0.03	1648	1647	1,2,3
		Total	95.20			
		Monoterpene hydrocarbons	24.20			
		Oxygenated monoterpenes	66.30			
		Sesquiterpene hydrocarbons	3.40			
		Oxygenated sesquiterpenes	1.30			

[a] The Kovats retention indices determined relative to a series of *n*-alkanes (C_{10}–C_{35}) on the apolar DB-5 [b] Theoretical Kovats index for each compound. Identification method: 1 = comparison of the Kovats retention indices with published data, 2 = comparison of mass spectra with those listed in the NIST 02 and Wiley 275 libraries and with published data, and 3 = co-injection with authentic compounds. t = traces < 0.05

Oxygenated monoterpenes represented 66.30% of the EO, while sesquiterpenes hydrocarbons accounted for only 3.40%. The major compound was carvacrol (57.70%), followed by *p*-cymene (20.20%) and *p*-cymene-2,5-diol (2.50%). Caryophyllene was the most abundant sesquiterpene, present at 1.40%. The chemical composition of this sample closely resembled that of *Origanum dictamnus* EOs previously studied by Liolios (2009) and Ntalli (2010) where carvacrol was also the predominant constituent, followed by *p*-cymene. Our data partially agreed with the available literature: in fact, carvacrol was consistently the main component (Mitropoulou et al., 2015; Solomou et al., 2024; Economakis et al., 1999) but the amounts of other components often differed from our sample. In these studies, the authors found a lower percentage of *p*-cymene and a higher amount of γ-terpinene and linalool, compared to the EO here reported ((Mitropoulou et al., 2015; Solomou et al., 2024; Economakis et al., 1999; Argyropoulou et al., 2014). Marrelli et al. (2016) reported a markedly different chemical composition for *O. dictamnus* EO. In their study, monoterpene hydrocarbons dominated the oil, with *p*-cymene as the major component (32.7%), followed by γ-terpinene (12.4%) and carvacrol (14.7%). Additionally,

an EO of *O. dictamnu*s analysed by Daferera and colleagues (2000) exhibited a different chemical composition compared to that reported here: specifically, thymol was the predominant constituent at 78%, followed by *p*-cymene at 10.8%, while carvacrol was absent. The variation in yield and chemical composition can be influenced by several factors, including geo-climatic conditions (such as soil fertilization), the duration of the drying process, and the timing of collection (Olivas et al., 2020). For example, Olivas and colleagues (2020) examined how harvesting time affects the chemical composition of *O. dictamnus* collected from Mexico: they found that the EO content was more diverse during the flowering stage, with the percentage of the main compound, carvacrol, varying between 47.99% and 79.84%.

4.1.1.2 Anti-enzymatic activities

To date, no studies were reported in the literature regarding the anti-enzymatic activities of *Origanum dictamnus* EO. Consequently, this section focuses on analyzing these activities. In Table 2, the IC_{50} values for the inhibition of cholinesterases, tyrosinase, α-glucosidase, and lipase by EO are reported.

Table 2. *IC_{50} (µg/mL) values for cholinesterases, tyrosinase, α-glucosidase, lipase inhibition by O. dictamus EO.*

Enzyme	EO	Galantamine	Kojic acid	Acarbose	Orlistat
AChE	14.19[b] ± 3.5	0.70 [a] ± 0.3	-	-	-
BChE	220.02 [b] ± 6.2	10.25 [a] ± 1.3	-	-	-
Tyrosinase (monophenolase reaction)	543.8 [b] ± 25.6	-	13.41 [a] ± 2.2	-	-
Tyrosinase (diphenolase reaction)	*n.a.*	-	66.73 ± 1.5	-	-
α-Glucosidase	358.7 [a] ± 23.8	-	-	1280.2 [b] ± 3.6	-
α-Amylase	n.a.	-	-	1.2 ± 0.2	-
Lipase	37.8 [b] ± 3.7	-	-	-	11.24 [a] ± 0.8

IC_{50}: inhibitor concentration that determined a 50% inhibition; n.a: not active (IC_{50}> 1 mg/mL). Data are expressed as mean ± standard deviation (n=3) Galantamine, kojic acid, acarbose or orlistat were used as reference standards. Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$. according to one-way ANOVA followed by Tukey's post hoc test

The IC_{50} value represents the concentration required to inhibit 50% of enzymatic activity. Among the tested enzymes, the EO showed the strongest inhibitory effect against AChE, followed, in order of increasing IC_{50}, by lipase, BChE, α-glucosidase, and tyrosinase involved in the monophenolase reaction. Conversely, the EO was not active against tyrosinase in the diphenolase reaction or against α-amylase, as the IC_{50} values for these enzymes exceeded 1 mg/mL (Table 2).

The IC_{50} values obtained in this study for both cholinesterases indicate good inhibitory activity. While several studies have described cholinesterase inhibition by EOs from the *Origanum* genus, none have specifically investigated the EO of *Origanum dictamnus*. Notably, the EO analyzed in this study showed a good inhibitory effect on acetylcholinesterase, with an IC_{50} value lower than those reported for *O. vulgar*e subsp. *glandulosum* Salzm. ex Benth. (IC_{50}: 139.5 µg/mL), *O. onites* L. (IC_{50} = 519 µg/mL), and *O. rotundifolium* Boiss. (IC_{50}: 32 µg/mL) (Özbek et al., 2017). However, our sample was less active respect to *O. syriacum* L. (IC_{50}: 1.7 µg/mL) and *O. ehrenbergii* Boiss. (IC_{50}: 0.3 µg/mL) (Loizzo et al., 2009). In any case, the IC_{50} value obtained in this study was higher than that of galantamine, which was used as a reference compound (0.7 µg/mL). The *O. dictamnus* EO also exhibited inhibitory activity against BChE, with an IC_{50}value comparable to those reported for other *Origanum* species, for which the literature describes IC_{50} values ranging from 0.3 to 538 µg/mL (Özbek et al., 2017; Loizzo et al., 2009; Tepe et al., 2016). *O. dictamnus* EO exhibited inhibitory activity against tyrosinase only in the monophenolase reaction. Few studies have investigated the tyrosinase-inhibiting properties of *Origanum* species to date, and none have specifically focused on *O. dictamnus* EO. The IC_{50} value of the EO in the monophenolase reaction was higher than those reported for *O. ehrenbergii* (IC_{50}: 44 µg/mL) and *O. syriacum* (IC_{50}: 25 µg/mL) by El Khoury et al. (2019). In contrast, another study reported that the EO of *Origanum compactum* Benth. was active against tyrosinase in the diphenolase reaction. This difference in activity was likely related to variations in chemical composition, as the EO of *O. compactum* was rich in carvacrol, as well as in thymol, β-pinene, *o*-cymene and 3-carene. Furthermore, the present study found that the EO of *O. dictamnus* exhibited lower inhibitory activity in the monophenolase reaction than kojic acid, the reference standard with an IC_{50} value of 14.4 µg/mL (Al-Mijalli et al., 2022). The EO was also tested for its inhibitory activity against enzymes involved in metabolic processes, specifically α-amylase, α-glucosidase, and lipase. The results showed that the EO had the strongest inhibitory effect on lipase, followed by moderate

activity against α-glucosidase, while it exhibited no inhibitory activity against α-amylase. To date, only two studies reported the potential inhibitory effects of *Origanum* EOs on α-amylase and α-glucosidase; no study investigated their activity against lipase. Bustanji and co-workers (2018) tested the effect of methanolic extract of *O. syriacum* against lipase, obtaining an IC_{50} of 254 μg/mL (Bustanji et al., 2018). According to the literature, the EO of *O. compactum* showed IC_{50} values between 119 and 150 μg/mL, indicating greater effectiveness than our sample against both α-amylase and α-glucosidase (Assaggaf et al., 2023).

4.1.1.3 Kinetic study

Given the inhibitory activity observed against various enzymes, a kinetic study was conducted to investigate the type of enzyme inhibition and to calculate the inhibition constant (Ki). As shown in Table 3, this constant represents the concentration of the inhibitor required to reduce the rate of the enzymatic reaction to half of its maximum value in the presence of a substrate, and it remains consistent across different experimental conditions.

Table 3. *V_{max}, K_m, K_i values for cholinesterases, α-glucosidase, lipase inhibition by O. dictamus* EO**.**

Enzyme	V_{max} (mmol/min*L)	K_m (mM/mL)	K_i (μg/mL)	$αK_i$ (μg/mL)	Type of inhibition
AChE	8.45 ± 0.78	3.41 ± 0.15	-	5.38	Uncompetitive
BChE	5.34 ± 0.75	2.06 ± 0.56	88.29	-	Mixed
α-glucosidase	7.63 ± 0.94	2.67 ± 0.59	134.8	-	Competitive
Lipase	0.04± 0.01	1.14 ± 0.15	14.09	-	Mixed

V_{max}: maximum rate of enzymatic reaction, K_m: substrate concentration where the corresponding reaction rate is ½ of V_{max}, K_i and $αK_i$: inhibition costant. Data are expressed as mean ± standard deviation (n=3)

Analysis of the Michaelis–Menten and Lineweaver–Burk plots presented in Figures 15–18 demonstrated that *O. dictamnus* EO could inhibit AChE through an uncompetitive mechanism. It could also act as a mixed inhibitor of

BChE and lipase and as a competitive inhibitor of α-glucosidase. Furthermore, the Ki values indicated that AChE was the most sensitive enzyme to the action of the EO, followed by lipase, BChE and α-glucosidase. For AChE, analysis of the Lineweaver–Burk (LB) plot (Fig. 15) showed that the lines were parallel at several points, indicating a mixed inhibition mechanism. Instead, for BChE and lipase, the LB plots (Figs. 16 and 18) showed lines intersecting at a point on the left side of the x-axis (second quadrant), indicating a mixed type of inhibition. Finally, as shown in figure 17, the lines intersect on the y-axis, indicating a type of competitive inhibition for glucosidase.

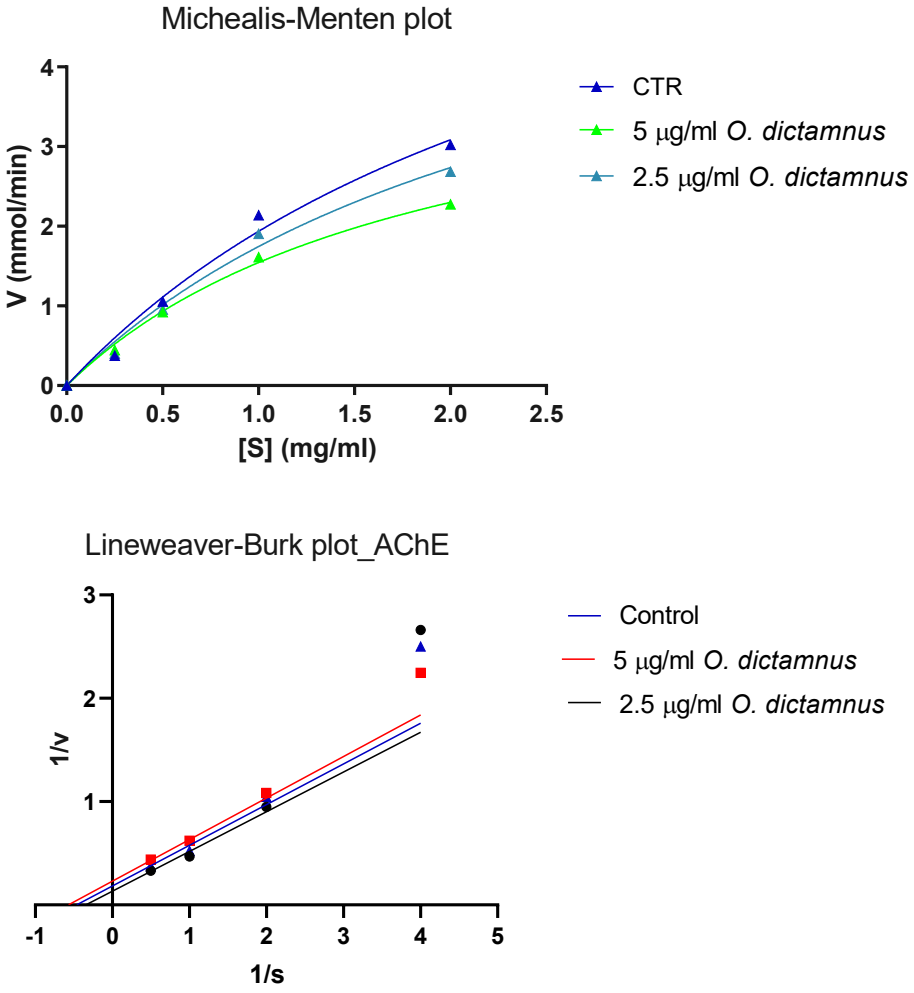


Figure 15. *Michealis- Menten and Lineweaver–Burk plots of AChE inhibition in absence (CTR) or presence of O. dictamnus EO.*

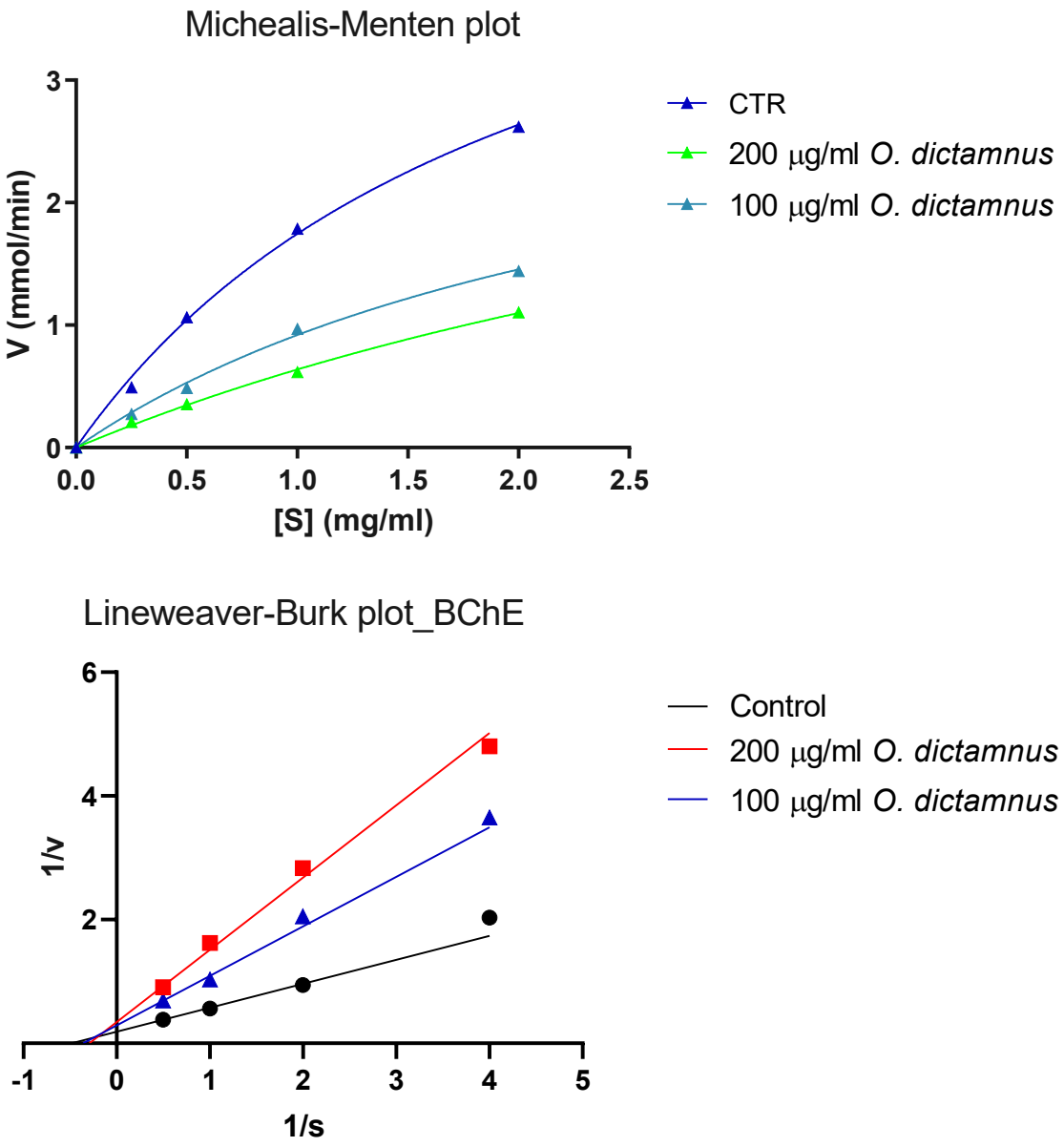


Figure 16. *Michealis- Menten and Lineweaver–Burk plots of BChE inhibition in absence (CTR) or presence of O. dictamnus EO.*

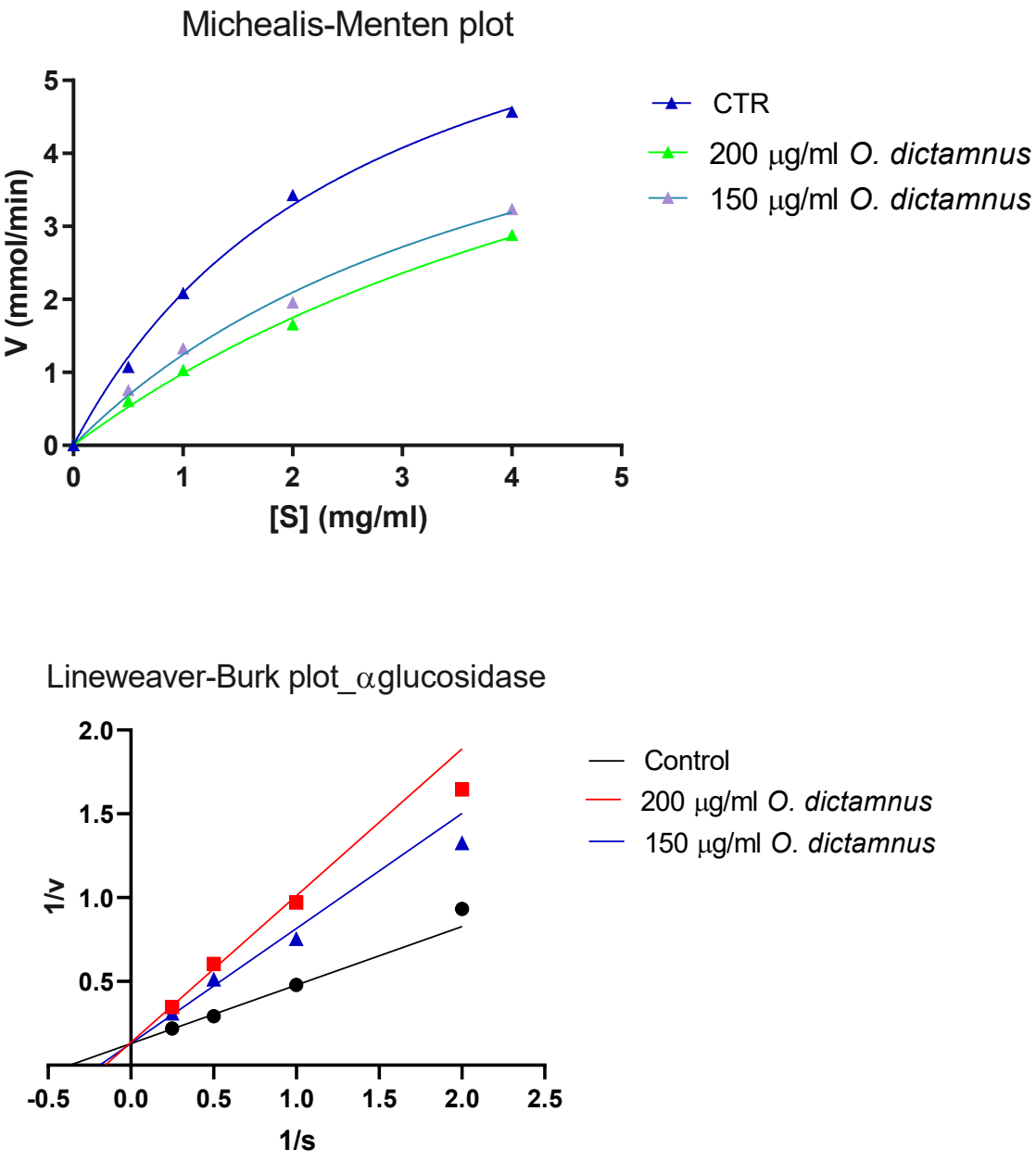


Figure 17. *Michealis- Menten and Lineweaver–Burk plots of α-glucosidase inhibition in absence (CTR) or presence of O. dictamnus EO.*

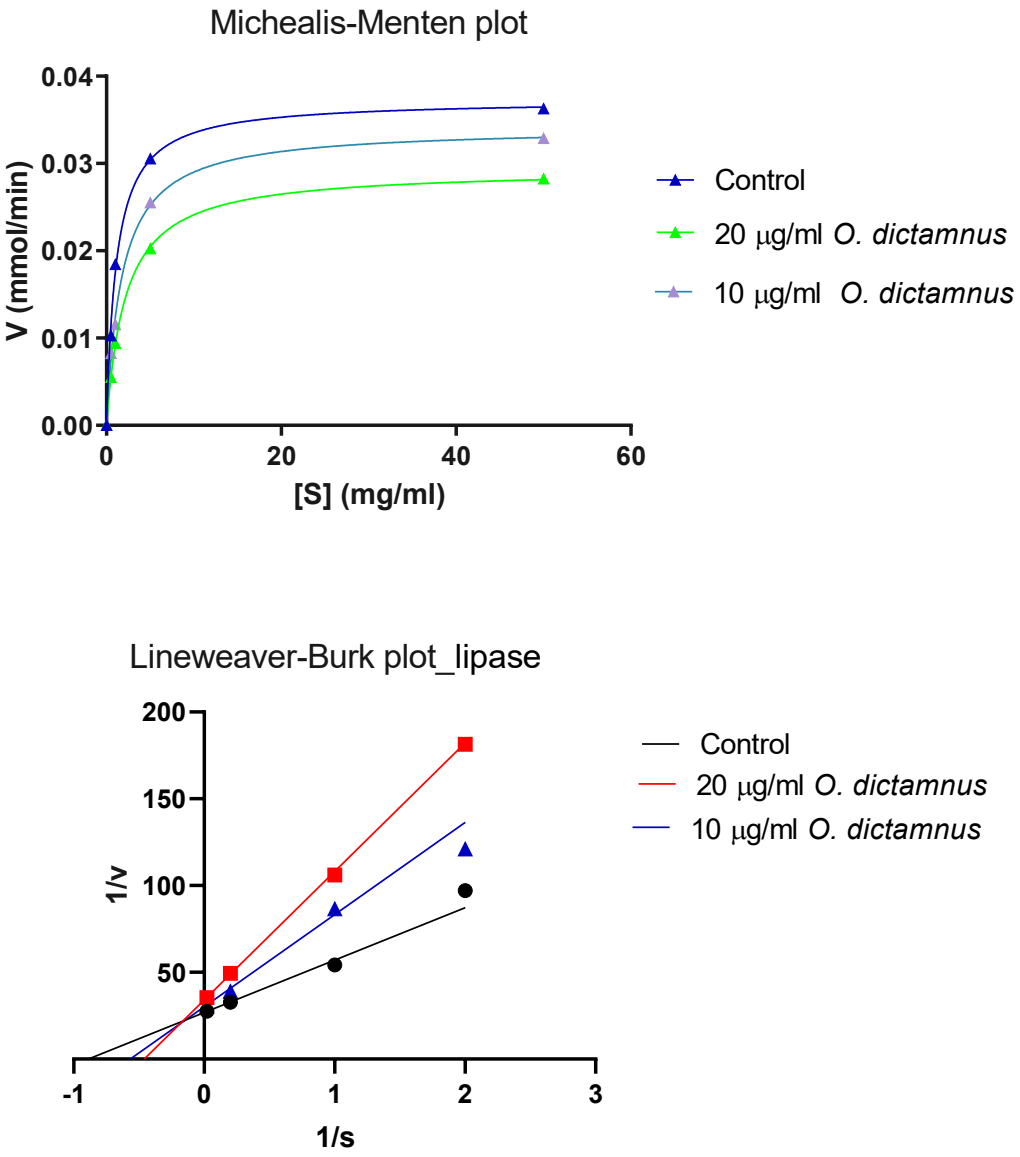


Figure 18. *Michealis- Menten and Lineweaver–Burk plots of lipase inhibition in absence (CTR) or presence of O. dictamnus EO.*

In general, mixed and/or non-competitive inhibitor can be advantageous because they are able to bind both the free enzyme and the enzyme-substrate complex, providing a broader mechanism of action and allowing for therapeutic effects at lower drug concentrations (Copeland, 2023)

4.1.1.4 Molecular Docking

A molecular docking was carried out to investigate the inhibitory effects of carvacrol and *p*-cymene towards AChE, BChE, α-glucosidase, tyrosinase, and lipase, and to estimate their binding affinities to the catalytic sites of the target enzymes. The table 4 summarizes the binding free energies, the number of hydrogen bonds, and the number of interactions in the complexes formed between carvacrol and *p*-cymene from one side, and the active amino acids of AChE, BChE, α-glucosidase, tyrosinase and lipase.

Table 4. *Free binding energies, hydrogen bonding, number of closest residues to the docked compounds carvacrol and p-cymene into the binding sites of acetylcholinesterase, butyrylcholinesterase, α-glucosidase, tyrosinase, and lipase.*

Compound	Free binding energy (kcal/mol)	H-Bonds (HBs)	Number of closest residues to the docked ligand in the active site/Number of Interactions
AChE			
Carvacrol	-6.26	1	3/3
p- Cymene	-5.49	0	4/5
BChE			
Carvacrol	-5.89	1	3/3
p- Cymene	-4.99	0	2/2
α-Glucosidase			
Carvacrol	-6.24	2	5/5
p- Cymene	-5.66	0	5/5
Tyrosinase			
Carvacrol	-5.71	1	10/12
p- Cymene	-5.58	0	10/12
Lipase			
Carvacrol	-5.47	1	6/7
p- Cymene	-5.14	0	3/5

The table 4 and the figs. 19, 20 and 21 reveal that carvacrol and *p*-cymene may possess inhibitory potency against the tested enzymes. They fit relatively well into the binding sites of these enzymes, leading to the formation of stable complexes with binding energies ranging from − 4.99 to − 6.26 kcal mol^{-1} (Table 4). The negative binding energies indicate that the inhibition process is thermodynamically favourable (Table 4). For every enzyme examined, carvacrol exhibits stronger affinity than *p*-cymene, probably due to the presence of a hydroxyl group. This group forms intermolecular hydrogen bond with amino acids of the targets (Figs. 20 and 21). The docking results also revealed that the binding affinities of carvacrol and *p*-cymene depend strongly on the enzyme type: AChE, BChE, α-glucosidase, tyrosinase, and lipase (Figs. 20 and 21). Both ligands interacted most favourably with tyrosinase: indeed, in tyrosinase-carvacrol and tyrosinase-*p*-cymene complexes, many interactions were established between carvacrol and *p*-cymene, and amino acids of tyrosinase (Fig. 19).

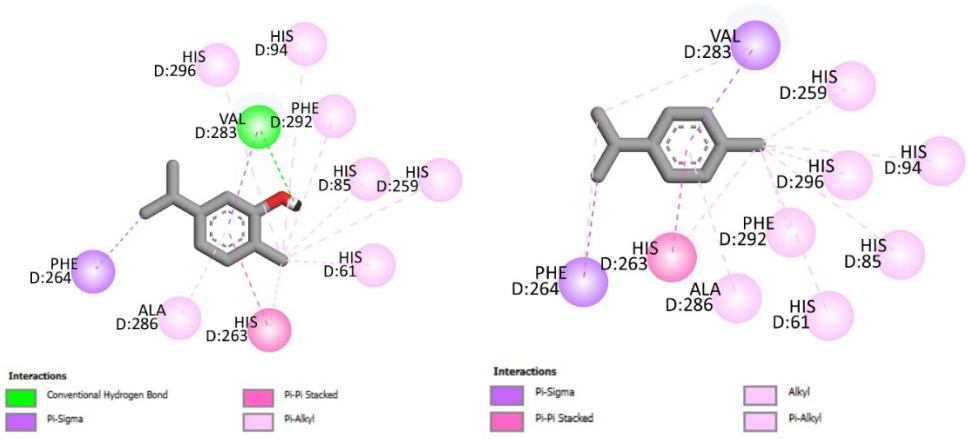
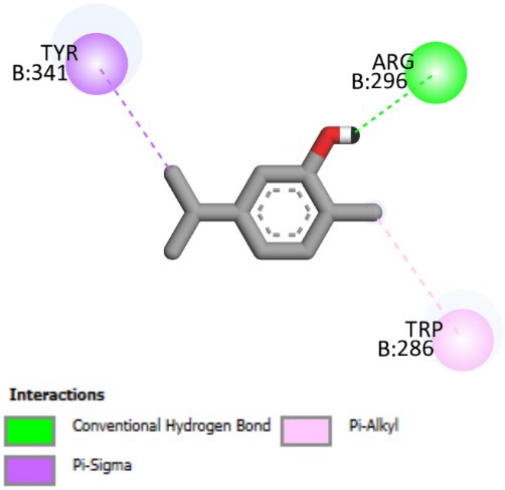
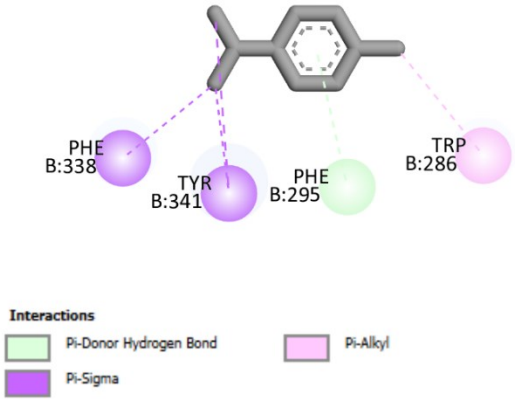


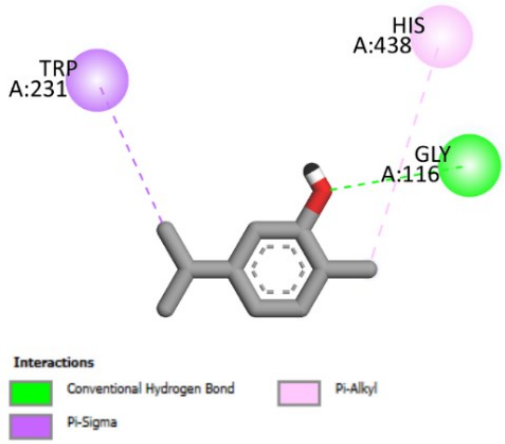
Figure 19. *2D representation of the main interactions between carvacrol and p-cymene and the active site of tyrosinase.*



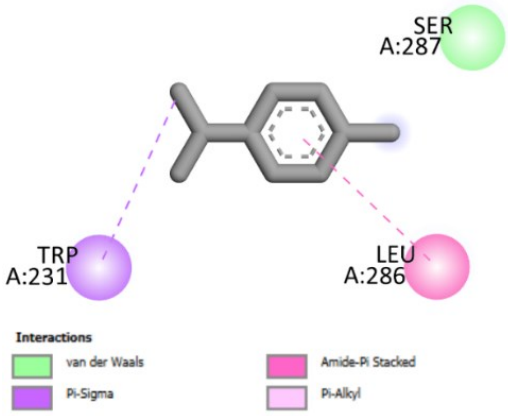
Acetylcholinesterase-Carvacrol complex



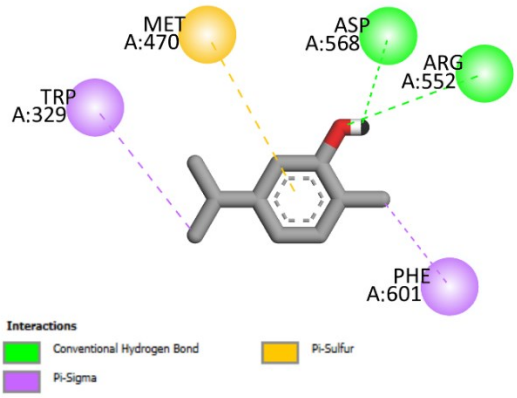
Acetylcholinesterase- *p*-cymene complex



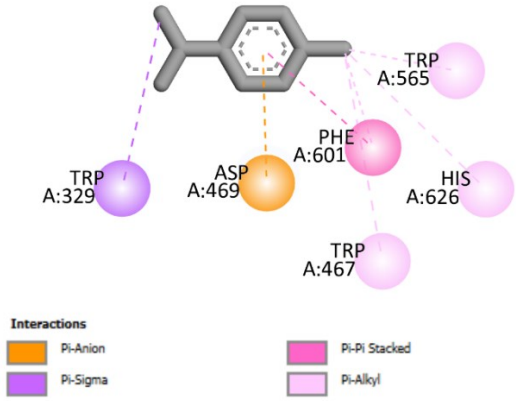
Butyrylcholinesterase-Carvacrol complex



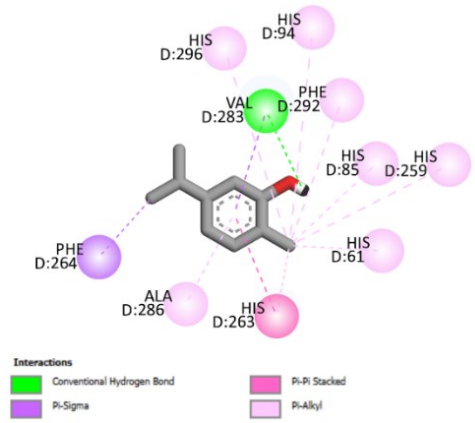
Butyrylcholinesterase- *p*-cymene complex



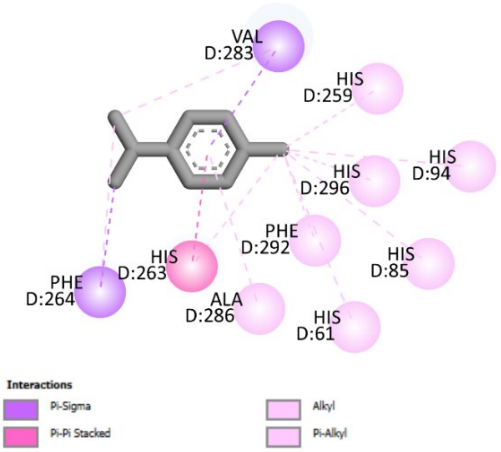
α-Glucosidase-Carvacrol complex



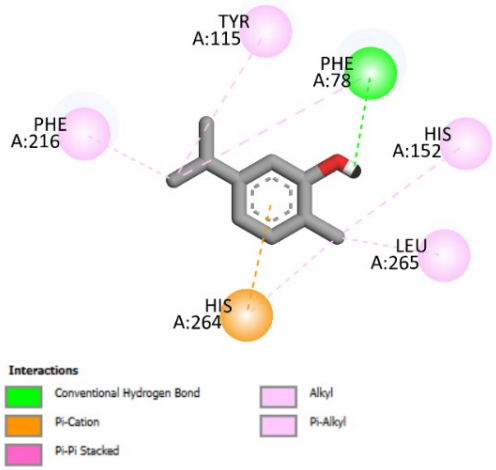
α-Glucosidase- *p*-cymene complex



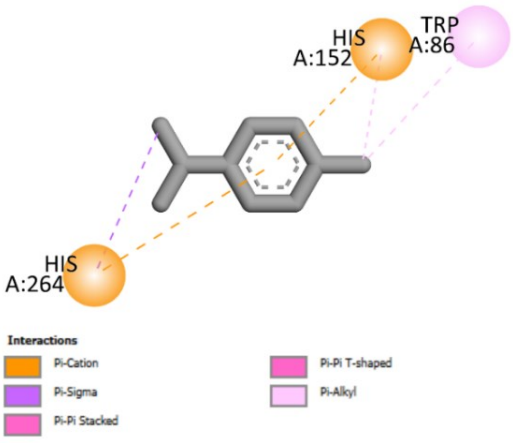
Tyrosinase-Carvacrol complex



Tyrosinase- *p*-cymene complex

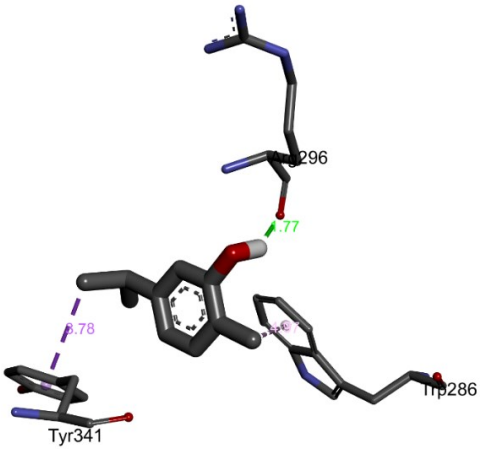


Lipase-Carvacrol complex

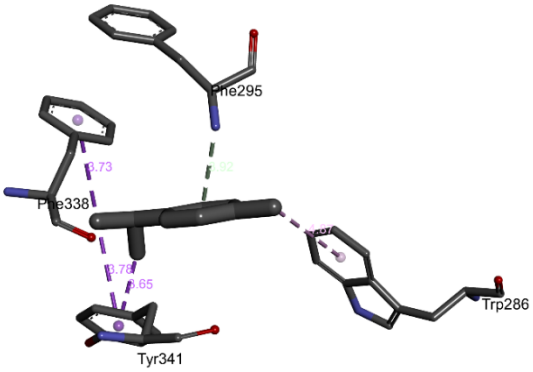


Lipase- *p*-cymene complex

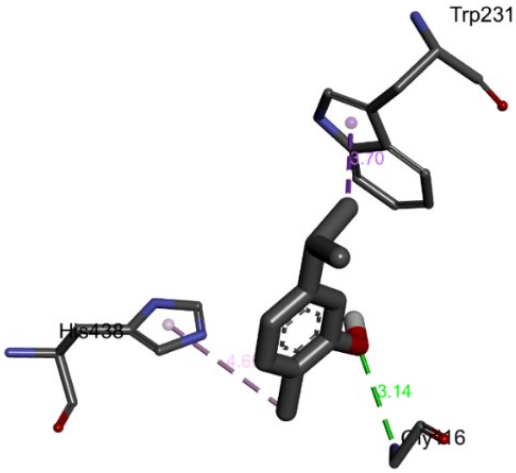
Figure 20. *2D representations of the closest interactions between carvacrol and p-cymene and the active-site residues of AChE, BChE, α-glucosidase, tyrosinase, and lipase.*



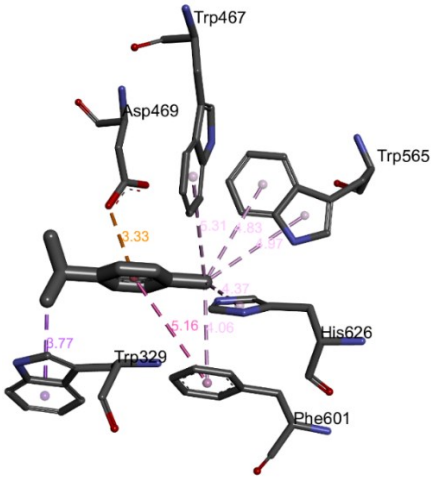
Acetylcholinesterase-Carvacrol complex



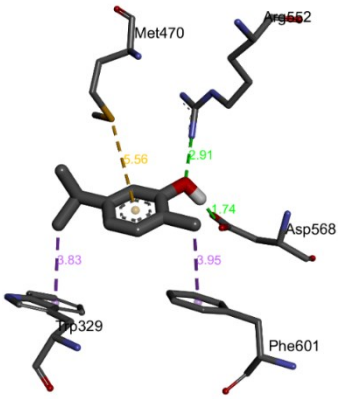
Acetylcholinesterase-*p*-cymene complex



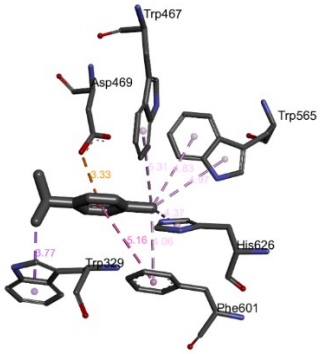
Butyrylcholinesterase-Carvacrol complex



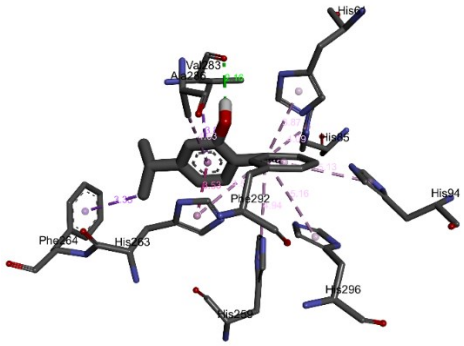
Butyrylcholinesterase- *p*-cymene complex



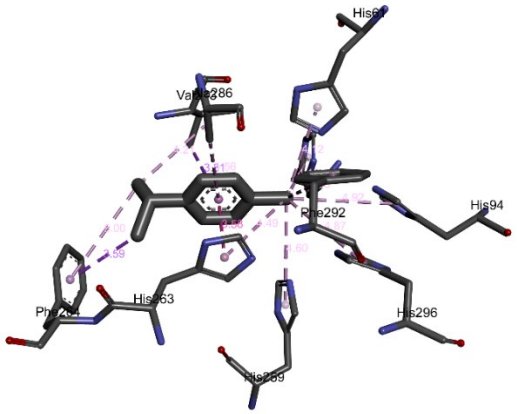
α-Glucosidase-Carvacrol complex



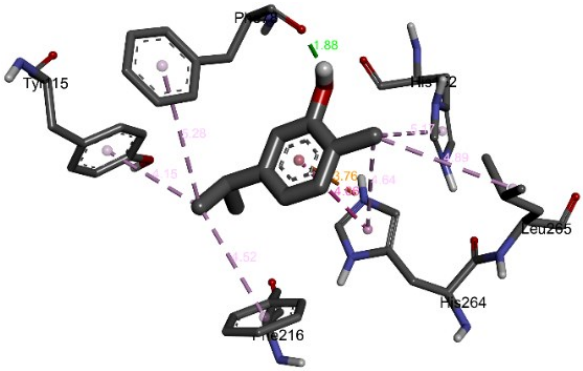
α-Glucosidase- *p*-cymene complex



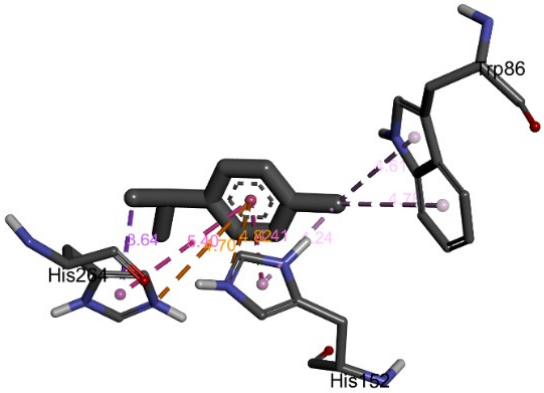
Tyrosinase-Carvacrol complex



Tyrosinase- *p*-cymene complex



Lipase-Carvacrol complex



Lipase- *p*-cymene complex

Figure 21. *3D representations of the closest interactions between carvacrol and p-cymene and the active-site residues of AChE, BChE, α-glucosidase, tyrosinase, and lipase.*

4.1.2 *Non-volatile fraction*

The polar fraction of *O. dictamnus* was studied by comparing two extraction methods: dynamic maceration (conventional method) and pressurized liquid extraction (unconventional method). In the preliminary phase, dynamic maceration was performed using two ethanol–water mixtures, EtOH/H_2O 7:3 (70% EtOH) and EtOH/H_2O 2:8 (20% EtOH) (Jacotet-Navarro et al., 2018), to determine which solvent provided the best antioxidant and enzymatic activities, as well as the highest total phenolic content (TPC), total flavonoid content (TFC), and total carbohydrate content (TCC). The extractions lasted 12 hours, using 4 g of plant material in 100 mL of solvent (ratio 1:25). After solvent removal and freeze-drying, the biological activities and phenolic metabolite content were evaluated. As shown in Tables 5 and 6, the extraction yield, TPC, and TFC were comparable between the two solvents. However, the extract obtained with 20% EtOH showed higher biological activities than that obtained with 70% EtOH. Based on these results, the 20% EtOH solvent was selected for subsequent pressurized liquid extractions.

Table 5. *Comparison of the extraction method by evaluating antioxidant activity, total flavonoid, phenolic and carbohydrate contents*

	Yield (%)	TPC (GAE mg/g)	TFC (QE mg/g)	TCC (GLUE mg/g)	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)
Mac. EtOH 70%	13.72^{a} ± 1.45	111.34^{a} ± 15.50	2.15^{a} ± 0.12	74.55^{a} ± 35.40	53.41^{c} ± 3.40	140.78^{c} ± 10.40
Mac. EtOH 20%	13.00^{a} ± 1.01	123.88^{a} ± 12.97	2.58^{a} ± 0.15	170.15^{b} ± 23.40	35.72^{b} ± 1.90	100.02^{b} ± 10.11
Trolox	-	-	-	-	3.65^{a} ± 0.90	2.05^{a} ± 0.45

Mac.: Maceration. Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$. according to one-way ANOVA followed by Tukey's post hoc test. GAE= Gallic acid equivalent; QE= Quercetine equivalent; GLUE= Glucose equivalent

Table 6. *Comparison of the extraction method by evaluating enzymatic activities. Results were expressed as IC_{50} (µg/mL)*

	AChE	BChE	α-amylase	α-glucosidase	Lipase
Mac. EtOH 70%	436.30^{c} ± 12.90	379.40^{c} ± 13.10	*n.a.*	170.70^{b} ± 12.98	19.95^{b} ± 1.40
Mac. EtOH 20%	349.90^{b} ± 15.30	248.70^{b} ± 17.28	*n.a.*	87.17^{a} ± 15.38	11.98^{a} ± 1.90
Galantamine	0.70 ^{a} ± 0.15	10.25 ^{a} ± 15.30	-	-	-
Acarbose	-	-	10.53 ± 15.30	928.12^{c} ± 15.30	-
Orlistat	-	-	-	-	11.00 ^{a} ± 0.85

Mac.: Maceration Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$. according to one-way ANOVA followed by Tukey's post hoc test. *n.a.: not active*

After identifying the solvent to be used, it was necessary to optimize the PLE extraction temperature before proceeding with the comparison between the two extraction methods (dynamic maceration and PLE). This step is essential because PLE method allows the use of high temperatures, which can favour the extraction of a greater number of bioactive compounds; however, excessive temperatures can lead to the degradation of thermolabile molecules, compromising the quality of the extract (Hossain et al., 2011; Ahmad et al., 2021). For this reason, three different extraction temperatures were tested, 50°C, 115°C, and 180°C, with the aim of optimizing the polyphenol yield and antioxidant capacity of the extracts. As shown in Table 7, 115°C proved to be the optimal temperature, producing extracts with the highest polyphenol and flavonoid contents, as well as the strongest antioxidant activity. Although the highest extraction yield was obtained at 180°C, this did not correspond to greater antioxidant activity, likely due to the degradation of compounds sensitive to high temperatures.

Table 7. *PLE extraction temperature optimization*

PLE Temperature	Yield (%)	TPC (GAE mg/g)	TFC (QE mg/g)	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)
50°	21.50^{a} ± 3.54	209.53^{a} ± 3.17	1.21^{a} ± 0.22	30.30^{d} ± 0.93	124.52^{d} ± 23.54
115°	23.67^{a} ± 2.52	245.83^{b} ± 4.61	4.52^{c} ± 0.12	22.49^{b} ± 1.90	75.25^{b} ± 22.63
180°	43.00^{b} ± 1.41	252.47^{b} ± 8.14	2.42^{b} ± 0.55	26.14^{c} ± 0.77	107.15^{c} ± 26.58
Trolox	-	-	-	3.65^{a} ± 0.90	2.05^{a} ± 0.45

Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$, according to a two-way ANOVA followed by Tukey's post hoc test. GAE= Gallic acid equivalent; QE= Quercetine equivalent.

After selecting the optimal temperature, the two extraction methods were compared. As shown in Tables 8 and 9, the extract obtained by PLE exhibited similar or higher values for all analyzed parameters compared to the extract obtained by dynamic maceration. In particular, the extraction yield was almost twice as high, and both the antioxidant activity and the inhibition of enzymes involved in metabolic disorders were significantly greater. Specifically, the extract obtained by PLE showed IC_{50} value lower than that of Orlistat, the standard drug used as a positive control. Only the activity against cholinesterases was comparable between the two extracts. There are no studies in the literature in which *O. dictamnus* extracts were obtained by pressurized liquid extraction, and in general, research on extracts from this species is quite limited. One of the few available studies, conducted by Letsiou and collaborators, reported a total polyphenol content of 200 mg GAE/g in a methanolic extract obtained by maceration, values comparable to those detected in the PLE extract. Regarding antioxidant activity, both the macerated extract and the PLE extract showed higher activity than the sample analyzed in this study, which had an IC_{50} of 18.69 mg/mL (Letsiou et al., 2023). As for enzymatic activities, no studies examined the effects of *O. dictamnus* extracts on the enzymes considered in the present work.

Table 8. *Comparison of the extraction method by evaluating antioxidant activity, total flavonoid, phenolic and carbohydrate contents*

	Yield (%)	TPC (GAE mg/g)	TFC (QE mg/g)	TCC (GLUE mg/g)	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)
Mac. EtOH 20%	13.00^{a} ± 1.01	123.88^{a} ± 12.97	2.58^{a} ± 0.15	170.15^{a} ± 23.40	35.72^{c} ± 1.90	100.02^{b} ± 10.11
PLE 115° EtOH 20%	23.67^{b} ± 0.94	245.83^{b} ± 17.21	4.52^{b} ± 0.10	183.95^{a} ± 12.38	22.49^{b} ± 0.96	75.25^{b} ± 22.63
Trolox	-	-	-	-	3.65^{a} ± 0.90	2.05^{a} ± 0.45

Mac. Maceration; PLE: Pressure Liquid Extraction. Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$. according to one-way ANOVA followed by Tukey's post hoc test. GAE= Gallic acid equivalent; QE= Quercetine equivalent; GE= Glucose equivalent

Table 9. *Comparison of the extraction method by evaluating enzymatic activities. Results were expressed as IC_{50} (µg/mL)*

	AChE	BChE	α-amylase	α-glucosidase	Lipase
Mac. (EtOH 20%)	349.90^{b} ± 15.30	248.70^{b} ± 17.28	*n.a.*	87.17^{b} ± 15.38	11.98^{ab} ± 1.90
PLE 115° (EtOH 20%)	377.20^{b} ± 17.20	236.60^{b} ± 15.45	*n.a.*	36.80^{a} ± 10.17	8.90^{a} ± 0.68
Galantamine	0.70^{a} ± 0.15	10.25^{a} ± 15.30	-	-	-
Acarbose	-	-	10.53 ± 15.30	928.12^{c} ± 15.30	-
Orlistat	-	-	-	-	11.00^{ab} ± 0.85

Mac. Maceration; PLE: Pressure Liquid Extraction. Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$. according to one-way ANOVA followed by Tukey's post hoc test. *n.a.: not active*

After finding that the extract obtained by PLE showed the most promising biological activities, its chemical composition was further investigated by liquid chromatography coupled to mass spectrometry, using electrospray ionization (ESI) and a QTOF analyzer. This analysis allowed the identification of approximately 30 secondary metabolites, mainly phenolic acids and flavonoids, as well as several organic acids (Figure 22 and Table 10).

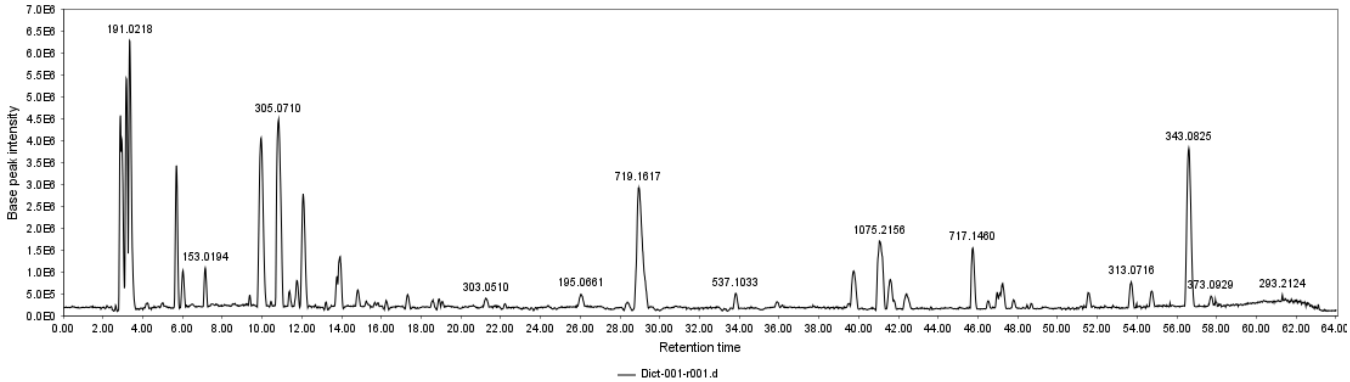


Figure 22. *LC- (-) HRMS base peak chromatogram of O. dictamnus PLE extract*

Table 10. LC-MS chemical composition of hydroalcoholic extracts of *O. dictamnus*

Rt (min)	[M-H]$^-$	Main fragments (m/z)	Putative identification	Peak Area	λ max (nm)	Formula (M)	Diff (ppm)	Family
3.2	133.0157	115.0505; 71.0146	Malic acid	5.91E^{+05}	280	$C_4H_6O_5$	1.56	Organic acid
5.69	395.0988	Dimerization						
	197.0463	179.0356; 135.0457; 72.9938	Syringic acid	1.61E^{+05}	280	$C_9H_{10}O_5$	0.27	Phenolic acid
6.02	167.0348	140.7979; 122.0357; 108.1121; 91.0187	Vanilic acid	1.43E^{+05}	280	$C_8H_8O_4$	-1.08	Phenolic acid
7.11	153.0196	109.0291; 91.0181	Protocatechuic acid	1.38E^{+05}	260	$C_7H_6O_4$	1.74	Phenolic acid
9.93	137.0246	108.0196; 91.0175; 75.0794	Hydroxybenzoic acid	8.26E^{+05}	270; 320	$C_7H_6O_3$	1.32	Phenolic acid
10.85	305.0704	225.1135; 96.9607; 79.9580	Gallocatechine	9.11E^{+05}	275;320	$C_{15}H_{14}O_7$	-4.02	Flavonoids
11.74	177.0198	133.0268; 105.0348; 77.0387	Aesculetin	7.17E^{+04}	270; 351	$C_9H_6O_4$	2.63	Phenolic acid
11.75	593.1504	575.142; 473.1082; 383.0769; 353.0672	Apigenin-di-*C*-hexoside	1.19E^{+05}	290;326	$C_{27}H_{30}O_{15}$	0.68	Flavonoids
12.01	373.1503	327.1449; 190.0974; 164.0841; 149.0603; 134.0375	Deoxyloganin	6.66E^{+04}	290;328	$C_{17}H_{26}O_9$	-0.28	Organic acid
12.07	179.035	135.0452; 107.0505; 107.0505; 79.0559	Caffeic Acid	4.65E^{+05}	290;327	$C_9H_8O_4$	0.1	Phenolic acid
13.2	813.1373	527.0875; 351.0567; 285.0392; 193.0349	Kaempferol-*O*-triglucuronide isomer	4.10E^{+04}	280; 340	$C_{33}H_{34}O_{24}$	0.71	Flavonoids
13.85	637.1041	351.0584; 285.0421; 193.0365; 175.0245	Luteolin-7-*O*-diglucuronide	2.36E^{+05}	280; 340	$C_{27}H_{26}O_{18}$	-0.84	Flavonoids
14.84	447.0927	357.0617; 327.0501; 299.0551; 133.0288	Homoorientin (Luteolin 6-*C*-glucoside)	1.04E^{+05}	270; 350	$C_{21}H_{20}O_{11}$	-1.31	Flavonoids
15.68	447.0931	357.0588; 327.0502; 387.0402; 193.0133; 133.0287	Orientin (Luteolin 8-*C*-glucoside)	5.24E^{+04}	270; 350	$C_{21}H_{20}O_{11}$	-0.41	Flavonoids
16.22	797.142	527.0889; 351.0558; 269.0455; 193.0362	Apigenin-*O*-triglucuronide isomer	5.55E^{+04}	260; 340	$C_{33}H_{34}O_{23}$	0.24	Flavonoids
17.31	621.1095	351.0569; 269.0463; 193.0347; 153.0604; 113.0243	Apigenin-7-*O*-diglucuronide	8.17E^{+04}	260; 340	$C_{27}H_{26}O_{17}$	-0.36	Flavonoids
18.6	431.0984	341.0675; 311.0561; 283.0614; 239.0724; 197.0596; 163.0399; 117.0357	Vitexin	7.10E^{+04}	270; 340	$C_{21}H_{20}O_{10}$	0.07	Flavonoids

21.24	303.051	285.0367; 241.0508; 223.038; 189.0547; 152.0113; 125.0254; 105.0359	Taxifolin	$9.77E^{+04}$	280	$C_{15}H_{12}O_7$	-0.09	Flavonoids
22.25	813.1525	635.1451; 527.1004; 461.0693; 443.0527; 351.0541; 333.0429; 285.038	Kaempferol-*O*-triglucuronide isomer	$5.23E^{+04}$	340	$C_{37}H_{34}O_{21}$	0.64	Flavonoids
29.09	719.1627	Dimerization						
	359.0769	197.0447; 179.0344; 161.0247; 133.0295; 109.0286	Rosmarinic acid	$5.91E^{+05}$	340	$C_{18}H_{16}O_8$	-0.95	Phenolic acid
33.82	537.1036	499.2755; 472.039; 359.0729; 295.0626; 251.0718; 197.0461; 161.0243; 135.0453	Salvianolic acid I	$1.06E^{+03}$	340	$C_{27}H_{22}O_{12}$	-0.46	Phenolic acid
39.72	491.0982	311.0542; 267.0642; 135.0443	Salvianolic acid C	$2.28E^{+05}$	260	$C_{26}H_{20}O_{10}$	-0.35	Phenolic acid
41.08	493.1137	422.7864; 359.0757; 251.0703; 197.0457; 179.0344; 161.0248; 135.0452	Salvianolic acid A isomer II	$4.57E^{+05}$	280; 320	$C_{26}H_{22}O_{10}$	-0.65	Phenolic acid
41.68	285.0405	243.0307; 218.0538; 197.0619; 151.0049; 133.0299; 107.0143	Luteolin	$1.76E^{+05}$	285	$C_{15}H_{10}O_6$	0.13	Flavonoids
45.72	717.1456	519.0932; 359.0779; 339.0507	Salvianolic acid E	$2.54E^{+05}$	260	$C_{36}H_{30}O_{16}$	-0.71	Phenolic acid
46.58	329.0669	300.0186; 178.9998; 117.0348; 67.0199	Cirsiliol	$6.00E^{+04}$	290; 340	$C_{17}H_{14}O_7$	0.68	Flavonoids
47.29	269.0456	225.075; 177.0159; 151.0031; 117.0345	Apigenin	$1.39E^{+05}$	285	$C_{15}H_9O_5$	0.2	Flavonoids
47.78	359.077	329.0278; 270. 0126; 180.9767; 151.0032; 132.0210	Thymonin	$6.79E^{+04}$	285	$C_{18}H_{16}O_8$	-0.67	Flavonoids
48.46	313.0714	313.071; 283.0244; 227.0346; 183.0436; 163.0034; 135.0073; 117.0341	Cirsimaritin	$3.60E^{+04}$	280; 340	$C_{17}H_{14}O_6$	-1.15	Flavonoids
56.59	343.0825	313.0347; 298.0114; 117.0340	Xanthomicrol	$8.14E^{+05}$	280; 340	$C_{18}H_{16}O_7$	0.5	Flavonoids

Carboxylic acids and simple phenols tend to be decarboxylated when present as negative ions (carboxylates or phenoxides). In this form, CO_2 loss is favourable, as the negative ion stabilizes the reaction intermediate. Among the identified organic acids, malic acid exhibited a fragmentation characterized by the loss of simple molecules such as water (133 → 115 Da). Phenolic acids were identified both through comparison with the literature and through characteristic fragmentations. For example, for protocatechuic acid, the 153 → 109 Da transition confirmed the loss of CO_2, while for caffeic acid, the 179 → 135 Da fragmentation was typical of catechol derivatives. In vanillic acid, however, cleavage of the $–OCH_3$ group was evident, consistent with what was reported for substituted aromatic structures (de Torre et al, 2022; Zengin et al., 2019; Petrakis et al., 2023). In glycosylated flavonoids, differences emerged between *O*- and *C*-glycosides. *O*-glycosylated or glucuronidated flavonoids, such as luteolin-7-O-diglucuronide and the triglucuronide derivatives of apigenin and kaempferol, exhibited typical losses of sugar units (-162 Da for glucose and -176 Da for glucuronic acid), resulting in the appearance of fragments corresponding to the aglycone (m/z 269 for apigenin, m/z 285 for kaempferol and luteolin) (Maietta et al., 2018). *C*-glycosides, such as orientin, homoorientin, and vitexin, exhibited characteristic losses of 120, 90, and 60 Da due to transverse fragmentation of the sugar ring, demonstrating the greater stability of the carbon-carbon bond compared to the *O*-glycosidic bond (de Torre et al, 2022; Petrakis et al., 2023). Flavonoid aglycones, including luteolin, apigenin, cirsimaritin, and cirsiliol, exhibited fragmentation patterns typical of flavones, with loss of CO and CO_2 and retro-Diels-Alder cleavages generating ions in the m/z region of 150–180 (Tuttolomondo et al, 2018; Petrakis et al., 2023). Finally, rosmarinic acid and salvianolic acids, consisting of condensed caffeic acid units, exhibited characteristic fragments at m/z 197, 179, 161, and 135, consistent with successive cleavages of the catechol moiety. In particular, the MS/MS spectrum of rosmarinic acid showed fragments at m/z 197 (loss of the caffeoyl group) and at m/z 161 (loss of two H_2O molecules) (de Torre et al, 2022; Zengin et al., 2019; Petrakis et al., 2023; Taamalli et al., 2015).

4.2 Conduct a comparative interspecific evaluation of EOs from Italian *Origanum* species (*O. majorana*, *O. vulgare*, and *O. heracleoticum*)

4.2.1 Chemical composition

GC-MS analysis allowed the identification of a total of 91 compounds. As reported in Table 11, the three EOs of *Origanum vulgare*, *Origanum majorana*, and *Origanum heracleoticum* showed differences in both chemical composition and yield. Specifically, the EO of *O. vulgare* exhibited a higher yield (0.52%) compared to *O. majorana* (0.29%) and *O. heracleoticum* (0.15%). The EO of *O. vulgare* was characterized by a predominance of aromatic monoterpenes, which accounted for 79.32% of its composition. Among these, the most abundant compounds were carvacrol (55.98%) and *p*-cymene (18.15%). However, the concentration of oxygenated monoterpenes was relatively low (2.44%), as well as that of hydrocarbon sesquiterpenes (2.40%) and oxygenated sesquiterpenes (0.40%). In contrast, the EO of *Origanum majorana* showed a predominance of hydrocarbon monoterpenes (61.33%) and oxygenated monoterpenes (28.39%). The main compounds were γ-terpinene (19.11%), terpinene-4-ol (16.74%), and α-terpinene (13.82%). However, aromatic monoterpenes, such as carvacrol (2.26%), were present in lower amounts compared to the other samples. The EO of *Origanum heracleoticum* exhibited a different chemical profile compared to the other two samples. Specifically, it was characterized by both aromatic monoterpenes (38.91%), such as carvacrol (15.79%) and thymol (12.21%), as well as hydrocarbon monoterpenes (28.85%) and sesquiterpenes (2.42%). Among the hydrocarbon monoterpenes, γ-terpinene (14.20%) was the most abundant, while among the sesquiterpenes, germacrene D (9.44%) and caryophyllene (7.33%) were the most represented in the EO.

Table 11. *Chemical composition of the EOs of O. vulgare, O. majorana and O. heracleoticum*

	RT	Compound	*O. vulgare*	*O. majorana*	*O. heracleoticum*	KI[a]	KI[b]	Identification
1	12.8	α-Thujene	0.85 ± 0.10	2.26 ± 0.25	1.01 ± 0.14	927	1110	1.2.3
2	13.1	α-Pinene	1.53 ± 0.50	0.73 ± 0.10	0.41 ± 0.05	932	932	1.2.3
3	14.0	Camphene	0.43 ± 0.20	0.04 ± 0.01	t	945	945	1.2.3
4	14.5	Dehydrosabinene	0.02 ± 0.01	-	-	951	953	1.2.3
5	15.9	Sabinene	-	6.76 ± 0.34	0.85 ± 0.05	970	968	1.2.3
6	16.0	β-Pinene	0.24 ± 0.05	-	-	971	971	1.2
7	16.7	1-Octen-3-ol	0.60 ± 0.07	-	0.70 ± 0.04	981	982	1.2
8	17.2	3-Octanone	-	-	0.46 ± 0.08	988	988	1.2.3
9	17.4	β-Myrcene	1.60 ± 0.05	1.58 ± 0.08	1.49 ± 0.04	991	991	1.2.3
10	17.9	3-Octanol	-	-	0.21 ± 0.07	997	999	1.2.3
11	18.1	α-Phellandrene	0.25 ± 0.07	1.40 ± 0.04	0.20 ± 0.03	1000	1000	1.2.3
12	18.5	3-Carene	0.11 ± 0.06	-	t	1005	1005	1.2.3
13	19.0	α-Terpinene	2.07 ± 0.14	13.82 ± 0.24	2.29 ± 0.17	1012	1022	1.2.3
14	19.8	*p*-Cymene	18.15 ± 0.80	1.89 ± 0.70	5.3 ± 0.21	1023	1023	1.2.3
15	19.9	Limonene	0.67 ± 0.04	4.91 ± 0.25	0.40 ± 0.04	1025	1025	1.2.3
16	20.0	1.8-Cineole	0.1 ± 0.01	0.22 ± 0.04	0.14 ± 0.02	1026	1026	1.2.3
17	20.9	*trans*-β-Ocimene	0.11 ± 0.04	0.04 ± 0.01	3.06 ± 0.10	1038	1038	1.2.3
18	21.6	*cis*-β-Ocimene	0.03 ± 0.01	0.06 ± 0.02	3.01 ± 0.07	1048	1042	1.2.3
19	22.1	γ-Terpinene	6.6 ± 0.21	19.11 ± 0.24	14.2 ± 0.23	1055	1055	1.2.3
20	22.7	*cis*-Sabinene hydrate	0.15 ± 0.04	1.91 ± 0.09	0.45 ± 0.07	1063	1058	1.2.3
21	24.3	Terpinolene	0.21 ± 0.09	4.70 ± 0.04	0.11 ± 0.02	1084	1084	1.2.3

22	24.3	*p*-Cymenene	0.05 ± 0.03	-	-	1085	1084	1.2
23	24.9	*trans*-Sabinene hydrate	-	4.01 ± 0.07	-	1094	1094	1.2
24	25.3	Linalool	0.19 ± 0.04	1.29 ± 0.09	2.59 ± 0.12	1099	1099	1.2
25	26.3	β-Thujone	0.03 ± 0.01	0.10 ± 0.01	-	1112	1101	1.2.3
26	26.6	*p*-2-Menthen-1-ol	0.05 ± 0.02	1.38 ± 0.14	-	1117	1116	1.2.3
27	27.5	1.3.8-*p*-Menthatriene	-	0.02 ± 0.01	-	1130	1118	1.2
28	27.7	Pinocarveol	-	0.04 ± 0.01	-	1132	1132	1.2.3
29	27.8	(4E,6Z)-*allo*-Ocimene	-	-	0.46 ± 0.03	1134	1134	1.2
30	28.0	*cis*-*p*-Mentha-2.8-dien-1-ol	-	-	t	1136	1135	1.2
31	28.0	2-Bornanone	-	0.17 ± 0.07	-	1137	1137	1.2
32	28.1	Camphor	-	0.05 ± 0.01	t	1138	1138	1.2.3
33	28.7	*trans*-2-Caren-4-ol	-	0.02 ± 0.01	-	1145	1153	1.2
34	29.7	*trans*-Borneol	0.62 ± 0.11	0.08 ± 0.03	0.08 ± 0.01	1160	1160	1.2
35	30.6	Terpinen-4-ol	1.15 ± 0.21	16.74 ± 0.14	0.49 ± 0.05	1172	1172	1.2
36	31.3	*p*-Cymen-8-ol	0.04 ± 0.01	-	-	1182	1182	1.2
37	31.8	α-Terpineol	0.21 ± 0.03	2.95 ± 0.19	0.22 ± 0.08	1189	1191	1.2
38	31.9	*trans*-Piperitol	-	0.32 ± 0.04	-	1191	1191	1.2
39	32.8	Dihydrocarvone	0.09 ± 0.02	0.16 ± 0.01	-	1204	1207	1.2.3
40	33.3	*cis*-Piperitol	0.03 ± 0.01	0.54 ± 0.09	-	1211	1211	1.2.3
41	33.6	*trans*-Carveol	-	0.02 ± 0.01	-	1216	1217	1.2.3
42	33.7	γ-Terpineol	-	0.06 ± 0.01	-	1217	1201	1.2.3
43	34.2	*cis*-Verbenyl, acetate	0.02 ± 0.01	0.02 ± 0.01	-	1225	1257	1.2.3
44	34.4	*cis*-Geraniol	-	0.03 ± 0.01	-	1227	1224	1.2.3
45	34.9	Thymol methyl ether	-	-	0.77 ± 0.07	1234	1240	1.2
46	35.4	Carvacrol methyl ether	0.53 ± 0.08	0.03 ± 0.01	4.84 ± 0.26	1242	1244	1.2

47	35.9	Carvone	0.05 ± 0.02	-	-	1249	1243	1.2
48	36.1	γ-Terpineol	-	0.94 ± 0.12	-	1252	1252	1.2.3
49	36.3	*p*-Menth-6-en-2-one	0.02 ± 0.01	-	-	1254	1256	1.2.3
50	36.3	Geraniol	-	0.03 ± 0.01	-	1255	1255	1.2
51	36.5	Linalyl acetate	-	1.94 ± 0.21	0.54 ± 0.09	1258	1260	1.2.3
52	38.1	Anethole	0.10 ± 0.02	0.26 ± 0.03	-	1282	1284	1.2
53	39.0	Thymol	4.49 ± 0.35	0.03 ± 0.01	12.21 ± 0.21	1294	1294	1.2.3
54	39.2	4-Terpinenyl acetate	-	1.27 ± 0.34	-	1298	1300	1.2.3
55	40.0	Carvacrol	55.98 ± 1.34	2.26 ± 0.21	15.79 ± 0.41	1310	1311	1.2
56	41.5	γ-Elemene	-	0.13 ± 0.04	0.17 ± 0.08	1332	1332	1.2.3
57	42.4	α-Terpinyl acetate	-	0.05 ± 0.01	-	1347	1350	1.2.3
58	42.9	Eugenol	0.02 ± 0.01	0.04 ± 0.01	-	1355	1355	1.2
59	43.5	Nerol acetate	-	0.04 ± 0.01	-	1364	1364	1
60	43.6	β-Ylangene	-	-	0.05 ± 0.01	1366	1366	1.2
61	43.9	Copaene	0.04 ± 0.02	0.04 ± 0.01	0.19 ± 0.03	1370	1370	1.2.3
62	43.9	Geranyl acetate	-	0.07 ± 0.02	-	1371	1372	1.2.3
63	44.4	β-Bourbonene	0.03 ± 0.01	-	0.67 ± 0.03	1378	1375	1.2.3
64	44.9	Carvacrol acetate	0.02 ± 0.01	-	t	1385	1367	1.2
65	44.9	α-Ylangene	-	-	0.15 ± 0.05	1386	1386	1.2.3
66	45.0	β-Elemene	-	-	0.11 ± 0.04	1388	1390	1.2.3
67	46.1	Methyleugenol	0.03 ± 0.01	-	-	1405	1403	1.2
68	46.5	Caryophyllene	1.02 ± 0.12	2.44 ± 0.19	7.33 ± 0.24	1412	1412	1.2.3
69	47.2	β-Copaene	-	-	0.34 ± 0.05	1423	1423	1.2
70	47.7	Aromandendrene	0.04 ± 0.01	0.09 ± 0.01	0.05 ± 0.01	1431	1435	1.2.3
71	48.1	Isogermacrene D	-	-	0.09 ± 0.01	1438	1438	1.2.3

72	48.6	Humulene	0.12 ± 0.04	0.11 ± 0.03	0.73 ± 0.14	1446	1446	1.2
73	49.1	Alloaromadendrene	-	-	0.22 ± 0.01	1453	1450	1.2.3
74	50.1	Germacrene D	-	-	9.44 ± 0.71	1470	1472	1.2.3
75	50.2	γ-Muurolene	0.03 ± 0.01	-	-	1471	1471	1.2
76	51.2	Ledene	0.03 ± 0.01	-	-	1488	1488	1
77	51.3	Bicyclogermacrene	-	0.95 ± 0.06	1.03 ± 0.12	1490	1495	1.2
78	51.6	α-Muurolene	0.02 ± 0.01	-	0.28 ± 0.07	1495	1508	1.2.3
79	52.2	β-Bisabolene	0.62 ± 0.08	-	0.77 ± 0.03	1506	1506	1.2.3
80	52.4	α-Farnesene	-	-	2.02 ± 0.24	1509	1509	1.2.3
81	52.9	Calamenene	0.10 ± 0.04	-	-	1516	1516	1.2
82	53.0	Cadina-1(10).4-diene	-	-	1.45 ± 0.14	1519	1519	1.2.3
83	55.1	Thymoquinol	0.15 ± 0.03	-	-	1555	1560	1.2.3
84	55.4	Caryophyllen alcohol	0.01 ± 0.01	-	-	1561	1562	1.2.3
85	55.9	Germacrene D-4-ol	-	-	0.75 ± 0.05	1569	1569	1.2
86	55.9	Spathulenol	0.02 ± 0.01	0.36 ± 0.03	-	1570	1570	1.2.3
87	56.2	Caryophyllene oxide	0.22 ± 0.05	0.33 ± 0.02	0.65 ± 0.07	1574	1574	1.2.3
88	57.7	α-Humulene epoxide	0.02 ± 0.01	-	-	1600	1614	1.2
89	59.6	τ-Cadinol	-	-	0.25 ± 0.01	1634	1634	1
90	60.3	α-Cadinol	-	-	0.28 ± 0.04	1648	1647	1.2
91	62.1	Shyobunol	-	-	0.49 ± 0.05	1681	1681	1
		Identified compounds	56	56	56			
		Monoterpene hydrocarbons (%)	15.52	61.33	28.85			
		Oxygenated monoterpenes (%)	2.44	28.39	4.52			
		Aromatic monoterpenes (%)	79.32	4.49	38.91			
		Sesquiterpenes hydrocarbons (%)	2.10	3.83	25.09			
		Oxygenated sesquiterpenes (%)	0.40	0.69	2.42			

Yield (%)	0.52	0.29	0.15

[a] The Kovats retention indices determined relative to a series of *n*-alkanes (C_{10}–C_{35}) on the apolar DB-5. [b] Theoretical Kovats index for each compound. Identification method: 1 = comparison of the Kovats retention indices with published data, 2 = comparison of mass spectra with those listed in the NIST 02 and Wiley 275 libraries and with published data, and 3 = co-injection with authentic compounds. t = traces < 0.05; - = absent. Peak area normalization was used to get the component relative concentrations.

In the literature, there are studies both supporting the chemical composition observed in this work and highlighting differences. For instance, Simirgiotis et al. (2020) analyzed an EO of *Origanum vulgare* with a chemical composition distinct from that reported here. In our analysis, carvacrol and *p*-cymene were identified as the major components, accounting for 55.98% and 18.15% of the composition, respectively. However, in the study by Simirgiotis and colleagues (2020), these compounds represented only 3.1% and 8.6%, respectively. Their sample was instead dominated by thymol (15.9%) and γ-terpinene (10.6%), both of which were present at lower levels (<10%) in our sample. Similarly, the study by Ilic et al. (2022) also reported a different chemical profile for the EO of *O. vulgare*. In their analysis, *p*-cymene was found in lower concentrations (1.5%) compared to our findings (18.15%). Moreover, their main constituents were caryophyllene oxide and germacrene D, which were detected in our sample but in much lower proportions. Conversely, our findings align with those of De Mastro et al. (2017) and Drăgan et al., (2022) where carvacrol was also identified as the primary constituent, although the reported percentages varied between studies.

For the EO of *O. majorana*, the composition obtained is consistent with that reported in the literature (Ragab et al., 2019; Partovi et al., 2018), although some differences in the relative percentages of the compounds were noted. In fact, the work conducted by Raina & Negi., (2012) analyzed an EO of *O. majorana* with a higher quantity of both terpinen-4-ol (31.15%) and sabinene hydrate (15.76%) compared to those analyzed in this work. The species *Origanum heracleoticum* is known to exhibit diverse chemotypes influenced by factors such as plant metabolism, climate, and the secretory activity of glandular hairs (Jerković et al., 2001). The *O. heracleoticum* EO analyzed in this study belongs to a carvacrol/γ-terpinene chemotype, which differs from the chemotypes reported by Sarikurkcu et al. (2015) and Schillaci et al. (2013). In the former study, the analyzed EO belonged to the linalool chemotype, while in the latter, it was of the thymol chemotype. In contrast, the EO of *O. heracleoticum* analyzed by Aytaç et al. (2022) was more similar to our findings, as it also displayed a carvacrol chemotype. However, their

sample contained a significantly higher concentration of carvacrol (72.2%) compared to our results (15.79%).

4.2.2 Antioxidant and enzymatic activities

The antioxidant activity was assessed using DPPH, ABTS, and FRAP assays, each targeting different reactive oxygen species. The DPPH assay quantifies the reducing power by measuring its ability to donate electrons and hydrogen atoms. In contrast, the FRAP assay specifically evaluates electron-donating substances. The ABTS assay, meanwhile, measures the antioxidant activity in both hydrophilic and lipophilic compounds across a wide pH range, providing a comprehensive assessment of the extract antioxidant potential. The results obtained are reported in Table 12.

Table 12. *Antioxidant activity of the EOs of O. vulgare, O. majorana and O. heracleoticum.*

	DPPH (IC_{50} mg/mL)	ABTS (IC_{50} mg/mL)	FRAP (mg Fe^{2+} eq./g EO)
O. vulgare	$0.28^{b} \pm 0.05$	$0.49^{b} \pm 0.10$	$3.84^{c} \pm 0.8$
O. majorana	$3.59^{d} \pm 0.08$	$2.95^{d} \pm 0.41$	$0.25^{a} \pm 0.05$
O. heracleoticum	$0.54^{c} \pm 0.04$	$0.61^{c} \pm 0.18$	$0.94^{b} \pm 0.07$
Trolox	$0.03^{a} \pm 0.01$	$0.02^{a} \pm 0.01$	$10.30^{d} \pm 0.08$

Results are the mean ± SD of three experiments. Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$, according to a one-way ANOVA followed by Tukey's post hoc test.

As shown in Table 12, *Origanum vulgare* showed the strongest antioxidant activity among the three species tested in all assays. Specifically, it exhibited an IC_{50} value of 0.28 mg/mL in the DPPH test, 0.49 mg/mL in the ABTS test, and 3.84 mg Fe^{2+} eq./g in the FRAP test. The literature confirms that the species analyzed in this study possess antioxidant activity, though the specific values often differ from those reported here. For example, the EO of *O. vulgare* analyzed in this study showed higher antioxidant activity compared to the results obtained by Simirgiotis et al. (2020), with better values in both DPPH (4.7 mg/mL) and ABTS (1.25 TE eq. mg/g) assays. Similarly, in the study conducted by Elansary et al. (2018) the EO of *O. vulgare* showed a higher IC_{50} value (2.8 mg/mL) than the one recorded in our analysis. By contrast, the IC_{50} reported by Han et al. (2017) (0.33 mg/mL) was comparable to the value obtained in this study. For *Origanum majorana*,

the values reported by Mossa & Nawwar (2011) differed from those obtained in this study: in their study the EO of *O. majorana* exhibited a lower IC_{50} value (58.67 μg/mL). A different scenario was observed for *O. heracleoticum*, for which the values obtained aligned with those of Sarikurkcu et al. (2015). Table 13 showed the enzymatic activities of the three species analyzed. As can be seen, the samples showed different activities depending on the enzyme studied. Regarding cholinesterases, the samples showed an inhibitory activity towards both AChE and BChE.

Table 13. *Enzymatic activity of the EOs of O. vulgare, O. majorana and O. heracleoticum. Results were expressed as IC_{50} (μg/mL)*

	AChE	BChE	Tyrosine	Dopa	α-amylase	α-glucosidase	Lipase
O. vulgare	$28.22^{c} \pm 1.2$	$390.23^{c} \pm 3.5$	$650.00^{c} \pm 2.5$	*n.a.*	*n.a.*	$331.50^{a} \pm 2.1$	$67.35^{b} \pm 1.2$
O. majorana	$56.40^{d} \pm 3.6$	$330.54^{c} \pm 2.8$	$466.90^{b} \pm 1.6$	$610.32^{b} \pm 3.1$	*n.a.*	$395.20^{ab} \pm 3.7$	$72.74^{c} \pm 0.9$
O. heracleoticum	$5.46^{b} \pm 0.5$	$201.28^{b} \pm 1.4$	$680.00^{c} \pm 4.8$	$1260.4^{c} \pm 4.2$	*n.a.*	$451.90^{b} \pm 4.2$	$14.94^{a} \pm 0.7$
Galantamine	$0.70^{a} \pm 0.3$	$10.00^{a} \pm 1.3$	-	-	-	-	-
Kojic acid	-	-	$13.40^{a} \pm 2.2$	$66.70^{a} \pm 1.5$	-	-	-
Acarbose	-	-	-	-	1.20 ± 0.2	$950.20^{c} \pm 3.6$	
Orlistat	-	-	-	-	-	-	$11.00^{a} \pm 0.8$

Results are the mean ± SD of three experiments. Different letters in the same column indicate mean values significantly different at $p < 0.05$, according to a one-way ANOVA followed by Tukey's post hoc test.

Specifically, *O. heracleoticum* was the most active towards AChE, with an IC_{50} value of 5.46 μg/mL. *O. vulgare* (28.22 μg/mL) and *O. majorana* (56.40 μg/mL) showed good activity with IC_{50} lower than 60 μg/mL. Similarly, *O. heracleoticum* was also the most active among the species analyzed in the inhibition of BChE, although with a higher IC_{50} (201.28 μg/mL). Regarding tyrosinase inhibition, both the monophenolase reaction (leading to the formation of dopa) and the diphenolase reaction (leading to the formation of dopaquinone and finally melanin) were evaluated. In the monophenolase assay, *O. majorana* was the most active species (466.90 μg/mL), compared to *O. vulgare* (650.00 μg/mL) and *O. heracleoticum* (680.00 μg/mL). Also, for

the diphenolase reaction, *O. majorana* was the most active sample with an IC_{50} value of 610.32 µg/mL. None of the three EOs showed inhibitory effects on α-amylase. In contrast, they showed a significant activity against α-glucosidase, with IC_{50} values significantly lower than acarbose, used as a control. Specifically, *O. vulgare* showed the highest activity (331.50 µg/mL) among the species analyzed, followed by *O. majorana* (395.20 µg/mL) and *O. heracleoticum* (451.90 µg/mL). For lipase inhibition, *O. heracleoticum* was the most effective (IC_{50}: 14.94 µg/mL), showing potency comparable to orlistat (11.00 µg/mL), used as a control. Both *O. vulgare* (67.35 µg/mL) and *O. majorana* (72.74 µg/mL) showed inhibitory activity with IC_{50} lower than 100 µg/mL. Only limited data are available in the literature on the enzymatic activities of EOs from *O. vulgare*, *O. majorana* and *O. heracleoticum*, with most studies focusing on cholinesterases. Orhan and coworkers (2008) analyzed the cholinesterase-inhibitory properties of *O. vulgare* and *O. majorana*, reporting IC_{50} values of 490 µg/mL (AChE) and 520 µg/mL (BChE) for *O. vulgare*. In both cases, the sample analyzed in this study showed lower IC_{50} than those reported by Orhan and collaborators. Conversely, the EO of *O. majorana* displayed an IC_{50} of 470 µg/mL for both AChE and BChE; also in this case, our sample showed lower IC_{50} values. The EO of *O. majorana* analyzed by Mossa et al. (2011), however, was more active towards AChE compared to our findings (36.40 *vs* 56.40 µg/mL).

Sarikurtcu et al. (2015) conducted a study evaluating the inhibitory effects of *Origanum vulgare* and *Origanum heracleoticum* EOs on various enzymes, including AChE, BChE, tyrosinase, α-amylase and α-glucosidase. This study is one of the few available sources of information on *O. heracleoticum*. However, our results concerning α-amylase inhibition differed from those reported in the study of Sarikurtcu and coworkers: none of our samples exhibited α-amylase inhibition, in contrast to the cited study. Regarding cholinesterases, our samples exhibited higher inhibitory activity. However, for tyrosinase inhibition, the *O. vulgare* and *O. heracleoticum* samples analysed by Sarikurkcu et al. displayed lower IC_{50} values than those obtained in our study. To date, no studies have investigated tyrosinase, α-amylase or α-

glucosidase inhibition in relation to the EO of *O. majorana*, nor are there any reports examining lipase inhibition by EOs from all three species.

4.2.3 Kinetic study

For each enzyme, a kinetic study was conducted using the EO that showed the strongest inhibitory activity. Table 14 reported the maximum rate of enzymatic reaction (Vmax), the substrate concentration where the corresponding reaction rate is ½ of V_{max} (Km) and the inhibition costant (Ki). This constant is indipendent from experimental conditions and is a measure of the affinity of the inhibitor for the enzyme (Copeland, 2023). Overall, the analysis of the Lineweaver–Burk plots (Figures 23–26) suggested that *O. heracleoticum* EO may inhibit AChE and lipase through a mixed mechanism, while it may act competitively toward BChE. The EO of *O. vulgare* appeared to behave as an uncompetitive inhibitor of α-glucosidase.

Table 14. *V_{max}, K_m, K_i values for cholinesterases, tyrosinase, α-glucosidase, lipase inhibition by O. vulgare, O. majorana or O. heracleoticum EOs.*

Enzyme	Essential oil	V_{max} (mmol/min*L)	K_m (mM/mL)	K_i (µg/mL)	αK_i (µg/mL)	Type of inhibition
AChE	*O. heracleoticum*	6.32 ± 0.85	2.16 ± 0.40	0.85	-	Competitive
BChE	*O. heracleoticum*	5.34 ± 0.97	2.06 ± 0.56	88.35	-	Mixed
α-glucosidase	*O. vulgare*	7.51 ± 0.76	2.50 ± 0.52	-	141.4	Uncompetitive
Lipase	*O. heracleoticum*	0.04 ± 0.01	1.12 ± 0.31	10.65	-	Mixed

V_{max}: maximum rate of enzymatic reaction, K_m: substrate concentration where the corresponding reaction rate is ½ of V_{max}, K_i and αK_i: inhibition costant. Data are expressed as mean ± standard deviation (n=3)

The Lineweaver–Burk plot (Figure 23) indicated that for AChE, the lines intercepted on the y-axis showing competitive inhibition for *O. heracleoticum* EO.

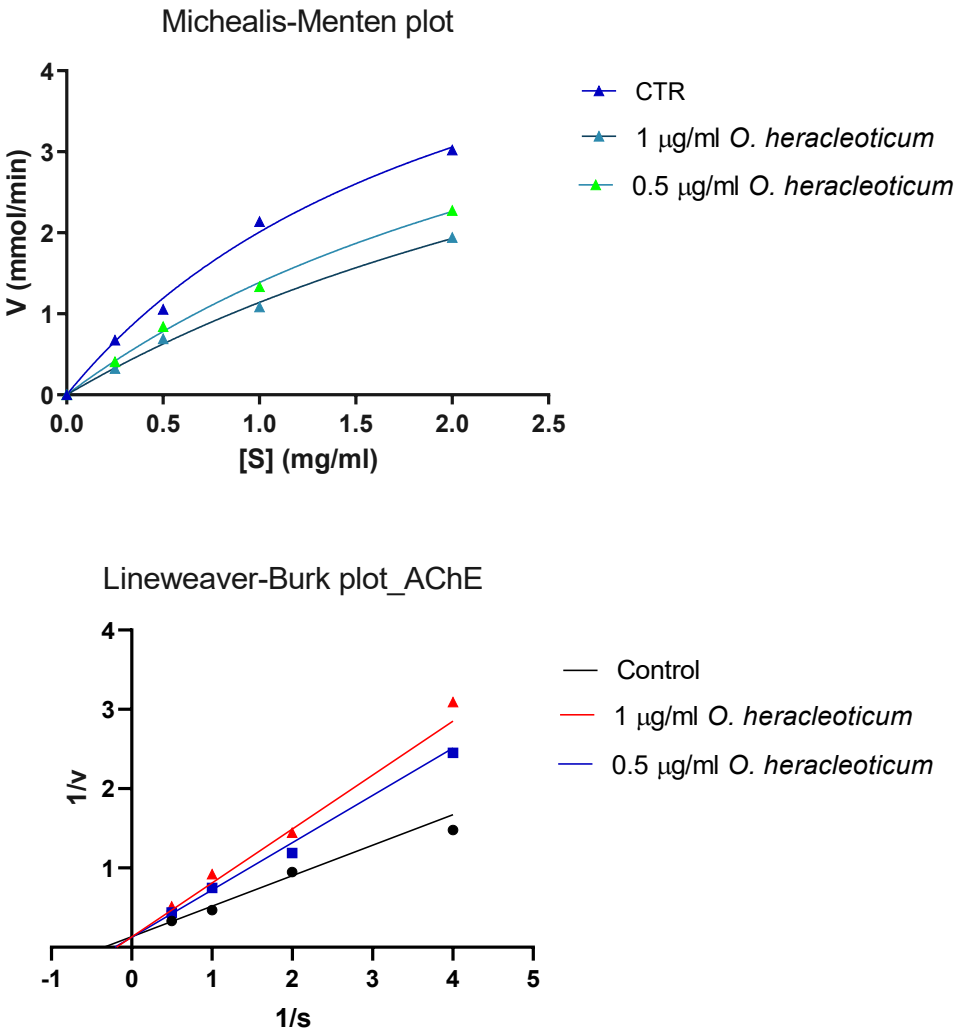


Figure 23. *Michealis- Menten and Lineweaver–Burk plots of AChE inhibition in absence (CTR) or presence of O. heracleoticum EO.*

For BChE, the Lineweaver–Burk plot (Figure 24) shows lines that intersect both the y- and x-axes at different points and are not parallel, indicating that *O. heracleoticum* EO may exhibit a mixed-type inhibition.

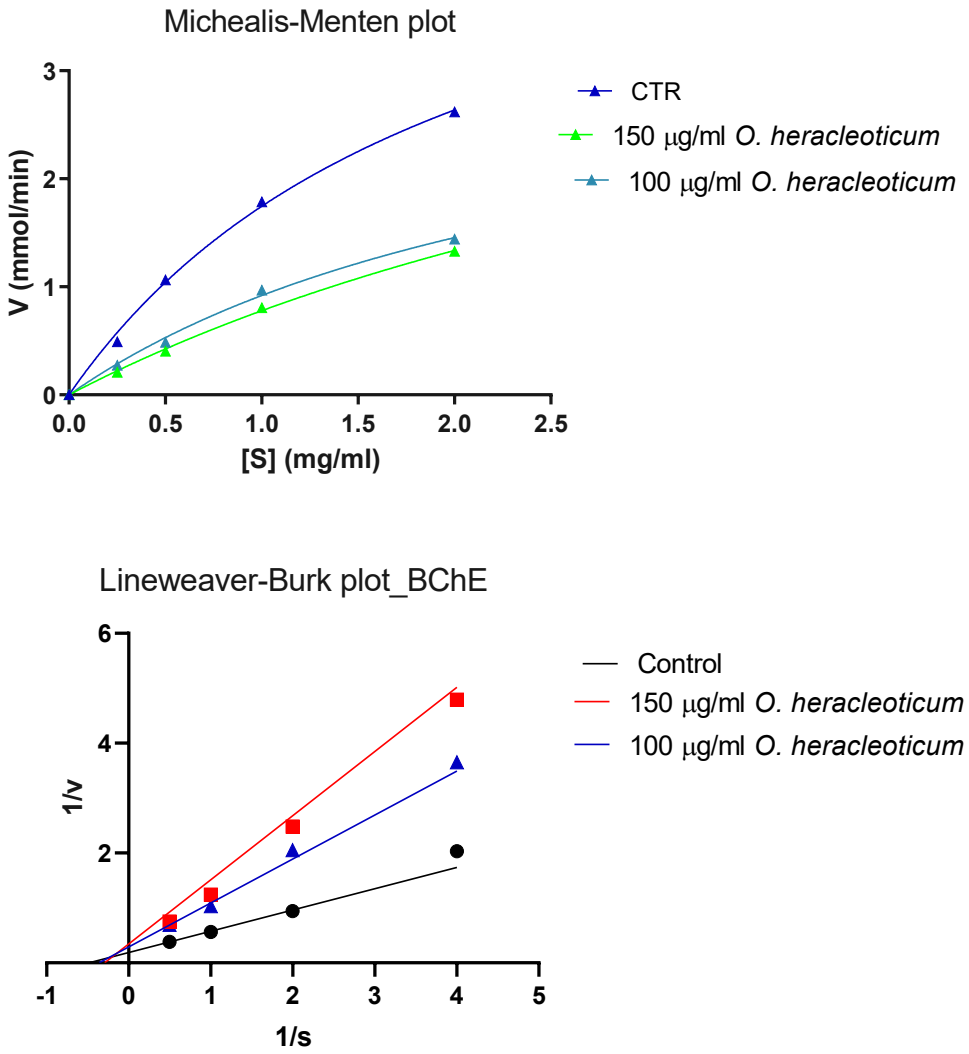


Figure 24. *Michealis- Menten and Lineweaver–Burk plots of BChE inhibition in absence (CTR) or presence of O. heracleoticum EO.*

The Lineweaver–Burk plot (Figure 25) showed that the lines obtained in the presence and absence of *O. vulgare* EO were parallel and intersect the y- and x-axes at different points. This pattern suggested that the EO may act as an uncompetitive inhibitor of α-glucosidase.

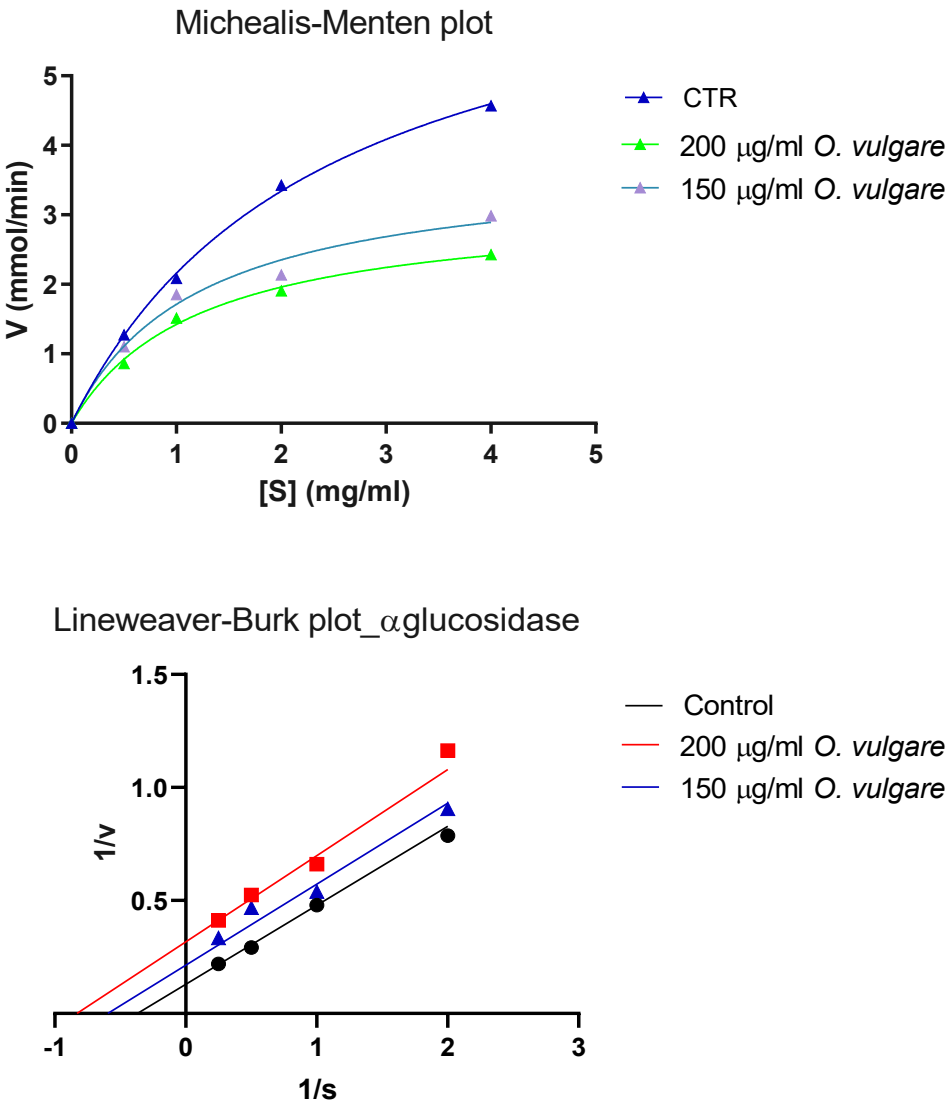


Figure 25. *Michealis- Menten and Lineweaver–Burk plots of α-glucosidase inhibition in absence (CTR) or presence of O. vulgare EO.*

For lipase (Figure 26), the lines obtained for the control and for *O. heracleoticum* EO intersect both the y- and x-axes at different points, suggesting a mixed-type inhibition.

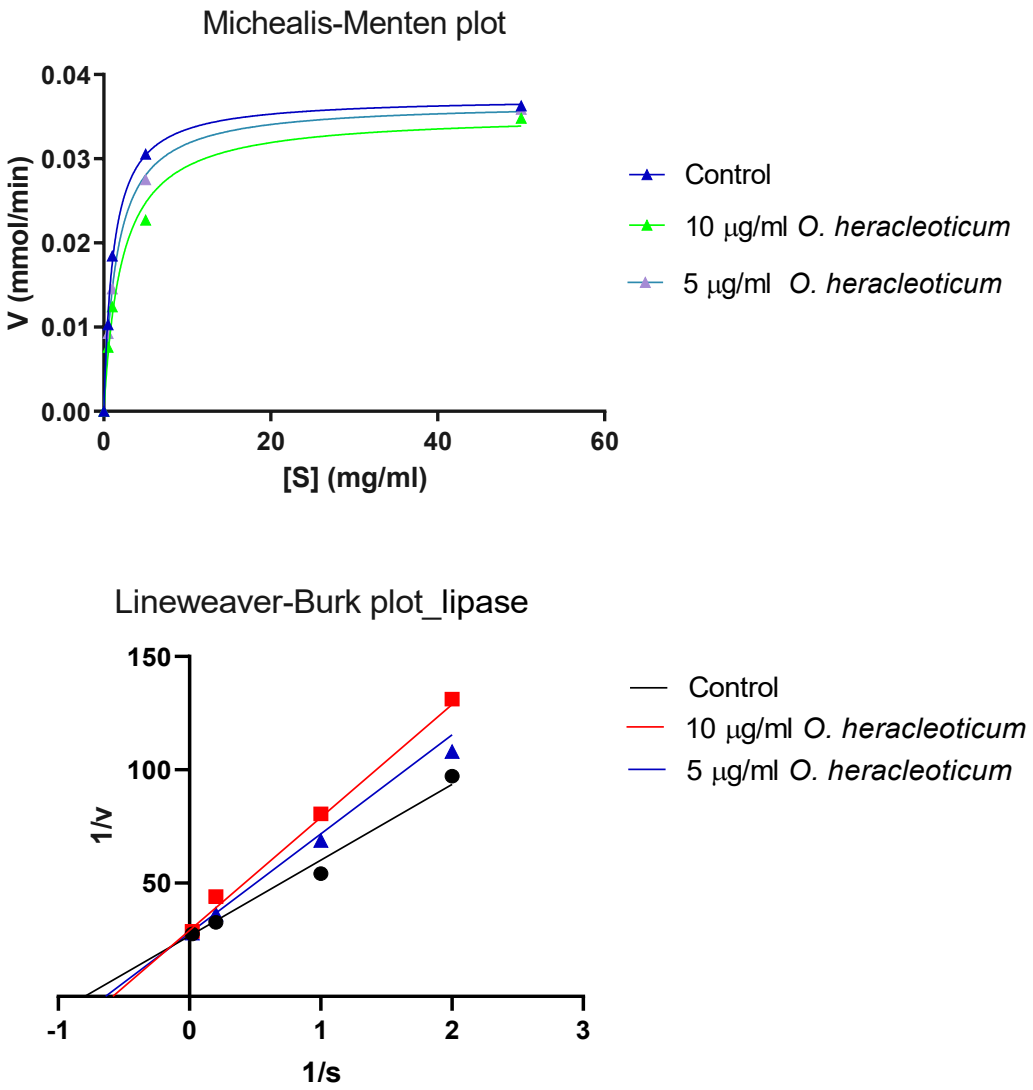


Figure 26. *Michealis- Menten and Lineweaver–Burk plots of lipase inhibition in absence (CTR) or presence of O. vulgare EO.*

4.3 Compare *O. heracleoticum* samples (intraspecies comparison) collected from different Italian localities analysing:

4.3.1 Essential oil

This section reports the results of an intraspecific comparison between different *Origanum heracleoticum* samples collected in different areas of Italy. The analysis was conducted to assess any variations in the composition of the volatile fraction, and consequently in the potential associated biological activities. After chemical characterization, a multivariate analysis was performed to highlight the relationships between the samples and to identify any geographic or chemotypic patterns. Samples were collected at the following location:

- Sicignano degli Alburni (Campania)
- Marsico Nuovo (Basilicata)
- Sila (Calabria)
- Grimaldi (Calabria)
- Sassari (Sardegna)
- Pollino (Basilicata)
- Nardò (Puglia)
- Agrigento (Sicilia)

4.3.1.1 Chemical composition and multivariate analysis of O. heracleoticum EOs

The aerial parts of *Origanum heracleoticum* samples were subjected to steam distillation for the extraction of EOs. Analysis of the volatile compound profiles of *O. heracleoticum*, collected from eight different Italian locations, revealed marked chemical variability between populations. A total of 108 compounds were identified, with the number of components varying from 49 to 59 for each sample (Table 15).

Table 15. *Chemical compostion of O. heracleoticum EOs*

			***O. heracleoticum* (%)**										
N°	**RT**	**Compound**	**M. N.**	**Gr.**	**Pol.**	**Na.**	**Sas.**	**Agr.**	**Sila**	**Sic.**	**KI[a]**	**KI[b]**	**Identification**
1	5.04	Methyl isovalerate	-	-	-	-	-	-	-	0.10 ± 0.01	822	795	1.2
2	12.79	α-Thujene	1.60 ± 0.12	1.61 ± 0.10	0.21 ± 0.03	1.70 ± 0.14	1.92 ± 0.15	1.81 ± 0.13	1.03 ± 0.08	1.62 ± 0.12	927	1110	1.2.3
3	13.13	α-Pinene	0.90 ± 0.07	0.72 ± 0.05	0.21 ± 0.02	0.81 ± 0.06	1.01 ± 0.07	0.82 ± 0.05	0.42 ± 0.03	0.70 ± 0.05	932	932	1.2.3
4	13.97	Dehydro-sabinene	0.10 ± 0.01	-	-	-	-	-	-	-	945	945	1.2.3
5	14.03	Camphene	0.41 ± 0.04	0.11 ± 0.02	t	0.11 ± 0.01	0.21 ± 0.03	0.12 ± 0.02	0.01 ± 0.01	0.10 ± 0.01	951	953	1.2.3
6	15.93	Sabinene	0.21 ± 0.02	0.71 ± 0.05	3.71 ± 0.33	-	0.31 ± 0.03	-	0.11 ± 0.01	0.91 ± 0.07	970	968	1.2.3
7	15.95	β-Pinene	0.21 ± 0.02	-	-	0.21 ± 0.02	0.21 ± 0.02	0.21 ± 0.02	0.11 ± 0.01	-	971	971	1.2
8	16.72	1-Octen-3-ol	0.81 ± 0.06	0.21 ± 0.02	0.11 ± 0.01	0.21 ± 0.02	0.41 ± 0.03	-	0.21 ± 0.02	0.41 ± 0.03	981	982	1.2
9	17.19	3-Octanone	0.41 ± 0.04	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	-	0.11 ± 0.01	0.21 ± 0.02	988	988	1.2.3
10	17.46	β-Myrcene	1.21 ± 0.10	2.01 ± 0.17	0.71 ± 0.06	2.21 ± 0.18	2.42 ± 0.19	1.91 ± 0.15	1.41 ± 0.12	1.91 ± 0.14	991	991	1.2.3
11	17.86	3-Octanol	0.11 ± 0.01	0.01 ± 0.01	0.11 ± 0.01	-	-	-	-	0.01 ± 0.01	997	999	1.2.3
12	18.10	α-Phellandrene	0.21 ± 0.02	0.41 ± 0.03	-	0.41 ± 0.03	0.41 ± 0.03	0.31 ± 0.03	0.31 ± 0.02	0.31 ± 0.03	1000	1000	1.2.3
13	18.49	δ-3-carene	-	0.11 ± 0.01	-	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	-	1005	1005	1.2.3
14	18.49	Cyclofenchene	0.11 ±	-	-	-	-	-	-	0.11 ±	1006	1008	

			0.01							0.01			
15	19.02	α-Terpinene	1.21 ± 0.11	3.71 ± 0.30	0.21 ± 0.02	3.51 ± 0.28	2.81 ± 0.25	3.11 ± 0.26	2.51 ± 0.22	3.11 ± 0.24	1012	1022	1.2.3
16	19.82	*p*-Cymene	19.21 ± 1.80	4.61 ± 0.40	0.71 ± 0.06	8.21 ± 0.70	6.51 ± 0.60	9.21 ± 0.80	3.81 ± 0.30	5.91 ± 0.50	1023	1023	1.2.3
17	19.86	*p*-Mentha-1(7).8-diene	-	-	-	-	-	-	-	0.51 ± 0.05	1025	1025	1.2.3
18	19.94	D-Limonene	0.41 ± 0.04	0.51 ± 0.05	0.41 ± 0.04	0.61 ± 0.05	0.51 ± 0.04	0.51 ± 0.04	0.41 ± 0.03	-	1026	1026	1.2.3
19	19.99	1.8- Cineole	-	0.21 ± 0.02	0.71 ± 0.06	-	-	-	0.41 ± 0.03	-	1038	1038	1.2.3
20	20.91	*trans*-β-Ocimene	0.11 ± 0.01	2.71 ± 0.25	2.61 ± 0.24	0.71 ± 0.06	0.31 ± 0.03	1.71 ± 0.15	0.81 ± 0.07	1.31 ± 0.11	1048	1042	1.2.3
21	21.39	*cis*-2-Caren-4-ol	t	-	-	-	-	-	-	-	1055	1055	1.2.3
22	21.61	*cis*-β-Ocimene	0.11 ± 0.01	0.91 ± 0.08	5.41 ± 0.40	0.21 ± 0.02	0.51 ± 0.04	0.21 ± 0.02	0.51 ± 0.04	1.91 ± 0.14	1063	1058	1.2.3
23	22.23	γ-Terpinene	5.61 ± 0.45	23.71 ± 1.80	0.71 ± 0.06	13.61 ± 1.10	14.21 ± 1.15	13.91 ± 1.10	14.01 ± 1.10	17.41 ± 1.35	1084	1084	1.2.3
24	22.72	*cis*- Sabinene hydrate	0.81 ± 0.06	0.41 ± 0.03	0.21 ± 0.02	0.51 ± 0.04	0.41 ± 0.03	0.11 ± 0.01	0.41 ± 0.03	0.71 ± 0.05	1085	1084	1.2
25	23.82	α-Pinene epoxide	-	-	0.11 ± 0.01	-	-	-	-	-	1094	1094	1.2
26	24.27	Terpinolene	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.21 ± 0.02	0.21 ± 0.02	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	1099	1099	1.2
27	24.33	*p*-Cymenene	0.11 ± 0.01	-	-	-	-	t	-	-	1112	1101	1.2.3
28	24.74	Methyl benzoate	t	-	-	-	-	-	-	-	1117	1116	1.2.3
29	24.93	*trans*-Sabinene hydrate	0.21 ± 0.02	-	-	-	-	-	0.11 ± 0.01	0.11 ± 0.01	1130	1118	1.2
30	25.34	Linalool	0.31 ± 0.03	0.81 ± 0.07	22.31 ± 1.70	0.61 ± 0.05	0.21 ± 0.02	0.51 ± 0.04	0.61 ± 0.05	1.11 ± 0.09	1132	1132	1.2.3
31	26.53	1-Octen-3-yl-acetate	-	-	0.71 ± 0.05	-	-	-	-	-	1134	1134	1.2

32	26.62	*p*-Menth-2-en-1-ol	0.11 ± 0.01	-	t	-	-	t	t	-	1136	1135	1.2
33	27.71	*trans*-Pinocarveol	0.11 ± 0.01	-	-	-	-	-	-	-	1137	1137	1.2
34	27.43	(4E.6Z)-*allo*-Ocimene	-	0.21 ± 0.02	0.31 ± 0.03	t	-	0.21 ± 0.02	0.11 ± 0.01	0.21 ± 0.02	1138	1138	1.2.3
35	27.96	*cis*-*p*-Mentha-2.8-dien-1-ol	-	-	t	-	-	-	-	-	1145	1153	1.2
36	28.44	β-Ocimene epoxide	-	-	0.11 ± 0.01	-	-	-	-	-	1160	1160	1.2
37	28.71	*trans*-2-Caren-4-ol	0.11 ± 0.01	-	-	-	-	-	-	-	1172	1172	1.2
38	28.09	Camphor	-	-	-	-	-	-	-	-	1182	1182	1.2
39	29.71	*endo*-Borneol	1.31 ± 0.10	0.11 ± 0.01	0.11 ± 0.01	0.31 ± 0.03	0.51 ± 0.04	0.21 ± 0.02	0.11 ± 0.01	0.11 ± 0.01	1189	1191	1.2
40	30.58	Terpinen-4-ol	0.81 ± 0.07	0.41 ± 0.03	0.21 ± 0.02	0.51 ± 0.04	0.71 ± 0.05	1.11 ± 0.09	0.61 ± 0.05	0.51 ± 0.04	1191	1191	1.2
41	31.27	*p*-Cymen-8-ol	0.11 ± 0.01	-	-	-	-	-	-	-	1204	1207	1.2.3
42	31.95	α-Terpineol	0.21 ± 0.02	0.31 ± 0.03	1.71 ± 0.14	0.11 ± 0.01	0.21 ± 0.02	0.11 ± 0.01	0.61 ± 0.05	0.41 ± 0.03	1211	1211	1.2.3
43	32.33	*cis*-Dihydrocarvone	0.11 ± 0.01	-	t	-	0.11 ± 0.01	t	0.11 ± 0.01	-	1216	1217	1.2.3
44	32.86	*trans*-Dihydrocarvone	0.11 ± 0.01	-	-	-	t	-	t	-	1217	1201	1.2.3
45	34.36	*trans*-3-Caren-2-ol	-	-	-	-	t	-	t	-	1225	1257	1.2.3
46	34.86	Thymol methyl ether	0.11 ± 0.01	2.51 ± 0.21	0.21 ± 0.02	1.91 ± 0.15	-	4.21 ± 0.35	1.01 ± 0.08	0.51 ± 0.04	1227	1224	1.2.3
47	35.47	Carvacrol methyl ether	1.51 ± 0.13	5.21 ± 0.42	1.21 ± 0.10	5.21 ± 0.41	0.81 ± 0.06	4.91 ± 0.39	3.51 ± 0.28	4.21 ± 0.33	1234	1240	1.2
48	36.01	Thymoquinone	0.21 ± 0.02	-	-	-	0.11 ± 0.01	-	-	-	1242	1244	1.2
49	36.07	Carvone	-	-	-	-	-	-	0.11 ±	-	1249	1243	1.2

								0.01					
50	36.41	Piperitone oxide	-	0.11 ± 0.01	-	-	-	-	-	-	1252	1252	1.2.3
51	36.62	Linalyl acetate	-	-	25.91 ± 1.80	-	-	-	-	-	1254	1256	1.2.3
52	38.12	Anethole	-	t	-	-	-	-	-	-	1255	1255	1.2
53	39.15	Thymol	13.31 ± 1.05	29.01 ± 2.10	10.21 ± 0.85	50.11 ± 3.20	2.21 ± 0.18	48.31 ± 3.10	34.21 ± 2.70	20.81 ± 1.65	1258	1260	1.2.3
54	40.05	Carvacrol	41.91 ± 3.30	11.71 ± 0.90	3.31 ± 0.25	3.71 ± 0.28	57.01 ± 4.10	0.71 ± 0.06	29.31 ± 2.20	23.51 ± 1.75	1282	1284	1.2
55	41.50	γ-Elemene	-	0.11 ± 0.01	0.11 ± 0.01	-	-	-	-	t	1294	1294	1.2.3
56	42.33	α-Cubebene	-	-	-	t	-	-	-	0.11 ± 0.01	1298	1300	1.2.3
57	42.84	Thymol acetate	-	0.21 ± 0.02	-	0.21 ± 0.02	-	0.11 ± 0.01	t	t	1310	1311	1.2
58	42.85	Eugenol	-	-	-	-	-	0.11 ± 0.01	t	-	1332	1332	1.2.3
59	43.61	β-Ylangene	-	-	0.11 ± 0.01	t	-	t	-	0.11 ± 0.01	1347	1350	1.2.3
60	43.88	Copaene	-	t	0.11 ± 0.01	0.11 ± 0.01	t	0.11 ± 0.01	t	0.21 ± 0.02	1355	1355	1.2
61	44.02	Carvacrol acetate	0.11 ± 0.01	0.21 ± 0.02	-	-	0.21 ± 0.02	-	0.11 ± 0.01	0.21 ± 0.02	1364	1364	1
62	44.42	β-Bourbonene	0.11 ± 0.01	0.11 ± 0.01	0.51 ± 0.04	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	t	0.31 ± 0.03	1366	1366	1.2
63	44.85	Geranyl acetate	-	-	0.31 ± 0.02	-	-	-	-	-	1370	1370	1.2.3
64	44.86	β-Cubebene	-	t	-	-	-	-	-	-	1371	1372	1.2.3
65	44.87	α-Ylangene	-	-	t	-	-	t	-	-	1378	1375	1.2.3
66	45.02	β-Elemene	-	t	0.11 ± 0.01	-	-	-	-	t	1385	1367	1.2
67	45.48	*cis*-Jasmone	-	t	-	-	-	-	-	-	1386	1386	1.2.3

68	46.56	Caryophyllene	2.01 ± 0.16	1.91 ± 0.15	4.91 ± 0.40	0.91 ± 0.07	2.91 ± 0.20	1.51 ± 0.12	1.01 ± 0.08	2.51 ± 0.20	1388	1390	1.2.3
69	48.22	*p*-Cymene-2.5-diol	0.51 ± 0.04	-	-	-	-	-	-	-	1405	1403	1.2
70	47.17	β-Copaene	-	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	t	0.11 ± 0.01	t	0.31 ± 0.03	1412	1412	1.2.3
71	47.72	Aromandendrene	-	t	-	-	0.11 ± 0.01	0.11 ± 0.01	-	-	1423	1423	1.2
72	47.76	α-Bergamotene	-	t	-	-	-	t	-		1431	1435	1.2.3
73	48.10	Isogermacrene-D	-	t	0.11 ± 0.01	-	-	-	-	t	1438	1438	1.2.3
74	48.24	γ-Cadinene	-	-	-	-	t	-	-	-	1446	1446	1.2
75	48.61	Humulene	0.21 ± 0.02	0.21 ± 0.02	0.81 ± 0.06	0.11 ± 0.01	0.31 ± 0.03	-	0.21 ± 0.02	0.41 ± 0.03	1453	1450	1.2.3
76	49.03	*epi*-β-Caryophyllene	-	-	0.11 ± 0.01	-	t	-	-	-	1470	1472	1.2.3
77	49.23	*cis*-Muurola-4(15).5-diene	-	-	-	-	t	-	-	-	1471	1471	1.2
78	49.05	*Allo*-aromadendrene	-	0.11 ± 0.01	-	-	-	0.21 ± 0.02	-	0.11 ± 0.01	1488	1488	1
79	49.95	δ-Cadinene	-	-	-	-	-	T	-	-	1490	1495	1.2
80	50.16	γ-Ylangene	0.11 ± 0.01	0.11 ± 0.01	-	0.31 ± 0.02	0.11 ± 0.01	0.11 ± 0.01	t	-	1495	1508	1.2.3
81	50.15	γ-Muurolene	-	-	-	-	-	0.31 ± 0.03	-	0.61 ± 0.05	1506	1506	1.2.3
82	50.32	γ-Amorphene	-	-	-	-	-	0.21 ± 0.02	-	-	1509	1509	1.2.3
83	50.58	β-Selinene	-	-	-	-	-	t	-	-	1516	1516	1.2
84	50.32	Germacrene D	0.31 ± 0.03	1.51 ± 0.12	5.91 ± 0.45	0.21 ± 0.02	0.11 ± 0.01	-	0.71 ± 0.05	3.01 ± 0.25	1519	1519	1.2.3
85	51.23	Ledene	-	-	-	-	0.21 ± 0.02	0.21 ± 0.02	-	-	1520	1524	1.2.3
86	51.29	Bicyclogermacre	-	0.51 ±	0.71 ±	-	-	-	0.11 ±	-	1523	1525	1.2

		ne		0.04	0.05				0.01				
87	51.62	α-Muurolene	-	t	0.11 ± 0.01	0.11 ± 0.01	t	0.11 ± 0.01	t	0.21 ± 0.02	1528	1528	1
88	51.63	β-Copaene	0.11 ± 0.01	-	-	0.01 ± 0.01	-	-	-	-	1529	1532	1.2
89	52.25	β-Bisabolene	1.21 ± 0.10	0.81 ± 0.07	0.91 ± 0.08	1.31 ± 0.11	1.31 ± 0.10	1.41 ± 0.12	0.71 ± 0.06	1.31 ± 0.10	1532	1535	1.2.3
90	52.32	γ-Cadinene	-	0.11 ± 0.01	-	-	-	-	0.11 ± 0.01	-	1534	1534	1.2.3
91	52.39	α-Farnesene	-	-	-	-	-	-	-	-	1535	1538	1.2.3
92	52.99	β-Cadinene	0.11 ± 0.01	0.31 ± 0.03	-	-	0.21 ± 0.02	-	-	0.11 ± 0.01	1539	1539	1.2
93	53.02	Cadina-1(10).4-diene	-	-	0.31 ± 0.03	0.51 ± 0.04	-	0.71 ± 0.05	0.11 ± 0.01	1.31 ± 0.11	1540	1542	1.2.3
94	53.43	Cubenene	-	-	-	t	t	0.11 ± 0.01	t	0.11 ± 0.01	1544	1544	1.2.3
95	53.73	α-Cadinene	-	t	t	t	t	0.11 ± 0.01	-	0.11 ± 0.01	1547	1549	1.2.3
96	54.00	α-Calacorene	-	-	-	-	-	t	-	t	1549	1551	1.2
97	54.25	α-Bisabolene	-	t	-	t	t	t	t	t	1551	1550	1.2.3
98	55.91	Germacrene D-4-ol	-	-	1.11 ± 0.08	-	-	-	-	-	1553	1553	1.2
99	55.92	Epicubebol	-	-	-	-	-	-	-	0.31 ± 0.03	1554	1558	1.2
100	55.95	Spathulenol	0.11 ± 0.01	0.31 ± 0.02	-	0.11 ± 0.01	t	0.11 ± 0.01	t	-	1555	1560	1.2.3
101	56.21	Caryophyllene oxide	1.11 ± 0.08	0.11 ± 0.01	1.01 ± 0.07	0.11 ± 0.01	0.11 ± 0.01	0.31 ± 0.02	t	0.11 ± 0.01	1561	1562	1.2.3
102	57.68	Humulene oxide II	0.11 ± 0.01	-	0.11 ± 0.01	-	-	-	-	-	1569	1569	1.2
103	58.09	Epicubenol	-	-	-	-	t	-	-	-	1570	1570	1.2.3
104	59.56	T-Cadinol	t	0.11 ± 0.01	0.21 ± 0.02	t	t	0.11 ± 0.01	t	0.11 ± 0.01	1574	1574	1.2.3

105	59.89	Cubebol	-	-	0.11 ± 0.01	-	-	-	-	-	1600	1614	1.2
106	60.3	α-Cadinol	t	0.11 ± 0.01	0.21 ± 0.02	t	t	0.11 ± 0.01	t	0.11 ± 0.01	1634	1634	1
107	62.02	α-Bisabolol	-	-	-	-	t	-	-	-	1648	1647	1.2
108	62.13	Shyobunol	-	0.21 ± 0.02	-	-	-	-	-	0.11 ± 0.01	1681	1681	1
		Monoterpens hydrocarbon	32.15	42.34	15.10	32.92	31.51	33.94	25.93	36.40			
		Monoterpens oxygenated	62.52	50.61	67.32	63.53	62.70	60.54	70.80	51.21			
		Sesquiterpens hydrocarbon	4.15	5.80	14.91	4.10	5.62	4.91	2.92	10.64			
		Sesquiterpens oxygenated	1.31	0.84	2.63	0.40	0.25	0.63	0.11	0.75			
		Others	-	0.61	-	-	-	-	0.31	1.12			
		Identified Compounds	55	59	58	49	56	53	56	58			

M.N: Marsico Nuovo; Gr: Grimaldi; Pol.: Pollino; Na: Nardò; Sas.: Sassari; Agr.: Agrigento; Sic.: Sicignano. T = traces < 0.01%; - = absent. [a] The Kovats retention indices determined relative to a series of *n*-alkanes (C_{10}–C_{35}) on the apolar DB-5. [b] Theoretical Kovats index for each compound. Identification method: 1 = comparison of the Kovats retention indices with published data, 2 = comparison of mass spectra with those listed in the NIST 02 and Wiley 275 libraries and with published data, and 3 = co-injection with authentic compounds. Peak area normalization was used to get the component relative concentrations.

Hydrocarbon monoterpenes were found to be the most abundant compounds in the EO ‘Grimaldi’ (42.34%), followed by those in ‘Sicignano’ (36.40%) and ‘Nardò’ (32.92%). In contrast, EOs from ‘Sila’ and ‘Pollino’ showed lower levels of hydrocarbon monoterpenes and higher levels of oxygenated monoterpenes, highlighting a chemical profile that was clearly distinct from the other samples. In particular, EO ‘Pollino’ had a completely different phytochemical profile, characterised mainly by the presence of linalyl acetate (25.91%) and linalool (22.31%), compounds found only in trace amounts or absent in the other samples. Thymol was found to be the main constituent in EOs ‘Nardò’ (50.11%) and ‘Agrigento’ (48.31%), while it was found in low percentages in the ‘Sassari’ sample (2.21%). Conversely, carvacrol was the predominant component in EOs ‘Sassari’ (57.01%) and ‘Marsico Nuovo’ (41.91%), indicating a clear carvacrol chemotype. These results confirmed the classification of ‘Nardò’ and ‘Agrigento’ as thymol chemotypes and ‘Sassari’ and ‘Marsico Nuovo’ as carvacrol chemotypes. The distribution of the biosynthetic precursors γ-terpinene and *p*-cymene further supported these chemotypic distinctions. γ-Terpinene reached its highest concentration in the ‘Grimaldi’ sample (23.71%), suggesting an active biosynthetic pathway towards the formation of phenolic monoterpenes. On the other hand, *p*-cymene, was most abundant in ‘Marsico Nuovo’ (19.21%) and least abundant in ‘Pollino’ (0.71%), contributing to the chemical differentiation between populations. Although sesquiterpenes were present in lower overall quantities, they also contributed to the variability of the chemical profiles. Hydrocarbon sesquiterpenes were most abundant in ‘Pollino’ EO (14.91%), followed by ‘Sicignano’ (10.64%), while ‘Sila’ showed the lowest values. Compounds such as β-caryophyllene, germacrene D, and cadinene varied across samples and may serve as potential chemical markers for distinguishing between different populations. Overall, the chemical profiles of the EOs of *Origanum heracleoticum* analysed in this study showed both similarities and differences compared to the data reported in the literature. Several previous studies highlighted the considerable variability in the main constituents, such as thymol, carvacrol, *p*-cymene and γ-terpinene.

Kokkini et al. (2004) reported that the EO of *Origanum vulgare* ssp. *hirtum* of Greek origin and *O. onites* of Turkish origin was mainly composed of carvacrol (33.5%), thymol (19.8%) and a high percentage of *p*-cymene (31.5%). In contrast, the ‘Sassari’ and ‘Marsico Nuovo’ samples analysed in the present study showed a carvacrol-type chemical profile, with a low thymol content, in accordance with Evrendilek (2015), who found 68.2% carvacrol, 11.8% *p*-cymene and 8.1% γ-terpinene in the EO of *O. vulgare* ssp *hirtum*. Similarly, several authors (Yuan et al., 2023; Veres et al., 2003) reported high concentrations of carvacrol (58.0% and 70.8%, respectively) and negligible amounts of thymol (0.1% and 0.3%), in line with the carvacrol-rich profiles observed in the ‘Sassari’ and ‘Marsico Nuovo’ samples.

Conversely, the 'Nardò' and 'Agrigento' samples, characterised by high levels of thymol and low levels of carvacrol, correspond to the thymol chemotype previously described by Tommasi et al. (2009). This study reported 54.7% thymol and 1.1% carvacrol in *O. heracleoticum* EO. Similarly, the concentrations of γ-terpinene and *p*-cymene detected in this study, 23.66% and 8.2%, respectively, in the 'Grimaldi' and 'Nardò' samples were consistent with the values reported in the aforementioned reference works (Tommasi et al., 2009). Indeed, Yuan et al. (2023) found 10.67% γ-terpinene and 17.32% *p*-cymene, whereas Tommasi et al. (2009) reported 8.5% and 13.4%, respectively. Overall, these results confirm the presence of multiple chemotypes within *Origanum heracleoticum*. Some populations exhibited profiles dominated by thymol or carvacrol, while others contained higher concentrations of the biosynthetic precursors γ-terpinene and *p*-cymene. Still others showed a distinct composition characterised by elevated levels of linalool and linalyl acetate. This chemical variability underscores the intraspecific complexity and diversity of the species.

After identifying the phytochemical constituents, a multivariate analysis was conducted, including both Principal Component Analysis (PCA) and Heatmap representation, with the aim of assessing the chemical variability and correlations between the different samples. The PCA biplot provides a comprehensive overview of the relationships among the eight *O. heracleoticum* samples (EOs from Marsico Nuovo, Grimaldi, Pollino, Nardò, Sassari, Agrigento, Sicignano, Sila) and the contributions of the main chemical variables to the first three principal components (Table 16 and Figure 27).

Table 16. *Loadings of the significant variables on three first principal components from data analysis*

Variables	**PC1**	**PC2**	**PC3**
Sabinene	0.3327	-0.0596	-0.1340
β-Myrcene	-0.2798	-0.0274	-0.2904
α-Terpinene	-0.2879	-0.1895	-0.2084
p-Cymene	-0.1370	0.2073	0.4014
cis-β-Ocimene	0.1616	-0.3627	-0.2298
trans-β-Ocimene	0.3283	-0.0761	-0.1239
γ-Terpinene	-0.2477	-0.1923	-0.3654
Linalool	0.3328	-0.0535	-0.0136
Thymol methyl ether	-0.1503	-0.3417	0.1517
Carvacrol methyl ether	-0.1810	-0.3802	0.0778
Linalyl acetate	0.3320	-0.0438	-0.0094
Anethole	-0.0564	-0.2378	-0.3894
Thymol	-0.1660	-0.3183	0.3353
Carvacrol	-0.0819	0.4149	-0.2122
Caryophyllene	0.3107	-0.1307	0.0748
p-Cymene-2,5-diol	-0.0662	0.3423	-0.3617
Germacrene D	0.3153	-0.1107	-0.1292
Eigenvalue	8.50	4.63	1.95
Total variance (%)	49.98	27.24	11.46

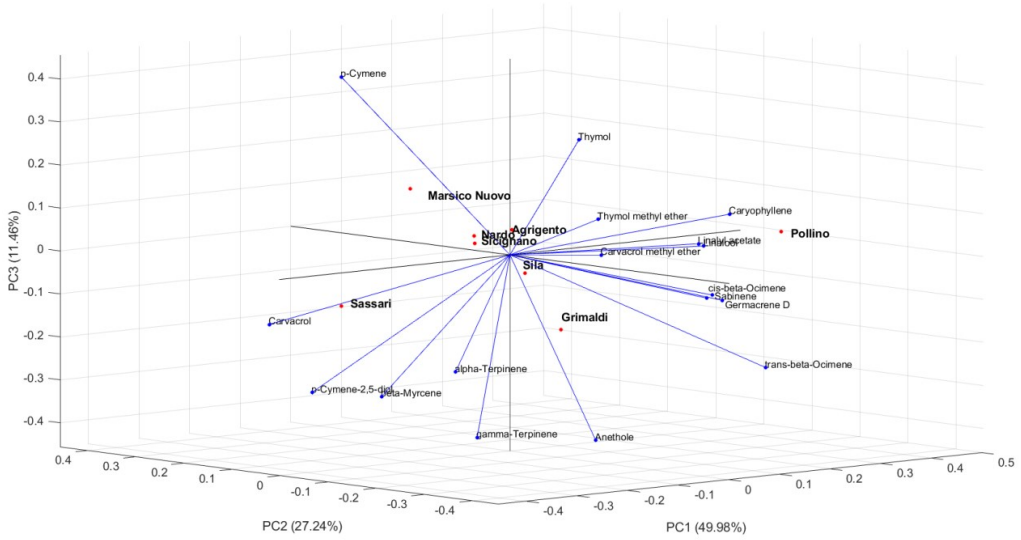


Figure 27. *Biplot (loading and scores plots) obtained by principal component analysis (PCA) of eight O. heracleoticum essential oils (Marsico Nuovo, Grimaldi, Pollino, Nardò, Sassari, Agrigento, Sicignano, Sila) based on the seventeen different variables (the main components of the EOs) in the three-dimensional space. The vectors shown are the eigenvectors of the covariance matrix.*

The three-dimensional Principal Component Analysis (PCA) biplot provided a multivariate overview of the chemical differentiation among EOs from all sampled Italian localities. The Principal Component 1 (PC1), which explained 49.98% of the total variance, distinguished the samples along a compositional gradient from monoterpene hydrocarbons and phenolic compounds (e.g., carvacrol methyl ether, *p*-cymene, carvacrol, and thymol methyl ether) toward high concentrations of oxygenated monoterpenes and sesquiterpenes (notably linalool, sabinene, *cis*- and *trans*-β-ocimene, and germacrene D). Thus, samples, like Pollino, were strongly projected along the positive PC1 axis due to their dominance in linalool and related compounds, whereas sites like Grimaldi, Agrigento, and Sila showed score lower, reflecting lower relative abundance of these compounds and a distinct chemotype. The Principal Component 2 (PC2), accounting for 27.24% of the variance, further discriminated samples based on the relative contributions of *p*-cymene and carvacrol, which were associated with the positive direction of PC2. This axis captured a biochemical differentiation orthogonal to PC1, essentially separating samples by their phenol-rich metabolite content:

Marsico Nuovo and Sassari score positively on PC2, highlighting their high contents of carvacrol and thymol, while Grimaldi and Agrigento showed negative values on this axis, indicating comparatively depleted phenolic monoterpenes. These variables distinguished samples such as Agrigento and Nardò, which appeared grouped due to their higher quantities of monoterpene hydrocarbons. The Principal Component 3 (PC3, 11.46% of variance) is defined largely by anethole, alpha-terpinene and p-Cymene-2,5-diol, enabling additional discrimination of samples like Marsico Nuovo and Sila along this axis.

In a hierarchical cluster heatmap (figure 28) depicting the chemical composition of EOs, color variability represents the relative abundance of each compound in each sample. A typical color scale ranges from blue (indicating low or negative relative abundance) through violet (intermediate levels) to pink (denoting high or positive abundance) for each matrix element. This visual encoding allowed rapid identification of patterns—such as which samples were rich in specific constituents or which compounds are particularly scarce. As a result, color gradients not only highlight compositional differences among samples, but also facilitate recognition of clustering structures and relationships associated with chemical diversity and distinguishing features among EO profiles.

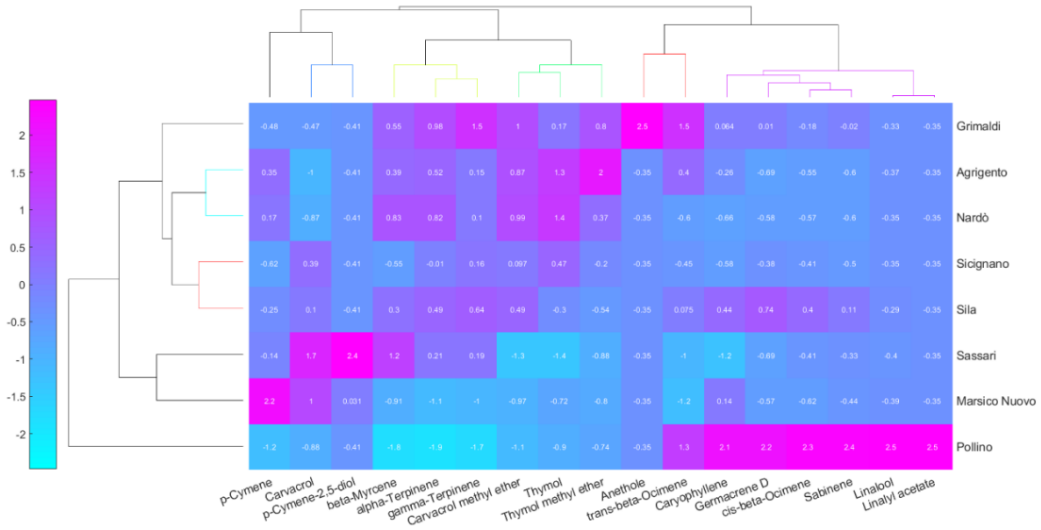


Figure 28. *Hierarchical Cluster Heatmap of 8 observations (rows) and 18 variables (columns), with normalized data values represented by a colour scale ranging from blue (low) to pink (high). The rows represent the EOs of O. heracleoticum distilled from plant collected in 8 Italian areas. The columns represent the tested main components of the EOs.*

Pollino EO is characterized by high (pink) values for linalool, linalyl acetate, sabinene, the ocimene isomers, and germacrene D, marking its distinct oxygenated monoterpene-rich profile. This cluster aligned with the high PC1 scores of Pollino samples, indicating a robust sesquiterpenic and oxygenated monoterpenoid chemotype distinct from others. Conversely, Sassari, and Marsico Nuovo EOs shared a cluster with markedly negative values (blue) for these constituents but elevated levels of carvacrol, corresponding well with their PCA groupings. Notably, a well-defined cluster was observed between the Sila and Sicignano EOs, which shared similar profiles characterized by intermediate to low relative amounts of *p*-cymene and thymol methyl ether, alongside modest levels of carvacrol, γ-terpinene, α-terpinene, and carvacrol methyl ether. This close grouping suggested a strong compositional affinity potentially related to geographic proximity or comparable environmental influences. Another clear cluster groups the Nardò, Agrigento, and Grimaldi samples, reflecting their shared chemical signatures. These EOs exhibited elevated levels of β-myrcene, α-terpinene, carvacrol methyl ether and γ-terpinene, coupled with moderate contributions

from thymol methyl ether. Such clustering underscored the complex variation in EO composition across different regions, revealing both local chemical homogeneity within clusters and differentiation between them. These patterns were of particular importance for understanding the phytochemical diversity and for potential applications requiring specific chemical profiles. Together, the PCA and heatmap analyses form a complementary framework to decipher chemotypic diversity. PCA reduced complex multidimensional chemical data into orthogonal axes representing major sources of variation, delineating sample separation based on metabolite groups. The heatmap provided a high-resolution matrix visualization where exact metabolite concentration gradients correspond to PCA-driven clusters. This integrated analysis enabled identification of discrete chemotypic signatures tied to geographic origins through both dimension reduction and detailed metabolite quantification, establishing a rigorous basis for further phytochemical, ecological, or applied research inquiries.

Based on the different chemotypes that emerged, confirmed by the formation of specific clusters in the multivariate analysis, and considering the data available in the literature (Caputo et al., 2018; Caputo et al., 2023), three representative samples were selected: 'Marsico Nuovo' (carvacrol chemotype), “Agrigento” (thymol chemotype) and “Pollino” (linalool chemotype), in order to evaluate their potential activity on the central nervous system (CNS). The other samples (“Sicignano”, “Sila”, “Grimaldi”, “Sassari” and “Nardò”) were chosen to investigate their potential use in nutraceuticals, with particular attention to their effects on metabolic disorders.

4.3.1.2 Evaluation of the activity of O. heracleoticum EOs on the central nervous system

The samples from ‘Agrigento’, ‘Marsico Nuovo’ and ‘Pollino’ were evaluated for their potential activity on the central nervous system, examining their inhibitory activity against cholinesterase and tyrosinase enzymes, together with their antioxidant activity and insecticidal activity against *Tenebrio molitor.*

The results obtained highlight significant differences between the samples from the three collection areas (Agrigento, Marsico Nuovo and Pollino) in terms of both antioxidant activity (table 17) and enzymatic inhibitory (table 18) capacity. The 'Marsico Nuovo' sample produced the best results in most of the conducted tests. It exhibited an IC_{50} value of 178.32 µg/mL, in the DPPH assay and IC_{50} value of 250.17 µg/mL, in the ABTS assay. The sample also demonstrated significant reducing activity in the FRAP assay (2.14 mM Fe^{2+} equivalent/g), confirming its overall antioxidant potential. When compared with literature data, the obtained values were consistent with previously reported results. For instance, Özcan and Erdoğan (2011) reported IC_{50} values of 80.00 µg/mL for antioxidant activity, whereas Mechergui et al. (2010) reported 60 µg/mL. Regarding cholinesterase inhibitory activity, 'Marsico Nuovo' displayed the lowest IC_{50} values for both acetylcholinesterase (AChE, 34.12 µg/mL) and butyrylcholinesterase (BChE, 220.4 µg/mL), making it significantly more active than the other EOs tested. Özbek et al. (2017) reported inhibition percentages of 38.66% (AChE) and 50.66% (BChE) for the EO of *O. heracleoticum* at 25 µg/mL, indicating higher activity than that observed in the present study. In contrast, Sadaoui et al. (2022) reported higher IC_{50} values: 103.56 µg/mL (AChE) and 68.89 µg/mL (BChE).

Table 17. *Antioxidant activities of EOs of O. heracleoticum*

	Agrigento	**Marsico Nuovo**	**Pollino**	**Trolox**
DPPH (IC_{50} µg/mL)	$451.02^{c} \pm 23.5$	$178.32^{b} \pm 17.4$	$1138.00^{d} \pm 86.2$	$3.65^{a} \pm 0.84$
ABTS (IC_{50} µg/mL)	$550.42^{c} \pm 45.31$	$250.17^{b} \pm 21.34$	$1045.10^{d} \pm 90.05$	$2.05^{a} \pm 0.45$
FRAP (mM Fe^{2+} eq./g ext.)	$2.52^{b} \pm 0.24$	$2.14^{b} \pm 0.33$	$0.39^{a} \pm 0.08$	$5.48^{c} \pm 0.41$

Results are the mean ± SD of three experiments. Different letters in the same line indicate mean values significantly different at $p < 0.05$, according to a one-way ANOVA followed by Tukey's post hoc test.

Table 18. *Enzymatic activities of EOs of O. heracleoticum. Results were expressed as IC_{50} (µg/mL)*

	Agr.	M. N.	Poll.	Galant.	Kojic a.
AChE	50.3 ^{c} ± 0.48	34.12 ^{b} ± 0.62	101.62 ^{d} ± 0.36	0.7 ^{a} ± 0.04	-
BChE	395.4 ^{c} ± 18.3	220.4 ^{b} ± 36.27	595.4 ^{d} ± 16.8	10.30 ^{a} ± 0.36	-
Tyrosinase (monophenolase)	311.2 ^{c} ± 12.5	233.50 ^{b} ± 1.45	497.1 ^{d} ± 2.47	-	13.44 ^{a} ± 1.39
Tyrosinase (diphenolase)	370.25 ^{b} ± 19.2	723.71 ^{d} ± 2.32	494.25 ^{c} ± 3.64	-	66.77 ^{a} ± 2.45

Agr.: Agrigento; M.N.: Marsico Nuovo; Poll.: Pollino; Galant.: Galantamine; Kojic a.: Kojic acid. Results are the mean ± SD of three experiments. Galantamine and Kojic acid were used as reference standards. Different letters in the same line indicate mean values significantly different at $p < 0.05$, according to one-way ANOVA followed by Tukey's post hoc test.

The insecticidal activity of the 'Marsico Nuovo' and 'Agrigento' samples against *Tenebrio molitor* was then assessed, given that they exhibited the highest cholinesterase inhibitory potential. (Thapa et al., 2017). The data (Figure 29) on the insecticidal activity of the EOs on *Tenebrio molitor* revealed a clear concentration-dependent effect for both analysed samples (Marsico Nuovo and Agrigento). Notably, the 'Marsico Nuovo' sample exhibited higher insecticidal efficacy than the 'Agrigento' sample at all concentrations tested. At 1 µL, 'Marsico Nuovo' induced 88.0% mortality, compared to 75.0% for 'Agrigento'. The same trend was observed at 0.75 and 0.575 µL, with mortality rates of 75.9% and 63.0% for 'Marsico Nuovo', versus 63.9% and 51.9% for 'Agrigento'. Overall, these results confirmed that the EO from 'Marsico Nuovo' possessed greater insecticidal potency, which was consistent with its superior antioxidant and cholinesterase inhibitory activities. This suggests a possible correlation between enzymatic inhibition and the insecticidal effect.

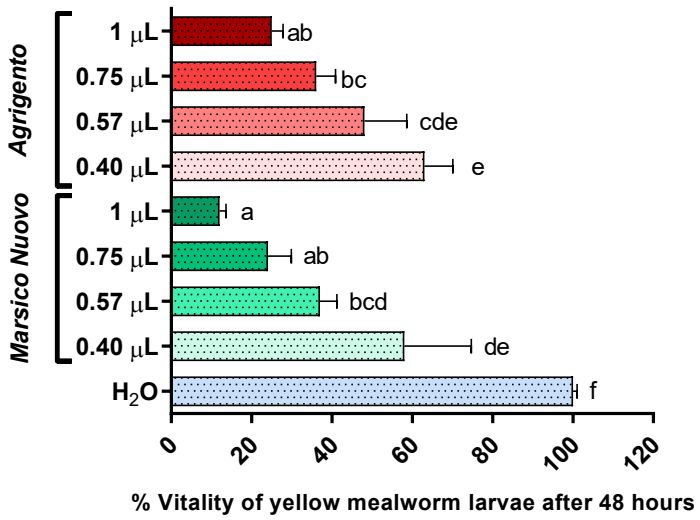


Figure 29. *Average vitality (mean ± standard deviation) of yellow mealworm larvae after 48 hours exposition to different dose of essential oil. Results are the mean ± SD of three experiments. Different letters indicate mean values significantly different at p < 0.05, according to a one-way ANOVA followed by Tukey's post hoc test*

Subsequently, an acute toxicity analysis was performed to determine the LD_{50}, LD_{90} and LD_{99} values for the EOs that were tested (Figure 30). The 'Marsico Nuovo' sample exhibited greater insecticidal potency, with an LD_{50} value of 4.30 µg/insect, which was lower than that of the 'Agrigento' sample (5.54 µg/insect). Similarly, the LD_{90} and LD_{99} values were lower for 'Marsico Nuovo' (9.67 and 18.73 µg/insect, respectively) than for 'Agrigento' (14.12 and 30.29 µg/insect), confirming its superior acute toxicity against *Tenebrio molitor*. There are several studies in the literature that evaluate the insecticidal activity of oregano EO (Xie et al., 2019; Plata-Rueda et al., 2021).

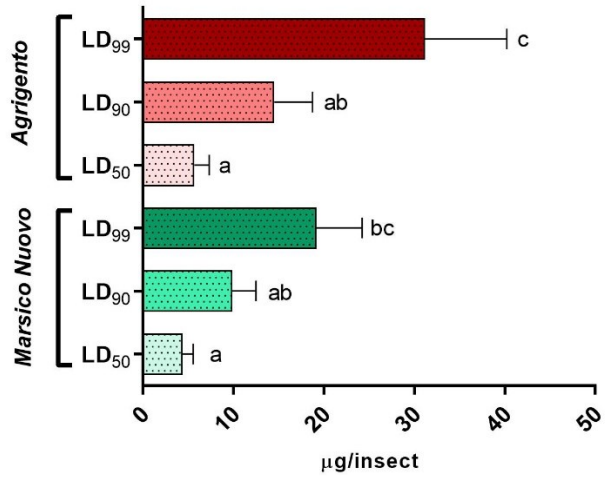


Figure 30. *Lethal doses of origanum essential oil (µg*Insect^{-1}) Results are the mean ± SD of three experiments. Different letters indicate mean values significantly different at p < 0.05, according to a one-way ANOVA followed by Tukey's post hoc test*

Based on the results obtained, for the Marsico Nuovo sample, the kinetics of inhibition were also studied, with the aim of understanding the mechanism by which it acts on cholinesterase enzymes. Table 19 reported the maximum rate of enzymatic reaction (Vmax), the substrate concentration where the corresponding reaction rate is ½ of V_{max} (Km) and the inhibition costant (Ki).

Tabella 19. *The kinetic parameters (V_{max}, K_m and K_i) were determined to assess cholinesterase inhibition*

Enzyme	V_{max} (mmol/min*L)	K_m (mM/ml)	K_i (µg/ml)	Type of inhibition
AChE	6.43 ± 0.35	0.96 ± 0.11	21.13	Competitive
BChE	10.51 ± 1.27	2.14 ± 0.31	82.70	Non-competitive

V_{max}: maximum rate of enzymatic reaction, K_m: substrate concentration where the corresponding reaction rate is ½ of V_{max}, K_i: inhibition costant. Data are expressed as mean ± standard deviation (n=3)

The Lineweaver-Burk plot (Figure 31) indicated that for AChE, the intercepted lines on the y-axis showing a possible competitive inhibition for the EO of *O. heracleoticum* 'Marsico Nuovo'. Regarding the BChE enzyme, as shown in the Lineweaver-Burk plot (Figure 32), the lines intersected on the x-axis in the second quadrant, suggesting a possible non-competitive inhibition.

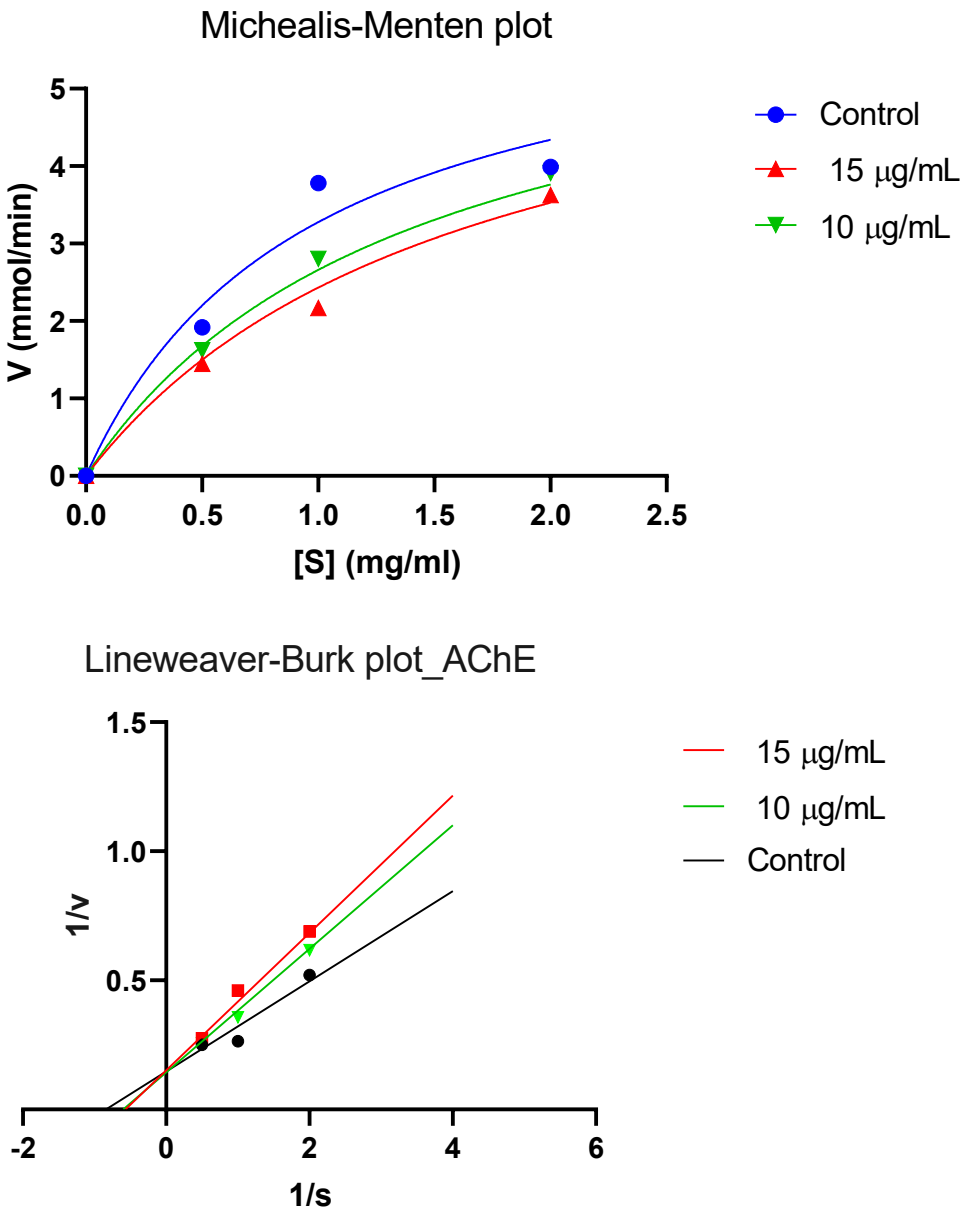


Figure 31. *Michealis- Menten and Lineweaver–Burk plots of AChE inhibition in absence (CTR) or presence of O. heracleoticum EO.*

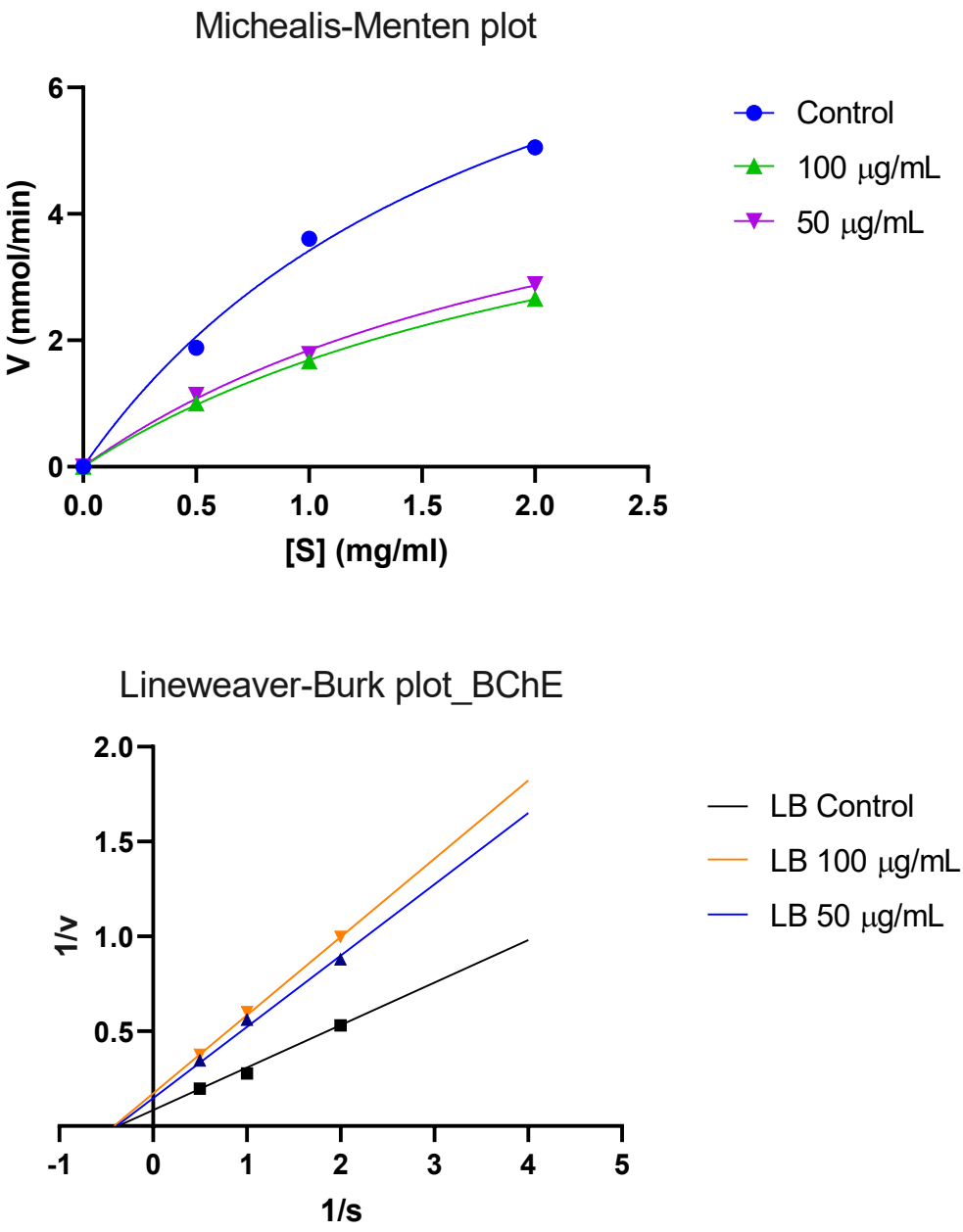


Figure 32. *Michealis- Menten and Lineweaver–Burk plots of BChE inhibition in absence (CTR) or presence of O. heracleoticum EO.*

4.3.1.3 Evaluation of the activity of O. heracleoticum EOs on the metabolic disorder

Samples from Sicignano, Sassari, Sila, Grimaldi and Nardò were analysed to evaluate their potential impact on diseases associated with metabolic disorders such as diabetes and obesity. For this reason, their inhibitory activity against key enzymes involved in these conditions, as well as their cytotoxic effects on two intestinal cell lines—a non-tumour line (IPEC-J2) and a tumour line (Caco-2)—were assessed. The ability of the samples to inhibit preformed biofilms from various foodborne pathogens, a phenomenon closely associated with the onset of foodborne and nosocomial infections, was also investigated.

- ***Antioxidant and enzymatic activities***

The antioxidant assays (table 20) revealed significant differences among the tested samples. In the DPPH assay, the Nardò, Sila, and Sassari samples showed comparable antioxidant activity, with IC_{50} values ranging from 216 to 231 µg/mL. In contrast, the Grimaldi and Sicignano samples displayed significantly higher IC_{50} values (297.26 and 303.41 µg/mL, respectively), indicating a weaker radical scavenging capacity. These values are lower than those reported in previous studies. Stamenic et al. 2014; Spagnoletti et al. 2016). Our findings suggested that the tested extracts may offer superior DPPH scavenging potential. Similarly, the ABTS assay confirmed higher antioxidant activity in the Nardò and Sila samples (319.54 and 321.44 µg/mL, respectively), while Grimaldi showed the lowest value (491.47 µg/mL), again indicating weaker antioxidant potential. In comparison with study conducted by Spagnoletti et al. (2016), who reported an antioxidant capacity of 32 µg TE/mg, the results of the present study agreed. The FRAP assay further supports these findings, highlighting a strong reducing capacity in the ‘Sicignano’ sample (5.72 mM Fe^{2} equivalents/g extract), comparable to the positive control Trolox (5.48 mM Fe^{2}/g). These values suggest that the ‘Sicignano’ extract, despite its lower DPPH and ABTS results, may contain compounds with strong redox properties that contribute to its antioxidant profile through different mechanisms.

Table 20. *Antioxidant activities of EOs of O. heracleoticum*

	Nardò	Sila	Grimaldi	Sassari	Sic.	Trolox
DPPH (IC_{50} µg/mL)	220.00^{b} ± 23.51	216.45^{b} ± 17.42	297.26^{c} ± 16.22	231.39^{b} ± 26.51	303.41^{c} ± 23.27	3.65^{a} ± 0.84
ABTS (IC_{50} µg/mL)	319.54^{b} ± 14.11	321.44^{b} ± 15.91	491.47^{d} ± 18.05	375.15^{bc} ± 12.08	401.12^{c} ± 18.06	2.05^{a} ± 0.45
FRAP (mM Fe^{2+} eq./g ext.)	3.19^{b} ± 0.24	3.43^{b} ± 0.33	2.10^{a} ± 0.22	2.20^{a} ± 0.19	5.72^{c} ± 0.38	5.48^{c} ± 0.41

Sic.: Sicignano. Results are the mean ± SD of three experiments. Different letters in the same line indicate mean values significantly different at $p < 0.05$, according to a one-way ANOVA followed by Tukey's post hoc test.

None of the tested EOs exhibited α-amylase inhibitory activity (table 21), this contrasts with results reported for other *Origanum* species, such as *O. compactum*, which exhibited α-amylase inhibition with an IC_{50} of 150.11 µg/mL (Assaggaf et al., 2023). To date, no studies have reported lipase or α-glucosidase inhibitory activity for the EO of *O. heracleoticum*, highlighting the relevance of the present findings. Notably, in our analysis, the degree of α-glucosidase inhibition differed substantially among the samples. The EO 'Sicignano' exhibited the strongest activity with an IC_{50} of 167.6 µg/mL, indicating potential for postprandial glycemic regulation. Although this activity was lower than that previously reported for *O. compactum* (IC_{50}: 119.84 µg/mL; Assaggaf et al., 2023) and *O. vulgare* (60% inhibition at 18 µg/mL; Salazar et al., 2020), it was significantly more active than the standard drug acarbose (IC_{50}: 920 µg/mL). Other EOs, including 'Grimaldi', 'Sila' and 'Sassari', exhibited lower inhibitory effects, with IC_{50} values ranging from 426 to 803 µg/mL. In the lipase inhibition assay, 'Sila' EO displayed the greatest activity (IC_{50}: 10.7 µg/mL), which was comparable to the pharmaceutical reference orlistat (IC_{50}: 11 µg/mL), and similar to the activity reported for *O. vulgare* EO (IC_{50}: 7.26 µg/mL, Quiroga et al., 2013). Conversely, the Sicignano sample displayed the lowest lipase inhibitory activity (IC_{50}: 87.03 µg/mL).

Table 21. *Enzymatic activities of EOs of O. heracleoticum. Results were expressed as IC_{50} (µg/mL)*

	Nardò	Sila	Grim.	Sassari	Sic	Acarb.	Orlis.
Amylase	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	11.42 ± 0.4	-
Glucosidase	200.6^{b} ± 23.21	426.4^{c} ± 36.27	433.4^{c} ± 30.3	803.3^{c} ± 24.36	167.6^{a} ± 12.25	920^{d} ± 5.85	-
Lipase	38.29^{d} ± 2.85	10.7^{a} ± 1.45	27.76^{c} ± 2.47	14.94^{b} ± 1.09	87.03^{b} ± 6.39	-	11^{a} ± 1.04

Grim.: Grimaldi; Sic.: Sicignano; Acarb.: Acarbose; Orlist.: Orlistat. IC_{50}: inhibitor concentration that determined a 50% inhibition; n.a: not active (IC_{50}> 1 mg/ml). Acarbose and orlistat were used as reference standards. Data are expressed as mean ± standard deviation (n=3 Different letters in the same line indicate mean values significantly different at $p < 0.05$, according to a one-way ANOVA followed by Tukey's post hoc test.

- **Cytotoxicity activity**

After observing the inhibitory effects of glucosidase and lipase enzymes, potential cytotoxicity was investigated using Caco-2 (human colon adenocarcinoma) and IPEC-J2 (porcine jejunal intestinal epithelium) cell lines. The cell lines were treated with EOs at concentrations of 350 and 860 µg/mL, after preliminary screening, for 24 hours. None of the tested samples induced cytotoxic effects on the IPEC-J2 cell line after 24 hours of incubation, compared to the untreated control group. In contrast, after 24 hours, a proliferative effect was noted for Sassari, Nardò, and Grimaldi EOs (Figure 33). Conversely, an opposite effect was observed in the Caco-2 tumor cell line. After 24 hours of incubation, the cytotoxic effect persisted for Sila and Sicignano at the same concentration active (860 µg/mL) for both EOs (Figure 34).

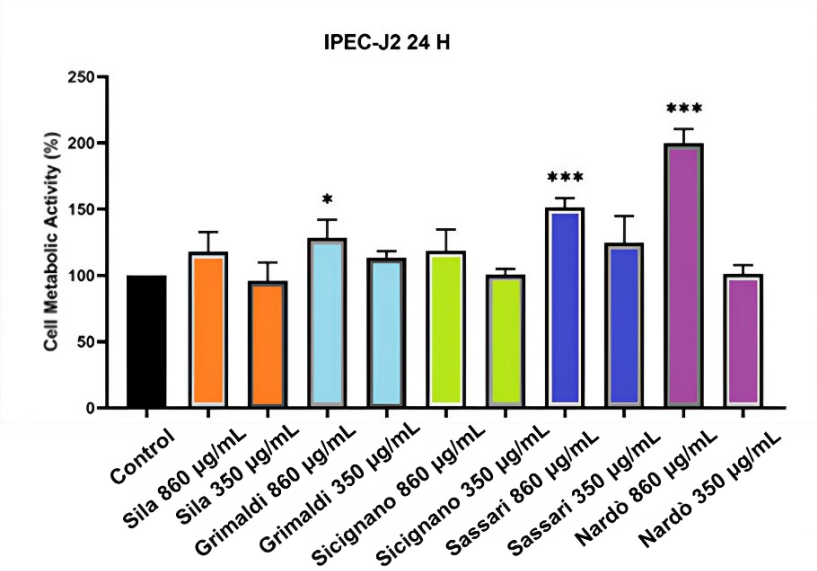


Figure 33. MTS assay showing cell proliferation and cytotoxicity in IPEC-J2 cells treated with essential oils from oregano for 24 h. The metabolic activity of cells treated with two different concentrations of essential oils was compared with a reference control of untreated cells; results are expressed as percentages. One-way ANOVA tests: the statistical differences between each oil with respect to the reference control; **p<0.01 and ***p<0.001 indicate statistically significant differences vs control

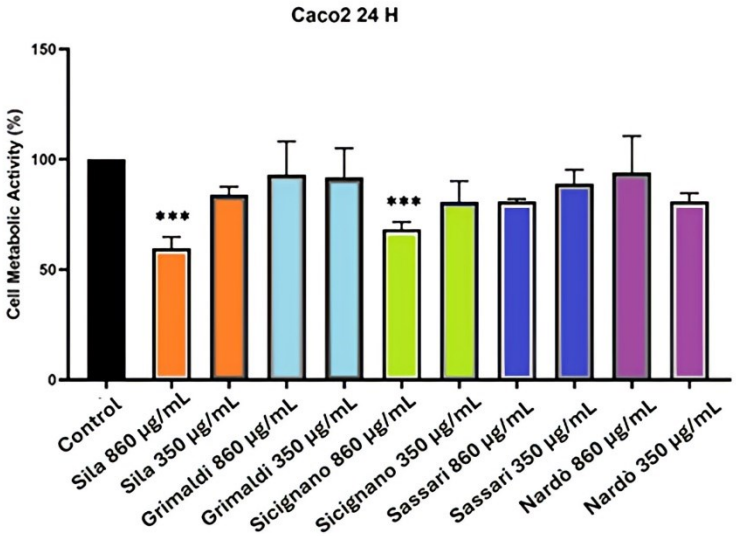


Figure 34. MTS assay showing cell proliferation and cytotoxicity in Caco2 cells treated with essential oils from oregano for 24 h. The metabolic activity of cells treated with two different concentrations of essential oils was compared with a reference control of untreated cells; results are expressed as percentages. One-way ANOVA tests: the statistical differences between each oil with respect to the reference control; **p<0.01 and ***p<0.001 indicate statistically significant differences vs control.

Although the biological activities of these EOs have been documented, cytotoxicity data, in the literature, remain limited. The toxicity assays revealed that, at tested concentrations, no cytotoxic effects occurred on the normal IPEC-J2 cell line after 24 hours of incubation. In contrast, the cancerous Caco-2 cells exhibited cytotoxicity under the same conditions. Comparable findings were reported in previous studies using different tumor hepatocell lines (HepG2) and the MTT assay (Elshafie et al., 2017; Becer et al., 2022). Elshafie et al. (2017) and Becer and coworkers (2022) reported that oregano EOs (*O. vulgare* subsp. *hirtum*) were dominated by the aromatic monoterpenes, mainly carvacrol and thymol, which together accounted for 74.8% of the EO composition. These two constituents showed promising cytotoxic activity against hepatocellular carcinoma cells, with IC_{50} values estimated at 236 µg/mL (Elshafie et al. 2017), 289 µg/mL and 48 µg/mL (Becer et al., 2022). Similar results were obtained in our study: the incubation of Caco-2 tumour cells with the EOs for 24 hours led to a significant reduction in metabolic activity, particularly with the ‘Sila‘ and ‘Sicignano‘ samples (350 µg/mL and 830 µg/mL).

- **Antibiofilm activity**

Furthermore, the potential inhibitory activity against biofilm formation was evaluated using MIC (Minimum Inhibitory Concentration), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) and CV (crystal violet) assays, testing different pathogenic bacterial strains.

The MIC values (µL/mL) of EOs of *O. heracleoticum* from five different localities (Sicignano, Sila, Grimaldi, Nardò, and Sassari) were evaluated against five clinically relevant bacterial strains: *Acinetobacter baumannii, Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Tetracycline, a well-known broad-spectrum antibiotic, was used as the reference antibiotic. The results, shown in table 22 were used to identify the concentration of oils to be used in the crystal violet assay. The oils from ‘Nardò’ and ‘Sassari’ exhibited the lowest MIC values across all bacterial strains (8.0–10.0 µL/mL), suggesting a higher

antimicrobial potency. Their activity was consistent across Gram-negative and Gram-positive species. The ‘Grimaldi’, ‘Sicignano’, and ‘Sila’ EOs demonstrated higher MIC values (ranging from 24.3 to 37.3 µL/mL), indicating moderate antimicrobial activity. Among them, ‘Grimaldi’ EO had the lowest MIC against *A. baumannii* (24.3 µL/mL), aligning with its anti-biofilm action observed. The ‘Nardò’ and ‘Sassari’ EOs were more effective against *P. aeruginosa* and *S. aureus*, with a MIC value of 8.00 µL/mL. The results confirmed that the geographic origin of the oregano could influence its antibacterial potency, likely due to variations in EO composition. The superior antimicrobial activity of Nardò and Sassari EOs might be attributed to their higher content of thymol and carvacrol, respectively, compounds well known for their strong bactericidal effects (Nazzaro et al., 2013). All tested EOs demonstrated promising antibacterial effects, reinforcing their potential as natural antimicrobial agents.

Table 22. *MIC (µL/mL) of the EOs.*

EOs	*A. baumannii*	*E. coli*	*L. monocytogenes*	*P. aeruginosa*	*S. aureus*
Sicignano	25.3 ^{b} ± 0.6	36.7 ^{c} ± 1.2	34.7 ^{b} ± 1.2	32.7 ^{c} ± 1.2	34.7 ^{c} ± 1.2
Sila	31.3 ^{c} ± 1.2	37.3 ^{c} ± 1.2	33.3 ^{b} ± 2.3	34.7 ^{c} ± 1.2	34.0 ^{c} ± 2.0
Grimaldi	24.3 ^{b} ± 0.6	36.7 ^{c} ± 2.3	32.7 ^{b} ± 2.3	34.0 ^{c} ± 2.0	35.3 ^{c} ± 1.2
Nardò	10.0^{a} ± 2.0	10.0 ^{a} ± 2.0	10.0 ^{a} ± 2.0	8.0 ^{a} ± 1.0	8.0 ^{a} ± 1.0
Sassari	10.0 ^{a} ± 1.0	10.0 ^{a} ± 2.0	10.0 ^{a} ± 1.0	8.0 ^{a} ± 1.0	8.0 ^{a} ± 2.0
Tetracycline	23.3 ^{b} ± 1.2	26.0 ^{b} ± 2.0	28.0 ^{b} ± 2.0	27.3 ^{b} ± 1.2	26.7 ^{b} ± 1.2

Results are the mean ± SD of three experiments. Different letters in the same line indicate mean values significantly different at $p < 0.05$, according to a one-way ANOVA followed by Tukey’s post hoc test.

Based on the values previously obtained from the MIC assay, the anti-biofilm activity of EOs at a concentration of 5 µL/mL was assessed. The data (shown in table 23) highlighted significant variability in the biofilm inhibition

capabilities depending on the EO. Notably, the Grimaldi EO exhibited the highest activity against *A. baumannii* (68.2%), showing minimal effect on *P. aeruginosa* (0.9%) and no inhibitory activity against the other pathogens tested. This indicates a strain-specific efficacy profile. Interestingly, the Nardò EO demonstrated the broadest spectrum of activity, inhibiting biofilm formation in *E. coli* (13.9%), *L. monocytogenes* (47.6%), *P. aeruginosa* (41%), and *S. aureus* (16.6%), with no effect on *A. baumannii*. The Sassari EO exerted moderate activity across most tested pathogens, particularly against *S. aureus* (28.3%) and *P. aeruginosa* (27.3%). These findings confirmed previous literature indicating that the chemical composition of EOs varies significantly depending on geographic, environmental, and edaphic factors (da Silva et al., 2021; Hou rt al., 2022; Nurzyńska-Wierdak & Walasek-Janusz, 2025). Moreover, the selective anti-biofilm effects observed reinforce the importance of strain-specific screening when evaluating EOs for antimicrobial applications, especially concerning ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) pathogens. These include *Staphylococcus aureus, Acinetobacter baumannii*, and *Pseudomonas aeruginosa* which were among the species evaluated in this study (Panda et al., 2022).

Table 23. *Inhibitory activity of EOs (5 µL/mL) on the mature biofilm. Results were expressed as percentage of inhibition.*

EOs	*A. baumanni*	*E. coli*	*L. monocytogenes*	*P. aeruginosa*	*S. aureus*
Sicignano	55.8^{c} ±1.2	*n.a.*	*n.a.*	30.0^{b} ± 0.8	*n.a.*
Nardò	*n.a.*	13.9^{b} ± 1.6	47.6^{b} ± 1.7	41.0^{c} ± 2.2	16.6^{a} ± 3.1
Sassari	9.3^{a} ±1.3	6.2^{a} ± 1.0	3.8^{a} ± 0.3	27.3^{b} ± 3.7	28.3^{b} ± 2.4
Sila	28.3^{b} ±1.3	*n.a.*	*n.a.*	0.5^{a} ± 0.2	*n.a.*
Grimaldi	68.2^{d} ±1.7	*n.a.*	*n.a.*	0.9^{a} ± 0.2	*n.a.*

Results are the mean ± SD of three experiments. n.a: not active. Different letters in the same column indicate mean values significantly different at $p < 0.05$, according to a one-way ANOVA followed by Tukey's post hoc test

The MTT assay was used to evaluate the metabolic activity of sessile cells present in the mature biofilms, after the addition of the EOs, at a

concentration of 5 µL/mL, 24 hours post bacterial growth in the multiwell plates. The assay provided a valuable complement to MIC and biofilm data by measuring not just bacterial growth inhibition, but also the suppression of metabolic acitivity in sessile cells. The results were expressed as the percentage of viable cells relative to untreated controls (table 24). EOs from 'Nardò' and 'Sassari' exhibited the strongest inhibitory effect on sessile cells, with viability reductions exceeding 50% for all strains, except for *S. aureus*. The highest inhibitory values were registered against *A. baumannii* (Nardò: 66.2%, Sassari: 83.8%) and *E. coli* (Nardò: 74.4%, Sassari: 70%). While 'Sicignano', 'Sila', and 'Grimaldi' EOs displayed limited activity on sessile cell metabolism, which was observed only against *P. aeruginosa* (Sicignano: 42.9%, Sila: 26.3%, Grimaldi: 35.9%). Among the tested bacteria, *P. aeruginosa* seemed the most resistant strain, although its metabolism was affected by Nardò and Sassari EOs. The results confirmed that the oregano EOs from Nardò and Sassari were more effective in reducing the metabolic activity of the bacterial mature biofilm. This finding was relevant because mature biofilms were typically more resistant to eradication than immature forms. Consequently, any mechanism that compromises biofilm stability or alters the metabolic activity of sessile cells may play a crucial role in mitigating the serious health implications posed by mature biofilms. Furthermore, the data confirmed that EOs rich in active aromatic monoterpenes such as carvacrol and thymol can exert inhibitory effects on pathogenic bacteria (Gavaric et al., 2015). The comparison between the crystal violet and MTT assays highlighted instances where the EOs displayed both anti-biofilm and metabolic inhibitory effects. Notably, Nardò EO significantly inhibited biofilm formation (Crystal violet assay) of *L. monocytogenes* (47.6%) and *P. aeruginosa* (41%), while also strongly reducing the metabolic activity of their sessile cells (52.8% and 66.4%, respectively). Sicignano EO strongly inhibited *A. baumannii* biofilm formation (55.8%) but lacked metabolic inhibition in the MTT assay, suggesting an anti-biofilm mechanism probably through interference with adhesion or EPS (extracellular polymeric substance) production. Sassari EO showed moderate biofilm inhibition (27–28%) against *A. baumannii* but, conversely, exhibited

high inhibitory activity against its sessile cell metabolism (83.8%). Grimaldi EO, while was very active against *A. baumannii* biofilm (68.2%), did not inhibit its sessile cell metabolism. This suggested a mechanism of action more focused on biofilm matrix disruption rather than on metabolism.

Table 24. *Inhibitory activity of the EOs (5 µL/mL) on the metabolism of the bacterial sessile cells in mature biofilms. Results were expressed as percentage of inhibition.*

EOs	*A. baumannii*	*E. coli*	*L. monocytogenes*	*P. aeruginosa*	*S. aureus*
Sicignano	*n.a.*	$1.9^{a} \pm 0.4$	*n.a.*	$42.9^{c} \pm 0.8$	*n.a.*
Nardò	$66.2^{b} \pm 0.6$	$74.4^{b} \pm 0.7$	$52.8^{a} \pm 1.2$	$66.4^{d} \pm 0.9$	$58.9^{b} \pm 0.8$
Sassari	$83.8^{c} \pm 0.6$	$70.0^{b} \pm 1.1$	$67.9^{b} \pm 0.6$	$61.2^{d} \pm 1.5$	$47.2^{a} \pm 0.8$
Sila	$12.5^{a} \pm 1.3$	*n.a.*	*n.a.*	$26.3^{a} \pm 2.3$	*n.a.*
Grimaldi	*n.a.*	*n.a.*	*n.a.*	$35.9^{b} \pm 2.9$	*n.a.*

Results are the mean ± SD of three experiments. n.a: not active. Different letters indicate in the same column mean values significantly different at $p < 0.05$, according to one-way ANOVA followed by Tukey's post hoc test.

4.3.2 Polar extract

This section presents the results of an intraspecific comparison between different *Origanum heracleoticum* samples collected from various Italian locations (Sicignano, Marsico Nuovo, Agrigento, Nardò, Sassari, and Sila). The objective of the analysis was to evaluate any differences in the composition of the non-volatile fraction and, consequently, in the potential associated biological activities. The 'Sicignano' sample was initially used to compare the effectiveness of different extraction techniques (dynamic maceration and PLE). This method was then used for extracting all samples. Subsequently, chemical characterization of the extracts and a multivariate analysis were performed, aimed at highlighting relationships between the samples and identifying any geographical or chemotypic patterns. Furthermore, the biological activities of the extracts were evaluated, with particular attention to enzymes involved in metabolic disorders and antibiofilm activity. As previously performed with *O. dictamnus*, an initial screening was carried out using the same extraction technique (dynamic maceration) with two different solvents: 70% EtOH and 20% EtOH (Jacotet-Navarro et al., 2018). As shown in Tables 25 and 26, different parameters were evaluated in order to identify the optimal extraction solvent, which is defined as the solvent that maximises the biological properties of the extract.

Table 25. *Comparison of the extraction methods by evaluating antioxidant activity, total flavonoid, phenolic and carbohydrate contents of 'Sicignano'.*

Method of extraction	Yield (%)	TPC (GAE mg/g)	TFC (QE mg/g)	TCC (GLUE mg/g)	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)
Maceration EtOH 70%	16.75^{a} ± 1.01	203.48^{a} ± 12.97	2.50^{a} ± 0.15	194.49^{a} ± 23.40	19.05^{c} ± 3.40	18.99^{c} ± 5.40
Maceration EtOH 20%	20.33^{b} ± 1.45	269.73^{b} ± 15.45	3.52^{b} ± 0.12	259.97^{b} ± 24.43	13.49^{b} ± 1.90	10.63^{b} ± 3.11
Trolox	-	-	-	-	3.65^{a} ± 0.90	2.05^{a} ± 0.45

Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$. according to one-way ANOVA followed by Tukey's post hoc test. GAE= Gallic acid equivalent; QE= Quercetine equivalent; GLUE= Glucose equivalent

Table 26. *Comparison of the extraction methods by evaluating the enzymatic activity of 'Sicignano'. Results were expressed as IC_{50} (µg/mL)*

	AChE	BChE	α-amylase	α-glucosidase	Lipase
Maceration EtOH 70%	415.20^{b} ± 12.92	430.20^{c} ± 13.07	*n.a.*	208.80^{b} ± 15.39	18.85^{b} ± 1.40
Maceration EtOH 20%	390.40^{b} ± 15.27	367.20^{b} ± 15.46	*n.a.*	49.65^{a} ± 12.98	13.85^{a} ± 1.90
Galantamine	0.70^{a} ± 0.15	10.25^{a} ± 15.30	-	-	-
Orlistat	-	-	-	-	11.15^{a} ± 1.04
Acarbose	-	-	10.25 ± 1.19	920.15^{c} ± 5.85	-

Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$. according to one-way ANOVA followed by Tukey's post hoc test. *n.a.: not active*

Table 25 highlighted how the different percentages of ethanol used in maceration influence both the extraction yield and the content of phenolic and flavonoid. The extract obtained with 20% EtOH showed a higher yield than that obtained with 70% EtOH (20.33% *vs* 16.75%), indicating better solubilization of the more polar compounds thanks to the greater aqueous component. This trend was also confirmed by the higher values of total phenols (TPC), flavonoids (TFC), and carbohydrates (TCC) in the 20% extract. Also, the antioxidant activity was greater in the 20% EtOH extract, both in the DPPH (IC_{50} = 13.49 µg/mL) and ABTS (10.63 µg/mL) assays. Overall, these results suggested that a more polar solvent favours the extraction of phenolic compounds with strong antioxidant activity. Significant differences in enzymatic inhibition shown in Table 26. The 20% EtOH extract exhibited higher activity against lipase and α-glucosidase. These results confirmed that a more polar solvent improves the extraction of bioactive compounds involved in the modulation of carbohydrate and lipid metabolism. AChE activity, however, is comparable between the two extracts, suggesting

that the compounds responsible for cholinesterase inhibition are recovered with similar efficiency under both conditions.

After identifying the optimal solvent (20% EtOH), a comparison was performed between two extraction techniques: the conventional method (dynamic maceration) and an unconventional technique (Pressurized Liquid Extraction, PLE). The comparison between maceration with 20% EtOH and PLE conducted at 115 °C showed a significant improvement in extraction performance with the PLE technique (Tables 27 and 28). PLE guaranteed a higher extraction yield (27.67%) and an increase in total phenol content (TPC: 368.31 mg GAE/g), while the flavonoid content, however, was similar between the two methods. Overall, the extract obtained via PLE exhibited significantly higher biological activity than the maceration extract. Notably, PLE enhanced the antioxidant and enzymatic inhibitory properties, demonstrating the superior efficiency of this extraction method. While PLE extract generally exhibited inhibition of cholinesterases and lipase (approaching the level of Orlistat), their α-glucosidase inhibitory activity was lower than that observed for maceration extract. These results clearly indicated an advantage of the PLE method, thanks to high temperature and pressure conditions that improve the extraction of bioactive compounds (Fraguela-Meissimilly et al., 2023; Dobroslavić et al., 2022).

Table 27. *Comparison of the extraction methods by evaluating antioxidant activity, total flavonoid, phenolic and carbohydrate contents of 'Sicignano'*

Method of extraction	Yield (%)	TPC (GAE mg/g)	TFC (QE mg/g)	TCC (GLUE mg/g)	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)
Maceration EtOH 20%	20.33^{a} ± 1.45	269.73^{a} ± 15.45	3.52^{a} ± 0.12	259.97^{a} ± 24.43	13.49^{b} ± 1.90	10.63^{b} ± 3.11
PLE 115° EtOH 20%	27.67^{b} ± 1.32	368.31^{b} ± 24.55	3.45^{a} ± 0.14	290.53^{a} ± 35.40	6.49^{ab} ± 1.36	5.12^{ab} ± 1.47
Trolox	-	-	-	-	3.65^{a} ± 0.90	2.05^{a} ± 0.45

Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$. according to one-way ANOVA followed by Tukey's post hoc test. GAE= Gallic acid equivalent; QE= Quercetine equivalent; GLUE= Glucose equivalent

Table 28. *Comparison of the extraction methods by evaluating the enzymatic activity of 'Sicignano'. Results were expressed as IC_{50} (µg/mL)*

	AChE	BChE	α-amylase	α-glucosidase	Lipase
Maceration EtOH 20%	390.40^{c} ± 15.27	367.20^{c} ± 15.46	*n.a.*	49.65^{a} ± 12.98	13.85^{ab} ± 1.90
PLE 115° EtOH 20%	287.00^{b} ± 24.61	268.60^{b} ± 24.38	*n.a.*	142.20^{b} ± 14.74	7.83^{a} ± 0.96
Galantamine	0.70^{a} ± 0.15	10.25^{a} ± 15.30	-	-	-
Orlistat	-	-	-	-	11.15^{ab} ± 1.04
Acarbose	-	-	10.25 ± 1.19	920.15^{c} ± 5.85	-

Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$. according to one-way ANOVA followed by Tukey's post hoc test. *n.a.: not active*

Once preliminary tests to optimize the extraction process were completed, pressurized liquid extraction was applied to all other *O. heracleoticum* samples. Theobtained extracts were then subjected to chemical characterization by LC-HRESIMS/MS and evaluated for different biological activities, with particular attention to enzymes involved in metabolic disorders. The extractive yields (table 29) ranged between approximately 25% and 31%, with higher values in samples from Nardò and Sila, while Sassari and Marsico Nuovo showed lower yields. Total phenolic content was highest in Sicignano (368 mg GAE/g), followed by Sassari and Nardò. Flavonoid content showed a different pattern, with Marsico Nuovo having the highest value (4.90 mg QE/g) and Agrigento the lowest. Carbohydrate content also varied between locations, being higher in Sila (301.99 mg GLUE/g) and lower in Sassari and Marsico Nuovo. Antioxidant activity, tested by DPPH and ABTS assays, partially reflected phenolic content. For DPPH, samples from Sila, Nardò, Sicignano, and Sassari show IC_{50} values between 6 and 7.5 µg/mL, while Marsico Nuovo showed higher values. The ABTS assay revealed less marked differences, with Nardò and Sassari showing the

highest antioxidant capacity. Currently, the scientific literature lacks any studies examining *O. heracleoticum* with pressurized liquid extraction (PLE). However, the work conducted by Miron et al., (2011) applied PLE to the species *O. vulgare*, reporting antioxidant activity values comparable to those obtained in the present study. Regarding *O. heracleoticum*, the study conducted by Kosakowska et al., (2020) analyzed a hydroalcoholic extract obtained by maceration: in this case, the antioxidant activity was lower than that evaluated in our samples extracted with PLE. In fact, the extract analyzed by Kosakowska and collaborators showed an IC_{50} of 68 µg/mL in DPPH and an ABTS activity of 381 µmol TE/g, values significantly higher than those obtained in the present work. The enzyme activities (table 30) indicated further differences between samples. For lipase, 'Sicignano' was the most active with an IC_{50} of 7.83 µg/mL, while 'Marsico Nuovo' and 'Nardò' were the least effective, with values above 40 µg/mL. 'Sila' showed intermediate activity (15.77 µg/mL), near to the reference inhibitor 'Orlistat' (11 µg/mL). For α-glucosidase, 'Sila' and 'Sassari' showed lower IC_{50} and therefore greater inhibition, while 'Agrigento' and 'Sicignano' showed lower activity, with IC_{50} above 140–180 µg/mL: all extracts showed higher activity than acarbose (IC_{50}: 920 µg/mL). α-amylase was not inhibited by any of the extracts, indicating a selective action towards α-glucosidase. Furthermore, no literature is available on the enzymatic activity of *O. heracleoticum* extracts obtained by PLE.

Table 29. *Antioxidant activity, total flavonoid, phenolic and carbohydrate contents of O. heracleoticum extracts from different Italian areas*

	Yield (%)	TPC (GAE mg/g)	TFC (QE mg/g)	TCC (GLUE mg/g)	DPPH (IC_{50} µg/mL)	ABTS (IC_{50} µg/mL)
Sassari	25.67^{a} ± 0.04	262.68^{c} ± 0.79	3.31^{b} ± 0.32	202.60^{a} ± 29.43	7.57^{ab} ±0.45	11.85^{a} ± 1.28
Nardò	31.3^{c} ± 0.09	256.55^{c} ± 2.72	4.48^{c} ± 0.28	260.46^{b} ± 2.24	6.68^{a} ±0.68	10.68^{a} ± 1.81
Agrigento	27.5^{b} ± 0.02	209.52^{b} ± 5.64	2.67^{a} ± 0.51	239.51^{ab} ± 27.25	11.59^{b} ±0.91	22.16^{b} ± 2.50
Sila	31.0^{c} ± 0.01	116.76^{a} ± 1.72	3.54^{b} ± 0.11	301.99^{c} ± 24.57	6.39^{a} ±0.27	16.61^{ab} ± 2.60
Marsico Nuovo	25.5^{a} ± 0.04	104.80^{a} ± 16.91	4.90^{d} ± 0.62	210.32^{a} ± 6.80	15.78^{c} ±0.45	21.96^{b} ± 1.81
Sicignano	27.6^{b} ± 0.02	368.31^{d} ± 18.85	3.46^{c} ± 0.22	259.97^{b} ± 44.71	6.49^{a} ±0.63	20.57^{b} ± 1.99

Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$. according to one-way ANOVA followed by Tukey's post hoc test. GAE= Gallic acid equivalent; QE= Quercetine equivalent; GLUE= Glucose equivalent

Table 30. *Enzymatic activities of extracts of O. heracleoticum from different Italian areas. Results were expressed as IC_{50} (µg/mL)*

	Sas.	Nar.	Agr.	Sila	M. N.	Sic.	Orlist.	Acar.
Lipase	29.46^{c} ± 3.00	42.75^{d} ± 4.00	32.67^{c} ± 3.50	15.77^{b} ± 1.50	50.65^{e} ± 5.00	7.83^{a} ±0.80	11.15^{ab} ± 1.04	-
α-glucosidase	38.30^{a} ± 4.00	58.22^{b} ± 6.00	183.50^{e} ± 18.00	34.45^{a} ± 3.00	76.83^{c} ± 7.50	142.20^{d} ± 14.00	-	920^{f} ± 5.85
α-amylase	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	*n.a.*	-	11.42 ± 0.4

Sas: Sassari; Nar: Nardò; Agr: Agrigento; M.N.: Marsico Nuovo; Sic: Sicignano; Orlist.: Orlistat; Acar.: Acarbose. Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$. according to one-way ANOVA followed by Tukey's post hoc test. *n.a.: not active*

Qualitative and semi-quantitative analysis (Figures 35-40 and Table 31) revealed significant differences in the chemical profiles of the samples, with the identification of approximately 50 secondary metabolites, primarily

phenolic acids (as hydroxybenzoic and hydroxycinnamic acids) and flavonoids.

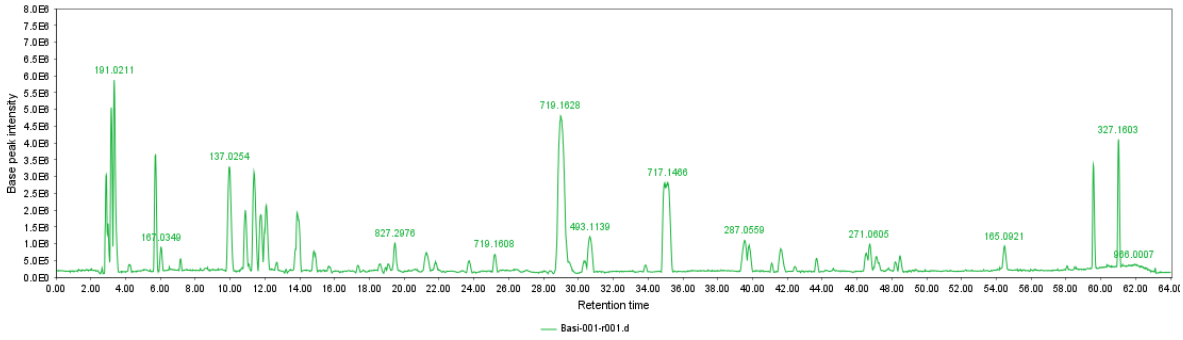


***Figure 35**. LC- (-) HRMS base peak chromatogram of 'Marsico Nuovo' PLE extract*

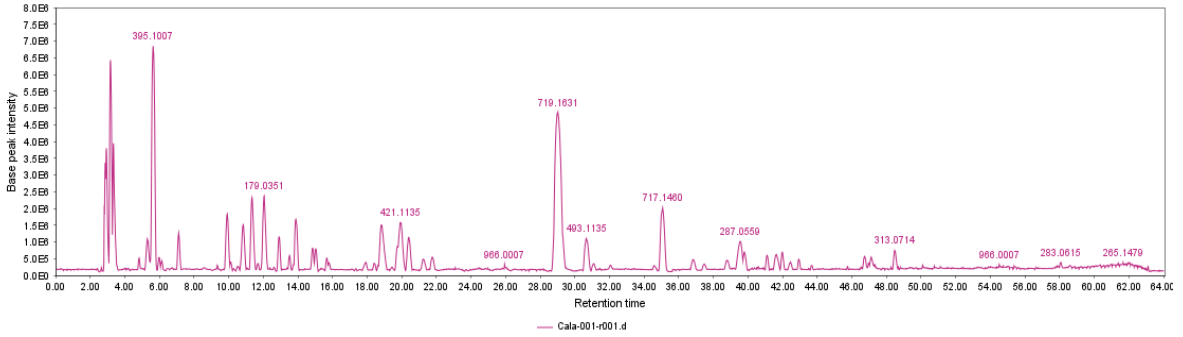


***Figure 36**. LC- (-) HRMS base peak chromatogram of 'Sila' PLE extract*

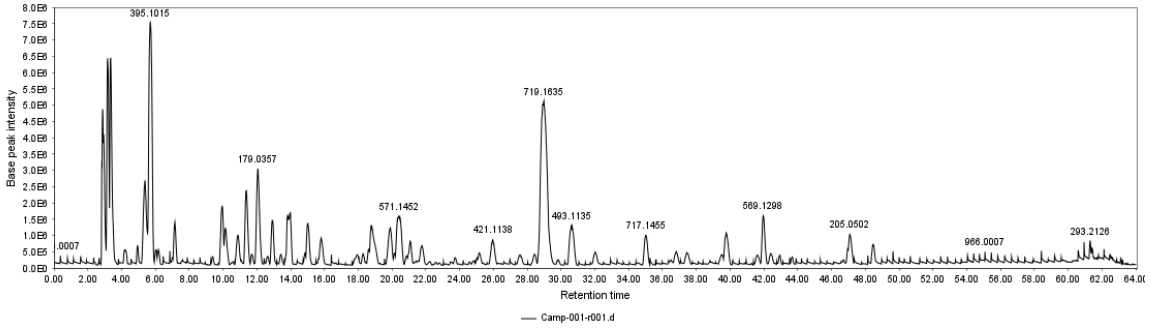


***Figure 37**. LC- (-) HRMS base peak chromatogram of 'Sicignano' PLE extract*

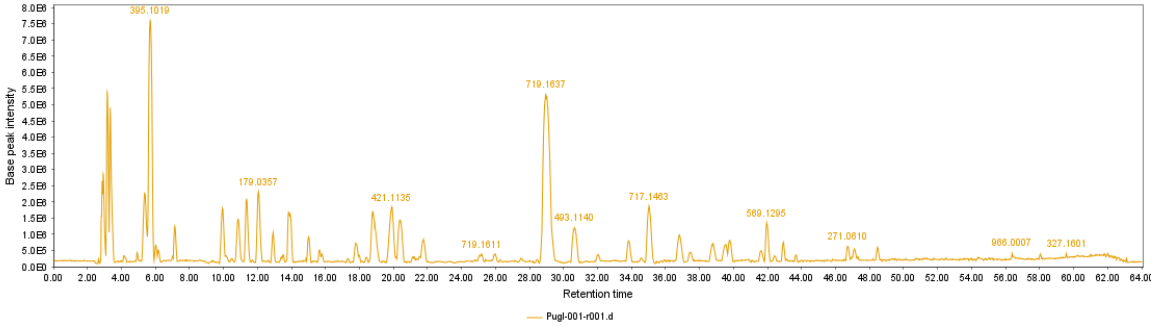


***Figure 38**. LC- (-) HRMS base peak chromatogram of 'Nardò' PLE extract*

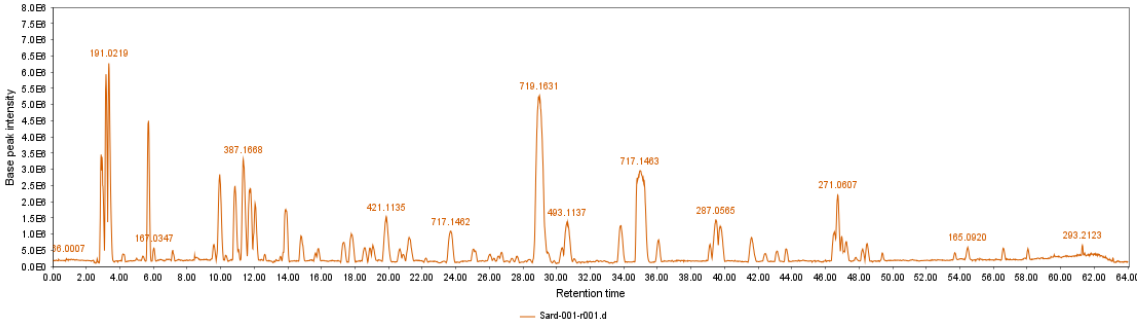


***Figure 39**. LC- (-) HRMS base peak chromatogram of 'Sassari' PLE extract*

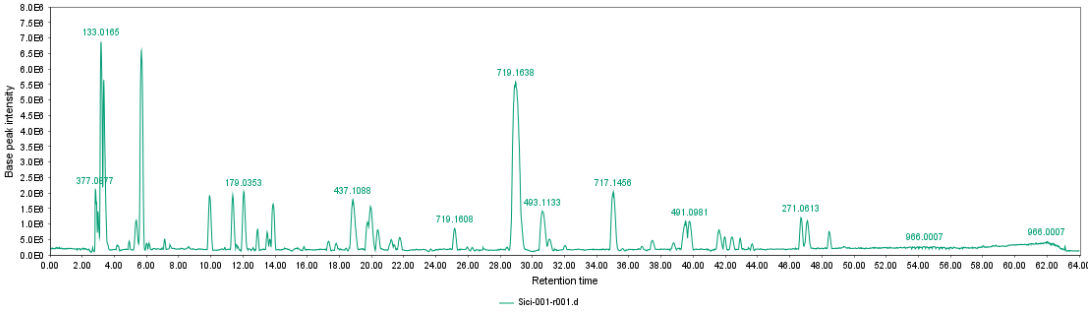


***Figure 40**. LC- (-) HRMS base peak chromatogram of 'Agrigento' PLE extract*

Structural assignment was obtained through the interpretation of diagnostic fragments in MS/MS, supplemented by literature consultation. Among the hydroxybenzoic acids, syringic acid was detected with a $[M–H]^-$ at m/z 197.0463 and with characteristic fragments at m/z 179 (H_2O loss), with fragments at m/z 161 (H_2O loss) and m/z 135 (CO_2 loss). (Taamalli et al., 2015). This compound was particularly abundant in the Sicignano and Nardò

samples, while it was less abundant in those from Marsico Nuovo and Sassari. Protocatechuic acid also showed wide variability: it had a $[M–H]^-$ at m/z 153.0196 and fragments at m/z 109 (CO_2 loss) and m/z 91 (de Torre et al., 2022) and it was more present in the Sila and Nardò samples. The hydroxycinnamic acids also showed well-defined diagnostic patterns. Caffeic acid exhibited a characteristic MS/MS fragmentation pathway, with the transition from m/z 179 to m/z 135, a cleavage pattern typically associated with catechol-type phenolic acids (Petrakis et al., 2023) Rosmarinic acid, on the contrary, showed a more complex fragmentation, with a $[M–H]^-$ at m/z 359 and ions at m/z 197 (unit derived from the caffeic acid), 179 (H_2O loss), 161 and 135 (CO_2 loss) (Zengin et al., 2019). It was the most abundant compound in most samples, with the sole exception of Sicignano, where salvianolic acid A ($[M−H]^-$ m/z 493) predominated, fragmenting to generate ions at m/z 197, 179, and 135 (Maietta et al., 2018). Regarding the flavonoid glycosides, fragmentation was dominated by the sequential loss of sugar residues, such as hexose (−162 u), deoxyhexose (−146 u), glucuronide (−176 u), and apioside (−132 u). The MS/MS analysis of Apigenin 7-O-(6''-malonyl-apiosyl-glucoside) revealed a molecular peak at m/z 649.141 $[M–H]^-$, corresponding to the fully acylated glycoside. Fragmentation was characterized by the sequential loss of the malonyl group and sugar residues, with the formation of intermediate fragments at m/z 321.0612 and 259.0617. The secondary fragments at m/z 233.082 and 215.0732 derived from retro-Diels-Alder cleavages typical of the flavonic structure, while those at m/z 189.0924; 161.0629; 135.045 represented fragmentation products of the apigenin aglycone, with progressive loss of CO and CO_2 (Slimestad et al., 2020). This compound was particularly abundant in some localities, especially in Sicignano. Another characterizing flavonoid was luteolin-7-O-diglucuronide ($[M–H]^-$ m/z 637.1047), which released the aglycone luteolin (m/z 285) after the loss of the two glucuronide units (Maietta et al., 2018).

Table 31. *Chemical composition of PLE extracts of O. heracleoticum through LC-HRESIMS/MS*

8				Peak Area								
Rt (min)	**[M-H]$^-$**	**Main fragments (m/z)**	**Putative identification**	**M.N**	**Sila**	**Sic.**	**Nardò**	**Sas.**	**Agr.**	**Formula (M)**	**Δppm**	**Code**
5.69	395.0988	Dimerization										
	197.0463	179.0356; 135.0457; 72.9938	Syringic acid	1.72E^{+05}	3.68E^{+05}	5.02E^{+05}	6.64E^{+05}	2.13E^{+05}	3.30E^{+05}	$C_9H_{10}O_5$	0.27	HB2
6.1	391.067	Dimerization										
	195.0299	167.6006; 123.0081; 95.0142; 68.9979	Hydroxycaffeic acid	2.57E^{+04}	6.54E^{+04}	2.92E^{+05}	7.61E^{+04}	2.49E^{+04}	5.33E^{+04}	$C_9H_8O_5$	0.02	HC2
7.11	153.0196	109.0291; 91.0181	Protocatechuic acid	7.06E^{+04}	1.74E^{+05}	1.33E^{+05}	1.65E^{+05}	5.78E^{+04}	5.96E^{+04}	$C_7H_6O_4$	1.74	HB3
9.41	175.0612	174.8652; 118.8871; 103.3055; 85.0661	2-Isopropylmalic acid	1.20E^{+02}	6.87E^{+03}	1.01E^{+05}	1.76E^{+04}	2.10E^{+02}	4.66E^{+03}	$C_7H_{12}O_5$	0.02	O2
9.56	353.0875	191.0554; 173.0438; 153.0168; 135.0448; 93.034	4-caffeoylquinic acid	4.93E^{+03}	3.58E^{+04}	3.28E^{+04}	2.77E^{+04}	1.17E^{+04}	7.31E^{+03}	$C_{16}H_{18}O_9$	-0.86	HC3
9.93	137.0246	108.0196; 91.0175; 75.0794	Hydroxybenzoic acid	8.15E^{+03}	4.25E^{+03}	2.57E^{+04}	6.34E^{+03}	1.15E^{+05}	4.49E^{+03}	$C_7H_6O_3$	1.32	HB1
10.13	649.141	321.0612; 259.0617; 233.082; 215.0732; 189.0924; 161.0629; 135.045	Apigenin 7-O-(malonyl-apiosyl-glucoside)	6.55E^{+05}	3.23E^{+05}	1.55E^{+05}	3.03E^{+05}	5.24E^{+05}	3.28E^{+05}	$C_{29}H_{30}O_{17}$	-0.04	FL7
10.28	329.0877	167.0357; 123.0457; 107.0121; 81.0341	Vanillic acid hexoside	6.09E^{+02}	8.54E^{+04}	3.10E^{+05}	1.39E^{+05}	-	2.02E^{+04}	$C_{14}H_{18}O_9$	-0.32	O5
10.58	633.1457	589.1579; 439.1213; 321.0633; 277.0726; 233.0823; 215.071; 189.0931	Origanine A	1.43E^{+04}	2.50E^{+04}	1.54E^{+05}	1.78E^{+04}	5.73E^{+04}	9.09E^{+02}	$C_{29}H_{30}O_{16}$	-0.64	PA1
10.85	305.0704	225.1135; 96.9607; 79.9580	Gallocatechine	1.19E^{+02}	6.86E^{+03}	1.00E^{+05}	1.76E^{+05}	2.10E^{+02}	4.65E^{+04}	$C_{15}H_{14}O_7$	-4.02	FL3
11.37	775.3394	Dimerization										
	387.163	207.1012; 163.11; 119.034	Hydroxyjasmonic acid *O*-hexoside	5.50E^{+05}	5.49E^{+05}	5.49E^{+05}	3.08E^{+05}	5.96E^{+05}	2.99E^{+05}	$C_{18}H_{28}O_9$	0.63	OA3
11.74	177.0198	133.0268; 105.0348; 77.0387	Aesculetin	4.17E^{+04}	5.41E^{+04}	1.04E^{+05}	3.21E^{+04}	1.99E^{+04}	3.76E^{+04}	$C_9H_6O_4$	2.63	O1
11.75	593.1504	575.142; 473.1082; 383.0769; 353.0672	Apigenin-di-*C*-hexoside	3.36E^{+05}	1.28E^{+03}	3.32E^{+04}	9.65E^{+03}	5.12E^{+05}	2.90E^{+03}	$C_{27}H_{30}O_{15}$	0.68	FL8
11.99	179.035	135.0452; 107.0505; 107.0505; 79.0559	Caffeic Acid	2.41E^{+05}	9.78E^{+04}	1.01E^{+04}	6.03E^{+04}	1.69E^{+05}	3.64E^{+04}	$C_9H_8O_4$	0.1	HC4
12.02	373.1503	327.1449; 190.0974; 164.0841; 149.0603;	Deoxyloganin	3.63E^{+05}	4.10E^{+05}	2.74E^{+05}	4.12E^{+05}	3.08E^{+05}	3.54E^{+05}	$C_{17}H_{26}O_9$	-0.28	O6

		134.0375										
12.61	377.0879	97.0435; 161.0209; 133.0286; 108.0209; 91.0556: 72.9934	Danshensuan C	$5.58E^{+04}$	$3.69E^{+04}$	$2.95E^{+05}$	$3.14E^{+04}$	$5.29E^{+04}$	$3.35E^{+04}$	$C_{18}H_{18}O_9$	0.25	PA2
13.49	329.1242	167.0726; 145.0309; 121.0292; 93.0337	Bartsioside	$5.40E^{+02}$	$3.23E^{+04}$	$1.53E^{+05}$	$4.82E^{+04}$	$3.96E^{+01}$	$1.74E^{+04}$	$C_{15}H_{22}O_8$	0.03	O3
13.81	637.1047	351.058; 285.0417; 193.0366; 175.0151	Luteolin-7-*O*-diglucuronide	$4.00E^{+05}$	$2.85E^{+05}$	$2.34E^{+05}$	$3.86E^{+05}$	$3.66E^{+05}$	$2.82E^{+05}$	$C_{27}H_{26}O_{18}$	0.1	FL9
14.84	447.0927	357.0617; 327.0501; 299.0551; 133.0288	Homoorientin (Luteolin 6-*C*-glucoside)	$1.35E^{+05}$	$3.71E^{+02}$	$2.26E^{+03}$	$8.43E^{+03}$	$1.72E^{+05}$	$2.18E^{+02}$	$C_{21}H_{20}O_{11}$	-1.31	FL10
14.85	225.113	148.0862; 120.0599; 95.0496; 59.0142	Tuberonic acid	$7.36E^{+03}$	$7.65E^{+03}$	$2.23E^{+05}$	$2.49E^{+03}$	$6.94E^{+03}$	$5.36E^{+02}$	$C_{12}H_{18}O_4$	-1.03	OA1
15.68	447.0931	357.0588; 327.0502; 387.0402; 193.0133; 133.0287	Orientin (Luteolin 8-*C*-glucoside)	$5.68E^{+04}$	$3.96E^{+01}$	$1.46E^{+05}$	$5.81E^{+02}$	$4.86E^{+04}$	$3.72E^{+01}$	$C_{21}H_{20}O_{11}$	-0.41	FL11
17.31	621.1095	351.0569; 269.0463; 193.0347; 153.0604; 113.0243	Apigenin-7-*O*-diglucuronide	$6.65E^{+04}$	$3.60E^{+04}$	$5.09E^{+04}$	$4.50E^{+04}$	$1.68E^{+05}$	$8.69E^{+04}$	$C_{27}H_{26}O_{17}$	-0.36	FL12
17.82	537.1037	339.052; 295.0605; 197.0454; 135.0452	Lithospermic acid	$2.72E^{+04}$	$2.96E^{+04}$	$3.20E^{+04}$	$1.53E^{+05}$	$2.34E^{+05}$	$6.82E^{+04}$	$C_{27}H_{22}O_{12}$	-1.9	HC1
18.6	431.0984	341.0675; 311.0561; 283.0614; 239.0724; 197.0596; 163.0399; 117.0357	Vitexin	$9.08E^{+04}$	$1.68E^{+02}$	$6.50E^{+04}$	$1.09E^{+03}$	$1.33E^{+05}$	$1.64E^{+02}$	$C_{21}H_{20}O_{10}$	0.07	FL13
18.79	437.1085	153.0195; 109.0295; 91.0194	Origanol A	$1.49E^{+04}$	$3.73E^{+05}$	$1.98E^{+05}$	$4.44E^{+05}$	-	$4.35E^{+05}$	$C_{20}H_{22}O_{11}$	-0.99	PA3
19.06	431.0979	342.0643; 283.0611; 253.051; 195.0433; 161.0241; 117.0341	Apigenin−7-*O*-glucoside	$8.26E^{+04}$	$1.53E^{+02}$	$1.03E^{+05}$	$1.30E^{+03}$	$1.26E^{+05}$	$1.10E^{+02}$	$C_{21}H_{20}O_{10}$	-1.09	FL14
19.74	651.1203	413.0882; 371.0748; 351.0586; 299.0581; 193.0358; 175.0249	Chrysoeriol 7.4'-diglucuronide	$1.13E^{+03}$	$1.81E^{+05}$	$3.08E^{+04}$	$9.87E^{+04}$	$1.80E^{+04}$	$2.36E^{+05}$	$C_{28}H_{28}O_{18}$	0.02	FL15
19.95	421.1136	153.0195; 109.0295; 91.0194	Desmethyloriganoside	$2.21E^{+04}$	$4.25E^{+05}$	$1.70E^{+05}$	$4.84E^{+05}$	$3.75E^{+05}$	$4.18E^{+05}$	$C_{20}H_{22}O_{10}$	-1	PA4
21.24	303.051	285.0367; 241.0508; 223.038; 189.0547; 152.0113; 125.0254; 105.0359	Taxifolin	$1.95E^{+05}$	$1.15E^{+05}$	$8.72E^{+04}$	$5.83E^{+04}$	$2.17E^{+05}$	$1.03E^{+05}$	$C_{15}H_{12}O_7$	-0.09	FL18
21.79	555.1499	449.1074; 287.0562; 243.0665; 225.053; 199.0408; 164.0106; 121.0292	Fustin-O-(hydroxymethylphenyl) -O-hexoside	$3.99E^{+02}$	$1.15E^{+05}$	$9.19E^{+04}$	$1.93E^{+05}$	$1.34E^{+04}$	$1.18E^{+05}$	$C_{28}H_{28}O_{12}$	-1.62	FL4
22.25	813.1525	635.1451; 527.1004; 461.0693; 443.0527; 351.0541; 333.0429; 285.038	Kaempferol-*O*-triglucuronide isomer 1	$3.84E^{+04}$	$6.47E^{+03}$	$9.38E^{+04}$	$2.60E^{+04}$	$5.01E^{+04}$	$1.41E^{+04}$	$C_{37}H_{34}O_{21}$	0.64	FL19

23.72	717.1461	537.1029; 519.0933; 475.1034; 431.1135; 365.0656; 339.051	Salvialonic Acid D	1.51E^{+03}	1.32E^{+03}	2.08E^{+04}	2.67E^{+03}	5.12E^{+03}	2.53E^{+03}	$C_{36}H_{30}O_{16}$	-0.01	PA5
25.21	719.1612	359.0745; 341.0648; 197.0446; 161.0232	Sagerinic acid isomer 1	1.29E^{+05}	2.82E^{+04}	6.15E^{+04}	7.42E^{+04}	9.24E^{+04}	1.69E^{+05}	$C_{36}H_{32}O_{16}$	-0.78	OA2
27.56	451.1245	403.7429; 167.0335; 152.0121; 123.045; 108.0216	3-hydroxyphlorizin	-	1.96E^{+03}	9.83E^{+04}	2.95E^{+04}	-	1.18E+03	$C_{21}H_{24}O_{11}$	-0.19	CA1
29.089	719.1627	Dimerization										
	359.0769	197.0447; 179.0344; 161.0247; 133.0295; 109.0286	Rosmarinic acid	8.58E^{+05}	8.86E^{+05}	1.42E^{+06}	9.32E^{+05}	8.99E^{+05}	9.61E^{+05}	$C_{18}H_{16}O_{8}$	-0.95	HC5
30.62	493.1139	295.0622; 185.0249; 159.0465; 135.0461; 109.0302	Salvianolic acid A	3.21E^{+05}	2.67E^{+05}	6.32E^{+05}	3.38E^{+05}	4.17E^{+05}	4.12E^{+05}	$C_{26}H_{22}O_{10}$	-0.24	PA6
31.98	621.146	561.1256; 501.0995; 417.0829; 328.059; 285.0409	Luteolin 7-O-pentosyl-acetyl-hexoside	3.53E^{+04}	7.23E^{+04}	1.17E^{+05}	9.12E^{+04}	2.35E^{+04}	6.87E^{+04}	$C_{28}H_{30}O_{16}$	-0.17	FL16
33.82	537.1036	499.2755; 472.039; 359.0729; 295.0626; 251.0718; 197.0461;161.0243; 135.0453	Salvianolic acid I	7.41E^{+04}	6.41E^{+03}	4.50E^{+03}	1.72E^{+05}	3.02E^{+05}	1.21E^{+04}	$C_{27}H_{22}O_{12}$	-0.46	PA7
35.16	717.1463	339.0495; 321.039; 295.0594; 279.028; 249.0539; 185.0232	Salvianolic acid B	1.22E^{+06}	4.96E^{+05}	1.12E^{+05}	4.53E^{+05}	1.60E^{+06}	5.19E^{+05}	$C_{36}H_{30}O_{16}$	0.27	PA8
39.49	571.1185	Dimerization										
	287.0558	151.0039; 135.045; 107.0132; 89.0397	Eriodictyol	3.01E^{+05}	2.91E^{+05}	1.10E^{+05}	1.88E^{+05}	3.69E^{+05}	2.99E^{+05}	$C_{15}H_{12}O_{6}$	-1.08	FL1
39.8	491.0982	311.0542; 267.0642; 135.0443	Salvianolic acid C	1.42E^{+03}	1.39E^{+03}	9.67E^{+02}	8.54E^{+02}	1.04E^{+03}	1.09E^{+03}	$C_{26}H_{20}O_{10}$	-0.35	PA8
41.08	493.1137	422.7864; 359.0757; 251.0703; 197.0457; 179.0344; 161.0248; 135.0452	Salvianolic acid A isomer II	6.67E^{+04}	9.68E^{+04}	8.11E^{+03}	3.33E^{+04}	6.92E^{+03}	1.95E^{+04}	$C_{26}H_{22}O_{10}$	-0.65	PA10
41.68	285.0405	243.0307; 218.0538; 197.0619; 151.0049; 133.0299; 107.0143	Luteolin	2.13E^{+05}	1.57E^{+05}	5.31E^{+04}	1.19E^{+05}	2.17E^{+05}	1.99E^{+05}	$C_{15}H_{10}O_{6}$	0.13	FL20
42.92	553.1346	447.0907; 391.0775; 285.0398; 241.0501	luteolin-O-glucoronide	8.89E^{+02}	7.20E^{+04}	7.33E^{+04}	1.16E^{+05}	8.37E^{+03}	8.01E^{+04}	$C_{28}H_{26}O_{12}$	-0.99	FL17
43.62	491.0975	311.0555; 293.0455; 265.0509; 249.0544; 197.0456; 135.0452	Salvianolic acid C isomer 1	9.60E^{+04}	4.73E^{+04}	5.35E^{+04}	5.53E^{+04}	9.78E^{+04}	5.97E^{+04}	$C_{26}H_{20}O_{10}$	-1.77	PA11
46.53	329.0669	300.0186; 178.9998; 117.0348; 67.0199	Cirsiliol	1.60E^{+05}	4.42E^{+04}	2.74E^{+04}	1.43E^{+04}	2.57E^{+05}	1.16E^{+03}	$C_{17}H_{14}O_{7}$	0.68	FL5
46.71	271.0603	253.0498; 208.0522; 177.0178; 151.003; 119.0499	Naringenin chalcone	1.79E^{+05}	1.04E^{+05}	2.43E^{+04}	1.19E^{+05}	4.01E^{+05}	2.32E^{+05}	$C_{15}H_{12}O_{5}$	-3.3	CA2

47.29	665.1721	619.1677; 577.1560; 283.0616; 268.0359	Naringin 6'-malonate	$5.95E^{+03}$	$4.45E^{+04}$	$3.08E^{+04}$	$4.43E^{+04}$	$1.51E^{+04}$	$1.24E^{+04}$	$C_{30}H_{34}O_{17}$	-0.34	FL2
48.46	313.0714	313.071; 283.0244; 227.0346; 183.0436; 163.0034; 135.0073; 117.0341	Cirsimaritin	$2.92E^{+02}$	$5.56E^{+02}$	$8.17E^{+03}$	$2.60E^{+02}$	$4.14E^{+02}$	$4.61E^{+02}$	$C_{17}H_{14}O_6$	-1.15	
59.56	329.1759	329.1742; 314.1487; 271.0947; 255.064; 227.0687; 209.0589	Carnosol	$3.47E^{+05}$	$3.70E^{+03}$	$5.93E^{+02}$	$3.46E^{+04}$	$2.57E^{+04}$	$3.70E^{+03}$	$C_{20}H_{26}O_4$	0.2	O4

M.N.: Marsico Nuovo; Sic.: Sicignano; Sas.: Sassari; HB: hydroxybenzoic acid derivative; HC: hydroxycinnamic acid derivate; O: Others; FL: Flavonoid; PA: Phenolic acid; OA: Organic acid; CA: Calcone. -: absent

After chemical characterization of the extracts and evaluation of their biological activities, a multivariate analysis was conducted to identify any distinctive patterns among the samples, both in terms of their metabolic composition and biological activities. The multivariate approach also allowed to explore the correlations between the different variables obtained, highlighting possible associations between specific metabolites and the observed enzymatic and antioxidant activities. The PCA biplot provided a comprehensive overview of the relationships among the six *O. heracleoticum* samples (PLE extracts from Marsico Nuovo, Nardò, Sassari, Agrigento, Sicignano, Sila) and the contributions of the main chemical variables to the first three principal components (Table 32 and Figure 41)

Table32. *Loadings of the significant variables on three first principal components from data analysis*

Variables	**PC1**	**PC2**	**PC3**
CA1	0.1645	0.1306	0.0099
CA2	-0.1599	0.0355	0.1373
FL1	-0.1762	-0.0424	0.0189
FL2	0.1285	-0.1155	0.1710
FL3	0.1637	0.1394	-0.0201
FL4	0.1286	-0.1663	0.0990
FL5	-0.1535	0.1306	0.1043
FL6	0.1487	0.1631	-0.0459
FL7	0.1828	0.0776	0.0284
FL8	-0.1568	0.1404	0.0853
FL9	-0.1460	-0.0331	0.0873
FL10	-0.1652	0.1270	0.0606
FL11	0.0746	0.2284	-0.0571
FL12	-0.1248	0.1125	0.1597
FL13	-0.1012	0.2112	0.0605
FL14	-0.0478	0.2416	0.0496
FL15	0.0448	-0.1954	-0.0592
FL16	0.1942	-0.0182	-0.0139
FL17	0.1480	-0.1428	0.0760
FL18	-0.1670	0.1149	-0.0112
FL19	0.0733	0.2315	0.0240
FL20	-0.1862	-0.0443	-0.0447
HB1	-0.1879	0.0268	-0.0603
HB2	0.1537	-0.0762	0.1407
HB3	0.1306	-0.1094	0.0930
HC1	-0.0782	0.0284	0.3294

HC2	0.1711	0.1243	-0.0283
HC3	-0.0837	0.1462	0.2405
HC4	-0.0301	-0.2353	0.0024
HC5	0.1578	0.1484	-0.0311
O1	0.1587	0.1057	-0.1319
O2	0.1541	-0.0428	0.1203
O3	0.1799	0.1010	-0.0050
O4	-0.1125	0.0423	-0.2093
O5	0.1193	0.1946	0.0483
O6	-0.1724	0.0420	-0.0505
OA1	0.1456	0.1676	-0.0447
OA2	-0.0906	0.0087	-0.1693
OA3	-0.0159	0.0935	0.1768
PA1	0.1075	-0.1938	0.1137
PA2	0.1348	0.1813	-0.0500
PA3	0.1090	-0.1988	0.0108
PA4	0.0281	-0.1637	0.2428
PA5	0.1356	0.1837	0.0053
PA6	0.1103	0.1911	0.0016
PA7	-0.1094	0.0552	0.2964
PA8	-0.1850	0.0693	0.0766
PA9	-0.0866	-0.0412	-0.2360
PA10	-0.0304	-0.1440	-0.1362
PA11	-0.1685	0.1261	0.0067
TPC (GAE mg/g)	0.1164	0.1400	0.1675
TFC (QE mg/g)	-0.0453	-0.0063	-0.0367
GLUE (mg/g)	0.1396	-0.1394	0.0020
DPPH (IC_{50} µg/mL)	-0.1180	0.0149	-0.2800
ABTS (IC_{50} µg/mL)	-0.0154	-0.0635	0.3525
Lipase (IC_{50} µg/mL)	-0.1337	-0.0650	-0.0472
α-glucosidase (IC_{50} µg/mL)	0.0775	0.0394	-0.2055
Eigenvalue	25.69	15.55	6.87
Total variance (%)	45.08	27.28	12.05

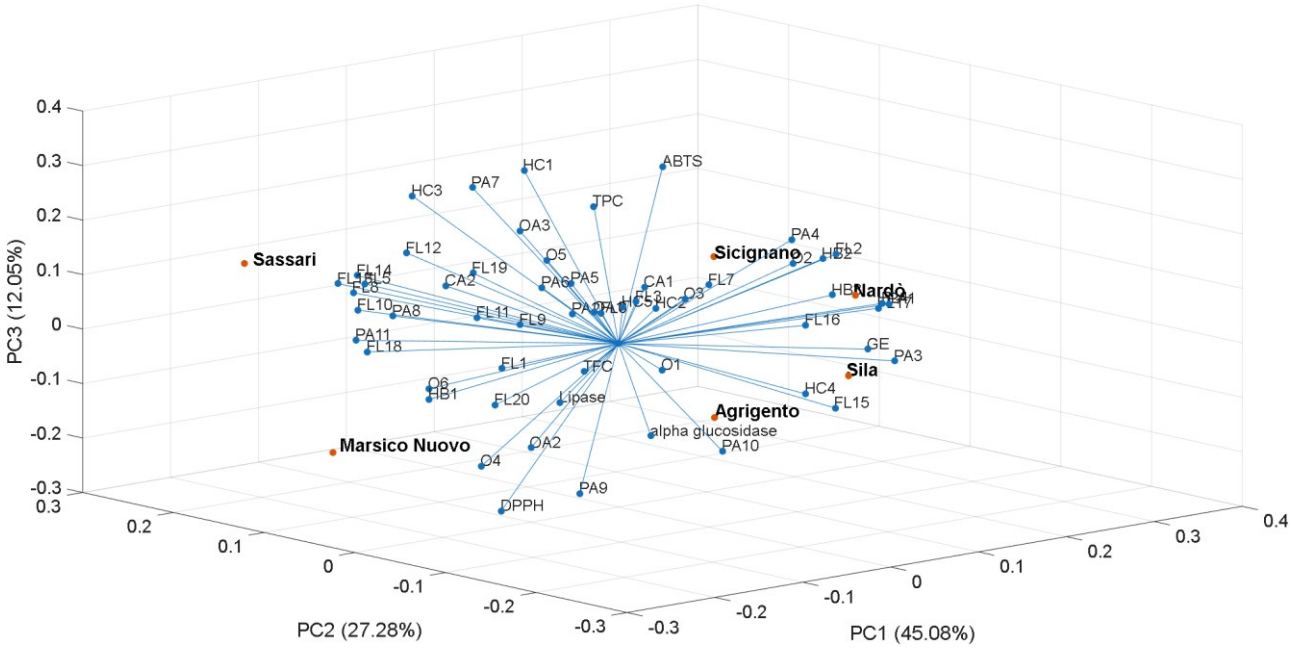


Figure 41. *Biplot (loading and scores plots) obtained by principal component analysis (PCA) of six O. heracleoticum extracts (Marsico Nuovo, Grimaldi, Pollino, Nardò, Sassari, Agrigento, Sicignano, Sila) based on their chemical and biological activity profiles in the three-dimensional space. The vectors shown are the eigenvectors of the covariance matrix.*

The 3D PCA plot provides a visualization of the relationship between the samples based on their chemical and biological activity profiles. Each dot represents a different sample, while the axes correspond to the first three principal components (PC1, PC2, and PC3), explaining 45.08%, 27.28%, and 12.05% of the variance, respectively. These principal components highlight the key sources of variation within the dataset.

PC1 is the most important component, explaining almost half of the variance in the data. Variables with the highest contribution to PC1 are likely driving the major differences between samples. Flavonoids and flavonoid glycosides are the primary contributors to PC1 , with compounds such as Eriodictyol (FL1), Naringin 6'-malonate (FL2), Taxifolin (FL3), and Apigenin 7-O-diglucuronide (FL12) showing a strong positive correlation with this principal component.in the PCA plot, are associated with high levels of flavonoids and flavonoid glycosides , suggesting these chemical compounds play a major role in shaping the chemical profile of these samples. Also, variables like TFC (Total Flavonoid Content) and DPPH (antioxidant capacity)

are strongly correlated with PC1. These antioxidant activities are elevated in Sassari and Marsico Nuovo, as indicated in both the PCA plot and the heatmap. The correlation between high flavonoid content and antioxidant activity explains why these samples group together along the positive axis of PC1, which represents strong antioxidant activity driven by flavonoids. Hydroxybenzoic acids and hydroxycinnamic acid derivatives contribute significantly to PC2 which explains 27.28% of total variance. Compounds such as syringic acid (HB2) and caffeic acid (HC4) show a positive correlation with this component. These acids are more abundant in Agrigento, Sila, and Nardò, indicating that these chemical compounds are linked to specific biological activities related to enzymes.

The presence of phenolic compounds like protocatechuic acid (HB3) and hydroxycaffeic acid (HC2) in the samples from Agrigento and Sila gives them a distinctive chemical composition that is reflected in their position on the positive side of PC2. PC2 is also primarily influenced by α-glucosidase activity, an enzyme involved in carbohydrate metabolism. Samples from Agrigento, Sila, and Nardò, which show higher alpha-glucosidase activity, are positively correlated with PC2. The observed enzymatic activity in these regions is therefore a significant factor contributing to the separation of these samples from others along PC2. Other compounds like hydroxycaffeic acid (HC2) and caffeic acid (HC4) are linked to biological activities such as oxidative stress defense, which are predominantly seen in Agrigento and Sila, contributing to their position along PC2. Phenolic acids, e.g. salvianolic acid A (PA6) and desmethyloriganoside (PA4), are the main contributors to PC3. These compounds, which are abundant in Agrigento, and Sicignano, influence the variability of the samples along this principal component. Additionally, lipase, an important enzyme involved in lipid metabolism, directly impacts the positioning of samples along PC3. This suggests that lipase activity is a key factor in differentiating Sicignano, which has higher lipase activity and is found on the positive side of PC3 from other samples, which show lower levels of lipase and higher antioxidant activity. Other enzymatic activities like DPPH (linked to antioxidant properties) and TFC (total flavonoid content) are slightly negatively correlated with PC3,

suggesting an inverse relationship between antioxidant activity and lipase in certain regions like Sicignano. By combining the contributions of both chemical compounds and biological activities, a clearer picture of the differentiation among samples was highlighted. Sassari and Marsico Nuovo are characterized by high levels of flavonoids (e.g., Eriodictyol, Naringin 6'-malonate) and flavonoid glycosides (e.g., Luteolin-O-glucuronide, Apigenin 7-O-glucoside), which are strongly correlated with PC1. The high antioxidant activity (observed through DPPH and TFC) correlates with the abundance of flavonoids and explain why Sassari and Marsico Nuovo are grouped on the positive axis of PC1, reflecting high antioxidant activity due to flavonoid compounds. Agrigento, Sila, and Nardò are characterized by higher concentrations of hydroxycinnamic acids (e.g., caffeic acid, hydroxycaffeic acid) and hydroxybenzoic acids (e.g., syringic acid, protocatechuic acid), which are expressed more in PC2 and are known to impact enzymatic activities. The high levels of α-glucosidase and other enzymatic activities related to carbohydrate metabolism are significant in differentiating these regions. The combination of these factors places these regions in the positive side of PC2 in the PCA plot. Sicignano has lower levels of antioxidants (e.g., DPPH, TFC) and flavonoids but higher levels of lipase and phenolic acids: the elevated lipase activity in this sample plays a central role in its position along the positive side of PC3. This highlights its distinct metabolic activity compared to other regions with higher antioxidant profiles.

The heatmap (figure 42) provided a detailed, visual representation of the relative abundance of various chemical compounds and biological activities across the samples. It served as a complementary tool to the PCA analysis, offering deeper insight into how these variables contribute to the observed variability in the dataset.

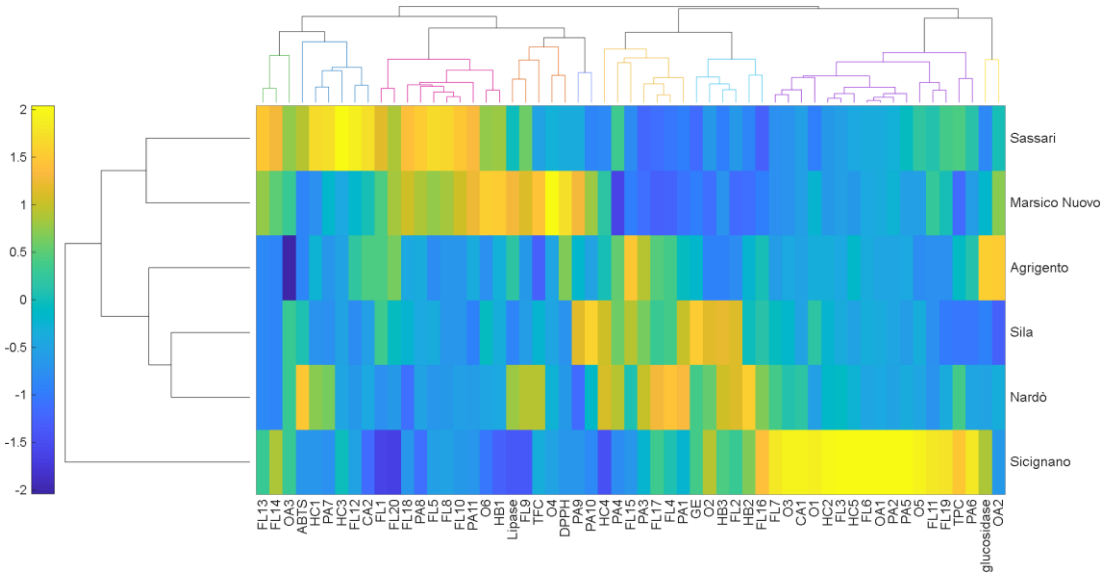


Figure 42. *Hierarchical Cluster Heatmap of 6 observations (rows) and variables (columns), with normalized data values represented by a colour scale ranging from blue (low) to yellow (high). The rows represent the extracts of O. heracleoticum distilled from plant collected in six Italian areas. The columns represent the main compound and biological properties of the extracts.*

The heatmap highlights the elevated concentrations of flavonoid glycosides and flavonoids (e.g., eriodictyol (FL1), taxifolin (FL3), luteolin derivatives like FL12, FL13) in Sassari and Marsico Nuovo. These compounds, which are strong antioxidants, are significantly higher in these two regions compared to others. This correlates with the strong positive correlation observed with PC1 in the PCA plot, where these samples are placed at the positive end of PC1, driven by the high antioxidant potential of these compounds. The presence of these flavonoids is directly linked to the high levels of TFC (Total Flavonoid Content) and DPPH (antioxidant capacity) observed in the same samples.

The heatmap clearly also shows that Agrigento, Sila, and Nardò are characterized by higher concentrations of hydroxycinnamic acids [e.g., caffeic acid (HC4), hydroxycaffeic acid (HC2)] and hydroxybenzoic acids [e.g., syringic acid (HB2)]. These compounds are integral to the biochemical activity of these regions, as they contribute to PC2, the axis primarily influenced by α-glucosidase activity. The heatmap also shows elevated α -glucosidase activity in Agrigento, Sila, and Nardò, confirming their position along the positive axis of PC2 in the PCA. The increased presence of

hydroxycinnamic acids in these regions suggests a biological function related to antioxidant and anti-inflammatory activity, which may help support the enzymatic functions linked to carbohydrate metabolism observed in these regions. The PCA and heatmap together suggest that these regions possess a higher degree of enzymatic activity, particularly in relation to carbohydrate breakdown, and this contributes to their grouping on PC2.

The heatmap reveals that Sicignano exhibits higher concentrations of phenolic acids [e.g., salvianolic acid A (PA6), desmethyloriganoside (PA4)] compared to other regions. These phenolic acids are key contributors to the variability seen along PC3, which is influenced by lipase activity. The heatmap highlights elevated lipase activity in Sicignano, positioning these samples on the positive side of PC3. This higher lipase activity, combined with the higher concentrations of phenolic acids, suggests that Sicignano may be metabolically distinct from the other regions. Its higher lipase activity is linked to lipid metabolism, and the heatmap reflects this metabolic distinction, with Sicignano showing increased enzymatic breakdown of fats. This makes it stand out from other regions, which are more characterized by flavonoid -based antioxidant activity.

The heatmap also shows the presence of organic acids [e.g., tuberonic acid (OA1), sagerinic acid (OA2)] in various regions, although their concentrations are generally lower than the aforementioned compounds. These compounds may play a supporting role in shaping the chemical profile of the samples, but they do not appear to have as pronounced an effect on the clustering of the samples compared to flavonoids, phenolic acids, or lipase activity. The heatmap reinforces the differentiation of the samples based on their chemical and biological profiles: Sassari and Marsico Nuovo are clustered on the positive side of PC1. The heatmap shows a clear dominance of flavonoid-related compounds and high antioxidant potential, confirming that these two regions have a distinctive biochemical signature.

Agrigento, Sila, and Nardò exhibit higher levels of hydroxycinnamic acids and hydroxybenzoic acids, as well as alpha-glucosidase activity. The heatmap confirms the PCA analysis, with these regions showing increased

enzymatic activity associated with carbohydrate metabolism, placing them on the positive side of PC2 in the PCA plot. Sicignano sample shows elevated lipase activity, as indicated by the heatmap, which also shows higher levels of phenolic acids. The heatmap positions Sicignano on the positive side of PC3, which reflects its distinct lipase activity and unique metabolic profile.

The heatmap provides a deeper understanding of the variability observed across the samples, reinforcing the patterns identified in the PCA plot. By visually displaying the concentrations of chemical compounds and biological activities, the heatmap shows how different regions group together based on their specific biochemical features. Together, the heatmap and PCA analysis provide a clear, integrated view of how chemical composition and biological activity shape the variability observed across the different samples.

5. MATERIAL AND METHODS

5.1 Plant material

The aerial parts of *Origanum dictamnus* L. were purchased in May 2023 from Minardi A. & Sons (Ravenna, Italy) through a licensed supplier. The species was identified by Prof. Vincenzo De Feo (University of Salerno) based on *Flora d'Italia*, and a voucher specimen (DF/738/2023) was deposited in the Herbarium of the Chair of Medical Botany, University of Salerno. The aerial parts of *O. vulgare*, *O. majorana* and *O. heracleoticum* collected in Calvanico (Salerno, Italy) were likewise identified by Prof. De Feo, and the corresponding voucher specimens (DF/740/2023; DF/741/2023; DF/742/2023) were deposited in the same herbarium. Additional *O. heracleoticum* samples were collected from various locations across Italy, including Sicignano (Salerno), Marsico Nuovo (Potenza), Agrigento, Sila (Crotone), Grimaldi (Cosenza), Pollino (Matera), Sassari, and Nardò (Lecce). All samples were authenticated by Prof. De Feo following *Flora d'Italia* (Pignatti et al., 2017), and the voucher specimens (DF/743/2023 to DF/751/2023) were deposited in the Herbarium of the Chair of Medical Botany, University of Salerno.

5.2 Extraction and analysis of EO

- *Hydrodistillation*

Aerial parts of each sample were subjected to hydrodistillation for 3 h, following the procedure described in the European Pharmacopoeia (2020). The resulting EOs were dried over anhydrous sodium sulphate and stored under N_2 at + 4◦C in the dark until tested and analyzed.

- *Gascromatography coupled with mass spectrometry (GC-MS)*

GC/MS analyses were performed on an Agilent 6850 Ser. II apparatus (Agilent, Roma, Italy), fitted with a fused silica DB-5 capillary column (30 m, 0.25 mm i.d., 0.33 μm film thickness, Agilent, Roma, Italy), coupled to an Agilent Mass Selective Detector MSD 5973; Ionization energy was set to 70 eV and the electron-multiplier voltage to 2000 V. Mass spectra were scanned in the range 40–500 amu, scan time 5 scans/s. Column temperature: 40 °C, with 5 min initial hold, and then to 270 °C at 2 °C/min, 270 °C (20 min); injection was splitless (1 μL of a 1:1000 *n*-hexane solution); injector and detector temperatures were 250 °C and 290 °C, respectively. Helium was used as the carrier gas (1.0 mL/min). Most constituents were identified by comparing their Kovats retention indices (Ri), which were determined relative to the retention times (Rt) of *n*-alkanes (C10-C35), to either those in the literature (Adams, 2017; Davies, 1990) and mass spectra on the column, or those of authentic compounds available in our laboratories via the NIST 02 and Wiley 275 libraries (Linstrom & Mallard, 2001). Peak area normalization was used to get the component relative concentrations. No response factors were calculated.

5.3 Extraction and analysis of extract

- *Dynamic maceration*

The plant leaves were cleaned and air-dried. Approximately 4 g of dried leaves per species were macerated with 100 mL of solvent (70% ethanol (*v*/*v*) and 20% ethanol (v/v)). This procedure was conducted three times, with a maceration period of 12 hours. The extracts were filtered, and the solvent was removed with rotary evaporator to yield the dry extracts.

- *Pressure liquid extraction (PLE)*

Analysis of plant leaves was performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA). Two different solvents (water and ethanol) were used, in a water: ethanol ratio (8:2). Extractions were performed using three different extraction temperatures (50, 115,

180°C). To test the influence of solvent composition, extractions using water/ethanol mixtures were performed at a fixed temperature of 115 °C. The extraction time was kept constant for all experiments (20 min). Before any extraction, a heating step of the extraction cell was performed for a predetermined time. The heating time varied according to the extraction temperature (5 min when the extraction temperature was 50, 7 min if the extraction temperature was 115 °C and 9 min if the extraction temperature was 180 °C). All extractions were performed using 11 mL extraction cells, containing 1 g of sample. When water was used for extraction, the extraction cell was filled with a sand mixture on top of the sample (2.0 g of sand) to prevent clogging of the system. The extraction procedure was as follows: (i) the sample was loaded into the cell, (ii) the cell was filled with solvent to a pressure of 1500 psi (1 psi = 6894.76 Pa), (iii) a heating period was applied, (iv) a static extraction (i.e., 20 min) was performed with all valves in the system closed, (v) the cell was rinsed (with 60% of the cell volume using the extraction solvent), (vi) the solvent was purged from the cell with gaseous N_2, and (vii) depressurization was performed. Between extractions, the entire system was rinsed to avoid any carryover effect. Once the extractions were completed, the solvents were removed. A Rotavapor R-210 (Buchi Labortechnik AG, Flawil, Switzerland) was used for ethanol evaporation. The aqueous extracts were freeze-dried using a freeze-dryer (Labconco Corporation, MO, USA).

- *High Performance Liquid Chromatography–Tandem Mass Spectrometry Analysis*

The samples obtained were extracted to recover and concentrate the bioactive compounds. For this purpose, 0.2 mL of cold water (H_2O) was added to the samples, and the samples were vortexed for 10 s. Then, 0.8 mL of ethanol was added and vortexed again for 10 s. Then, the mixture was centrifuged at 14,000 rpm for 5 min, and the supernatant was carefully transferred to a 1.5 mL Eppendorf tube and dried in a SpeedVac device at 40 °C and 13 bar pressure. Finally, the dried samples were resuspended in 25 µL of pure ethanol for their subsequent analysis. Each extract was dissolved

in EtOH to a final concentration of 1 mg/mL. Subsequently, the samples were vortexed for 30 s, centrifuged at 14,800 rpm for 5 min at 4 °C, and the supernatants were collected and stored at −80 °C until analysis. Aliquots of 2 µL were injected into a LC-MS/MS system consisting of a HPLC model 1290 coupled to a Q-TOF series 6540 using an Agilent Jet Stream thermal orthogonal ESI source (Agilent Technologies, Waldbronn, Germany). Compounds were separated using Kinetex® (5 µm C18, 150 × 4.6 mm, Phenomenex) from Agilent Technologies (Wadbronn, Germany). Milli-Q water was used as mobile phase (A), while acetonitrile (ACN) was employed mobile phase (B), and 0.1% formic acid was used as the mobile phase modifier. The column temperature was maintained at 40 °C, and the flow rate was set to 0.5 mL/min with the following gradient: 0–17% B in 10 min; 25% B in 35min; 50% B in 55 min; 100% B in 60 min; 100% B for 3 min. The mass spectrometer was operated in ESI (−) modes using the following parameters: capillary voltage of −3000 V for ESI negative; mass range from 25 to 1100 m/z; nebulizer pressure of 40 psi; and drying gas flow rate of 8 L/min and 300 °C. The sheath gas flow was 11 L/min at 350 °C. MS/MS analyses were performed employing the auto MS/MS mode using five precursors per cycle, dynamic exclusion after two spectra (released after 0.5 min), and collision energies of 20 and 40 V.

- *MZmine Data Processing*

Raw MS data were processed using Mzmine 3.4.27 with adjustments to the method outlined by Francolino et al. (2025). Centroid mass detection was applied to both MS1 and MS2 levels. Parameters for the ADAP chromatogram builder, including the minimum number of consecutive scans, minimum intensity for consecutive scans, minimum peak intensity, and *m*/*z* (mass-to-charge ratio) tolerance, were carefully configured. The wavelet algorithm was used for the local minimum feature resolver, with optimized settings for the signal-to-noise (S/N) threshold, intensity window S/N, minimum feature height, coefficient area threshold, peak duration range, and retention time (RT) wavelet range. Chromatograms were deisotoped using a ^{13}C isotope filter, adjusting the *m*/*z* and RT tolerances, with a

maximum charge of 1 and a selection of the most intense isotopes as representatives. Additional steps included identifying isotopic peaks, aligning features with the join aligner, and gap filling for peak detection.

5.4 Cholinesterases inhibition assay

Cholinesterase inhibitory activity was assessed by the colorimetric assay of Ellman et al. (1961) with some modifications. Briefly, 10 µL of the sample dissolved in MeOH, at various concentrations (100, 10, 1 and 0.1 mg/mL), was combined with 25 µL of 0.28 U/mL AChE (or BChE) solution and 415 µL of Tris-HCl solution (0.1 M, pH 8). The mixture was put for 15 min at 37 °C. Then, a solution of 1.83 mM of acetylthiocholine iodide (AChI) or butyrylcholine iodide (BChI) (75 µL) and 475 µL of 5,5-dithio-bis-(2-nitrobenzoic acid (DTNB) were added. Following a further 30 min incubation at 37 °C, the absorbance was read at 405 nm using a UV spectrophotometer (Thermo Fischer Scientific, Vantaa, Finland Varian, CARY 100 Bio, USA). Galantamine (0.1-100 µg/mL) was used as a reference drug.

The enzyme activity was calculated as a percentage using the formula (1):

$$\%=[(AC-AE)/Ac]*100 \quad (1)$$

where AC represented the absorbance of enzyme solution without EO, AE represented the absorbance of the enzyme solution after contact with the EO. The experiments were carried out in triplicate, and the results were expressed as IC_{50} value for each enzyme. The IC_{50}, which represents the EO concentration that causes 50% inhibition, was derived by plotting the percentage inhibition *versus* various concentrations.

5.5 Tyrosinase Inhibition assay

The inhibitory effects on tyrosinase (TYR) were determined by evaluating both the monophenolase and diphenolase activities of the enzyme, using the method of Khatib et al. (2024), with minor modifications. The reaction mixture was prepared in a 96 multiwell plate: in each well, aliquots 8 µL of sample (0.1–1 mg/mL) were mixed with 400 µL of substrate, 120 µL of tyrosinase (200 U/ml) and 280 µL of sodium acetate buffer (50 mM, pH 6.8). The

reaction mixture was incubated for 5 min at 37 °C; thereafter, 400 µL of L-DOPA or L-tyrosine (2 mM) were added. The optical density of the reaction mixture was recorded at 492 nm (Thermo Fischer Scientific, Vantaa, Finland Varian, CARY 100 Bio, USA), after an additional incubation of 20 min at 37 °C. Kojic acid (1–50 µg/mL) was used as positive control.

The results were calculated with formula (2) and expressed as IC_{50} values.

$$\%=[A_0-A_1]A_0*100 \quad (2)$$

where A_0 is the absorbance of the control without the sample and A_1 the absorbance of the sample. The experiments were carried out in triplicate. The sample concentration producing 50% inhibition (IC_{50}) was gained by calculating the inhibition against sample concentrations.

5.6 α-Amylase inhibition assay

Jaradat's approach, with minor modifications, was used to assess the amylase activity (Jaradat et al., 2020). The mixture, composed by 100 µL of different concentrations of the sample (previously dissolved in methanol or in ethanol to obtain solution of 5–1000 µg/ml), 200 µL of 20 mM sodium phosphate buffer (pH = 6.9), and 100 µL of amylase solution (10 U/mL), were placed in a block heater at 37 °C for 10 min. Then, 180 µL of a 1% substrate solution was added and placed in a block heater at 37 °C, for 20 min. Following this, 180 µL of a 3,5 dinitrosalycyclic acid (DNSA) water solution (96 mM) was added to the mixture and boiled in a block heater at 100 °C for 10 min. Then, the absorbance of the solution was read at 540 nm using a UV Spectrophotometer (Thermo Fischer Scientific, Vantaa, Finland Varian, CARY 100 Bio, USA). Acarbose (1-100 µg/mL) was used as a reference drug. All experiments were carried out in triplicate, and the results were expressed as IC_{50} value.

5.7 α-Glucosidase inhibition assay

α-Glucosidase inhibitory activity was assessed following the method proposed by Si et al. (2010) with some modifications. Briefly, the assay was performed in a 96-well plate, by first adding 150 µL of 0.1 M phosphate buffer

at pH 7.0, followed by the addition of 10 µL of the sample dissolved in MeOH or in EtOH to obtain various concentrations (5–1000 µg/mL). Then, 15 µL of the α-glucosidase enzyme water solution (1 U/mL) were added to each well to initiate the reaction, and the plate was put for 5 min at 37 °C; next, 75 µL of the substrate (2.0 mM) 4-nitrophenyl α-D-glucopyranoside were added; then, the plate was incubated for 10 min at 37 °C. The absorbance was measured at 405 nm using a UV Spectrophotometer (Thermo Fischer Scientific, Vantaa, Finland Varian, CARY 100 Bio, USA). Acarbose (10-500 µg/mL) was used as a reference drug, and phosphate buffer served as a negative control. The results were expressed as IC_{50} values, and each assay was repeated three times.

The inhibition of the enzymatic activity was determined as a percentage using the formula (3).

$$\%=[AC-AEAc]*100 \quad (3)$$

where AC represented the absorbance of negative control (in this reaction mixture, the EO was substituted with buffer), whereas AE represented the absorbance of the enzyme solution after the addition of the EO. The IC_{50}, which indicated the sample concentration that causes 50% inhibition, was derived by plotting the percentage inhibition *versus* various samples concentrations.

5.8 Lipase inhibition assay

Lipase inhibition was evaluated according to Bendicho et al. (2001), with some modification. Briefly, porcine pancreas type 2 lipase (Sigma product L3126, 200 U/g) was dissolved in ultrapure water at a concentration of 5 mg/mL and then centrifuged at 6000 rpm for 5 min; the supernatant was used for the assay. A 5 mM solution of 4-nitrophenyl decanoate (NPD) in dimethylsulphoxide was prepared. The composition of the reaction mixture included: 20 µl of 5 mM NPD, 800 µl of Tris-HCl buffer (pH 8.5), 20 µl of sample at different concentrations (5–1000 µg/ml, obtained by dissolving the sample in methanol), and 20 µl of the enzyme solution. The mixture was pre-incubated at 37 °C for 20 min, before substrate addition, and then incubated

for an additional 30 min at 37 °C. In the control, the EO was replaced with the same volume of distilled water. The absorbance was measured at 412 nm (Thermo Fischer Scientific, Vantaa, Finland Varian, CARY 100 Bio, USA) and a blank sample, without the enzyme, was also measured. Orlistat (10-100 µg/mL) was used as positive control. All experiments were carried out in triplicate, and the results were expressed as IC_{50} value.

5.9 Enzyme kinetics

Enzyme-kinetic studies were undertaken only when the EO displayed an IC_{50} value <1 mg/mL. Lineweaver-Burk plots were used to linearize the substrate-velocity data and to investigate the kinetic parameters determining the type of inhibition, as well as the Michaelis-Menten constant (Km), maximum velocity (Vmax) values. Additionally, the inhibition constant (Ki) was determined to express the affinity of the EO for binding to enzymes (Lineweaver & Burk, 1934). For the kinetics study, the experiments were carried out as described in previous paragraphs with the exception that several substrate concentrations were used. For cholinesterases, the concentration of two enzyme was kept constant at 0.28 U/mL, while various concentrations of acetylthiocholine iodide (AChI) or butyrylcholine iodide (BChI) was increased from 0.250 to 2.5 mM, in absence (control) and in the presence of 5 µg/mL of EO. The reaction was started by the addition of substrate, and monitored with UV spectrophotometer at 412 nm (Thermo Fischer Scientific, Vantaa, Finland Varian, CARY 100 Bio, USA), at 1 min intervals over a period of 30 min. All experiments were carried out in triplicate. For tyrosinase, the concentration of the enzyme was kept constant at 200 U/ml and different concentrations of tyrosine (0.5–2.5 mM) in the absence (control) and in the presence of 400 µg/mL of EO were used. The reaction was started by the addition of substrate, and monitored with UV spectrophotometer at 412 nm (Thermo Fischer Scientific, Vantaa, Finland Varian, CARY 100 Bio, USA), at 1 min intervals over a period of 30 min. All experiments were carried out in triplicate. For α-glucosidase, the concentration of the enzyme was kept constant at 1 U/mL, and different concentrations of 4- pNPG (0.5–4 mM) were used in the absence (control)

and in the presence of 200 µg/mL of EO. The reaction was started by the addition of substrate, and monitored with UV spectrophotometer at 415 nm (Thermo Fischer Scientific, Vantaa, Finland Varian, CARY 100 Bio, USA), at 1 min intervals over a period of 6 min. All experiments were carried out in triplicate. For lipase, the concentration of the enzyme was kept constant at 5 mg/mL, while various concentrations of 4-nitrophenyl decanoate (0.5–50 mM) in the absence (control) and in the presence of 30 µg/mL of EO were used. The reaction was started by the addition of substrate, and monitored with UV spectrophotometer at 412 nm (Thermo Fischer Scientific, Vantaa, Finland Varian, CARY 100 Bio, USA), at 1 min intervals over a period of 30 min. All experiments were carried out in triplicate.

5.10 Molecular docking

The inhibitory potency of carvacrol and *p*-cymene against acetylcholinesterase, butyrylcholinesterase, α-glucosidase, tyrosinase, and lipase as potential inhibitors was estimated by investigating their binding affinities to the corresponding binding sites using the Autodock4 (AutoDock version 4.2.6 http://autodock.scripps.edu/) package (Morris et al., 2009; Noumi et al., 2020). X-ray coordinates for the targeted enzymes along with their corresponding original docked ligands, were downloaded from the RCSB Protein Data Bank website using the PDB files 4EY762, 1P0I633W3764, 2Y9 ×65 and 1ETH66 respectively (Nicolet et al., 2003; Tagami et al., 2013). The stable geometries of carvacrol and *p*-cymene were minimized using the MMFF94 (Merck Molecular Force Field 94) level44. Then, they are saved as PDB files. The molecular docking stepwise may be found in previous reported studies (Hammouda et al., 2022; Othman et al., 2020). The binding interaction modes of docked carvacrol and *p*-cymene within the binding sites of α-acetylcholinesterase, butyrylcholinesterase, α-glucosidase, tyrosinase, and lipase were illustrated employing the Discovery Studio Client (BIOVIA Discovery Studio Visualizer v21.1.0.20298 https://discover.3ds.com/discovery-studio-visualizer-download).

5.11 Antioxidant Activity

- *DPPH Assay*

The antiradical activity was determined using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) following the protocol of Brand-Williams (Brand-Williams et al., 1995) with some modifications reported by De Martino and coworkers (2021).

- *FRAP Assay*

The FRAP assay (FRAP is an acronym for “Ferric Ion Reducing Antioxidant Power”) was performed following the protocol of Benzie and Strain (Benzie & Strain, 1996**)**. A FRAP reagent is a solution consisting of 23 mM acetate buffer (pH 3.6), 10 mM of tripyridyl triazine (TPTZ) in 40 mM of HCl, and 20 mM of $FeCl_3$ (in a 10:1:1 ratio). Different concentrations of ferrous sulfate heptahydrate, $FeSO_4$ $7H_2O$, in a range from 1 mM to 0.1 mM were prepared to obtain the calibration curve. The reaction was carried out for each sample in a final volume of 272 µL in wells. The reaction mixture was incubated at 37 °C for 30 min in dark conditions. The absorbance of the blank, consisting of FRAP alone and monitored spectrophotometrically at the wavelength of 593 nm, was subtracted from the absorbance of the FRAP with the sample to determine the FRAP value for each sample. The FRAP values were determined using the $FeSO_4$ $7H_2O$ calibration curve (Amamcharla & Metzger, 2014**)** and expressed as mmol Fe^{2+}/g of EO. Trolox was used as positive control.

- ABTS Test

The ABTS test was performed using the method described by Xiang and coworkers (Xiang et al., 2019). In ultrapure water, it made a solution of ABTS 2,20-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (7 mM) and ammonium persulfate (2.45 mM) (Sigma-Aldrich, Milan, Italy). To create the ABTS radical (ABTS•), ammonium persulfate was added to the ABTS solution until the final ammonium persulfate concentration was 2.45 mM. The sample was incubated at room temperature in the dark for 12–16 h. At 734 nm, the concentration of the ABTS radical (ABTS•) stock solution was

determined to have an absorbance of 0.700 using a Multiskan GO spectrophotometer (Thermo Fisher Scientific, Vantaa, Finland). 6-hydroxy 2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma-Aldrich, Milan, Italy) was utilized as an antioxidant standard. Trolox (2.5 mM) was produced and used as a stock standard in methanol. The working standards were generated and diluted with methanol on a regular basis. In triplicate, 10 µL of the standard solution or samples and 190 µL ABTS• were added to the wells for analysis. Amounts of 10 µL of PBS and 190 µL of ultrapure water were added to the wells for the control.

5.12 Antibacterial Activity

- *Microorganisms and Culture Conditions*

The bacterial strains *Acinetobacter baumannii* (ATCC 19606), *Escherichia coli* (DSM 8579), *Pseudomonas aeruginosa* (DSM 50071), *Listeria monocytogenes* (ATCC 7644), and *Staphylococcus aureus subsp. aureus* Rosebach (ATCC 25923), purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), were used in the experiments. Before the antimicrobial assays, they were cultured in Luria broth (LB, Sigma Aldrich Italia, Milano, Italy) for 18 h at 37 °C (*A. baumannii* was grown at 35 °C) and 80 rpm (Corning LSE, Pisa, Italy).

- *Minimal Inhibitory Concentration (MIC)*

The resazurin microtiter plate assay determined the MIC (Fratianni et al., 2023). The tests were performed in flat-bottomed 96-well microtiter plates placed at 37 or 35 °C (depending on the strain) for 24 h. The MIC value was identified by the color change from dark purple to colourless. Sterile DMSO and tetracycline (dissolved in DMSO, 1 mg/mL) were used as negative and positive controls, respectively. Experiments were carried out in triplicate; the results were expressed as the mean ± standard deviation.

- *Inhibition of Mature Biofilm*

The capacity of the EOs to affect a mature bacterial biofilm was assessed in flatbottomed 96-well microtiter plates (Falcon, VWR International, Milano,

Italy) (Fratianni et al., 2023). The overnight bacterial cultures were adjusted to 0.5 McFarland with fresh culture broth. Afterward, 10 µL of the bacterial cultures was added to each well, and 240 µL sterile Luria–Bertani broth (Sigma Aldrich Italia, Milano, Italy) was added. After 24 h, the culture broth was eliminated, and 10 µL/mL and 20 µL/mL of each oil were added, and with sterile Luria–Bertani broth, a final volume of 250 µL was reached. The plates were closed with parafilm tape to avoid the evaporation of material in the wells and incubated for 24 h at 37 or 35 °C. After the elimination of the planktonic cells, sessile cells were washed twice with sterile phosphate-buffered saline (PBS). Then, the plates were placed for 10 min under a laminar flow hood before adding 200 µL of methanol in each well for 15 min to allow the fixation of the sessile cells. Methanol was removed, and each plate was left to dry; then, 200 µL of crystal violet solution 2% w/v was added to each well. After 20 min, the staining solution was removed, and the plates were washed with sterile PBS and left to dry. The bound dye was released by adding 200 µL of glacial acetic acid (20% w/v) to each well. The absorbance was measured at 540 nm (Cary50Bio, Varian). The inhibitory action on the mature biofilm (expressed as percent value) was calculated with respect to the control (represented by the bacterial cells grown without the presence of the samples, where inhibition was considered 0%).

- *Inhibition of Bacterial Metabolism*

The effects of the EOs on the metabolic activity of the sessile cells within the bacterial biofilm after 24 h were also evaluated through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method (Fratianni et al., 2023). After another 24 h of incubation, the culture broth was eliminated; then, 150 µL of PBS and 30 µL of 0.3% of MTT (Sigma, Milano, Italy) were added, keeping the microplates at 37 or 35 °C (depending on the strain). After two hours of incubation, the MTT solution was removed, and each well was washed with a sterile physiological solution. Two hundred microliters of dimethylsulfoxide (DMSO) were added to dissolve the formazan crystals, which were read at 570 nm (Cary50Bio, Varian) after two hours at 37 or 35 °C (depending on the strain).

5.12 Culture conditions and MTS assay

The IPEC-J2 (porcine intestinal epithelial cells) and the Caco-2 (human colorectal adenocarcinoma cells) cell lines were used to study the metabolic activity and cytotoxicity of oregano EOs. Cells were maintained exactly as previously described in the study by Gruľová et al., (Gruľová et al., 2024). IPEC-J2 cells were used from the 30th to 35th passages, and Caco-2 cell from 22 to 28th passages. For this study the cell cultures were seeded onto a 96- well plate; after overnight cultivation, the cells became confluent and were washed with sterile phosphate buffered solution (PBS) (1X, pH 7.4). The samples of EOs from oregano were dissolved in 100 % DMSO (Sigma-Aldrich, St. Louis, MO, USA) and then diluted for the required concentrations of oils in DMEM/F12 media (830 µg/mL or 35 µg/mL); then, the obtained samples were added to each cell lines for 24 h. After this incubation period, the cells were washed with PBS. DMEM/F-12 media (100 µL) was added to each well after cell washing and supplemented with MTS solution (20 µL/ well) incubated for 1 h at room temperature and then the absorbance was measured at 490 nm with a 96-well microplate reader (Synergy HTX Multi-Mode Reader spectrophotometer, Agilent, Santa Clara, CA, USA).

5.13 Larvicidal activity

Larvicidal (lethal) activity of oregano EOs was assessed in contact-toxicity test following Plata-Rueda et al. (2021), using larvae of the yellow mealworm (*Tenebrio molitor* L., Coleoptera: Tenebrionidae). Individuals were obtained from own breeding (Department of Ecology, FHaNS, University of Prešov, Slovakia), a stock culture was kept in plastic trays (35 cm long × 19,5 cm wide × 11,5 cm), maintained at room temperature, fed with a mixture of semolina, bread-crumb and wheat flour and regularly offered by fresh pieces of vegetable. Larvae were expossed to four different concentrations of *Origanum* EOs: 1 µL, 0.75 µL, 0.575 µL and 0.4 µL, causing different mortality. LD_{50}, LD_{90} and LD_{99} in µg/Insect were consequently determined on the base of the specific EOs weight. Healthy larvae of yellow mealworm without obvious damage were used in the experiment. EOs were applied on the thorax of *T. molitor* larvae using a micropipette. In each test, 3-

experiments were undertaken for each EO concentration. Three independent experiments were run completely (total n=108 specimens). Bioassays were controlled after 24 and 48 hours and the specimens were checked for movement and necrosis. *Tenebrio molitor* insecticidal data (48 hours mortality) from three independent tests were pooled together and subjected to Finney's probit analysis for determining the LD_{50}, LD_{90}, LD_{99} and 95% confidence intervals of upper/lower confidence limit (UCL/LCL). To compare between *Origanum* EO samples effectivity, LD_{50} was determined for the separated tests, muttual comparison was provided using ONE-way ANOVA in Past. The crude mortality obtained from the larvicidal bioassay was corrected using Abbott's formula. Means of corrected mortality from all replicates were determined using Univariate statistics in statistical programe PAST.

5.14 Statistical analysis

For EOs of *O. heracleoticum* the components identified in the samples were considered as original variables and subjected, after normalization, for doing Principal Component Analysis (PCA). Hierarchical Cluster Heatmap analysis of the same components, was also conducted. The statistical analyses were performed using Matlab software with three principal components (PC) and the number of clusters was determined using scaled distances in the Hierarchical Cluster Heatmap. PCA and Hierarchical Cluster Heatmap were used to understand the similarity between the tested samples (EO from Nardò, EO from Sassari, EO from Grimaldi, EO from Sicignano, and EO from Sila), in relation to the variables considered above. While for PLE extracts fifty components identified in the samples and the main biological activities were considered as original variables and subjected, after normalization, for doing Principal Component Analysis (PCA). Hierarchical Cluster Heatmap analysis of the same components, was also conducted. The statistical analyses were performed using Matlab software with three principal components (PC) and the number of clusters was determined using scaled distances in the Hierarchical Cluster Heatmap. PCA and Hierarchical Cluster Heatmap were used to understand the similarity between the tested samples (PLE extracts from Marsico Nuovo, Nardò, Sassari, Agrigento, Sicignano,

Sila), in relation to the variables considered above. GraphPad (GraphPad Prism version 8.0 https://www.graphpad.com) for Windows was used to do analyses of data obtained with t-test analysis (three times for each determination, at p level < 0.05).

6. CONCLUSION

This study explored the phytochemical and biological potential of several species of the *Origanum* genus, integrating chemical analyses, bioassays, kinetic studies, and *in silico* evaluations, with the aim of identifying natural resources useful in nutraceuticals and phytotherapy. The study was structured around three main points:

- the study of *Origanum dictamnus* (volatile and non-volatile fractions)
- an interspecies comparison between species belonging to the Italian flora (*O. majorana, O. vulgare, O. heracleoticum*),
- intraspecies study on *O. heracleoticum* samples from different locations in Southern Italy, analyzing both the volatile and non-volatile fractions.

The EO of *O. dictamnus* was rich in carvacrol and *p*-cymene, in agreement with the literature. The inhibitory activity against acetylcholinesterase and lipase suggests the species' potential interest in both neurodegenerative and metabolic disorders. Kinetic studies highlighted mixed or competitive inhibition mechanisms depending on the enzyme, while molecular docking confirmed the high affinity of the main constituents for the catalytic sites, strengthening the interpretation of the experimental data. The non-volatile fraction, investigated using both maceration and pressurized liquid extraction (PLE) with hydroalcoholic solvents, showed that PLE yielded extracts richer in polyphenols and with enhanced biological activity, highlighting the greater efficiency of process intensification strategies rather than differences in extraction sustainability.

The interspecific comparison between *O. majorana, O. vulgare*, and *O. heracleoticum* highlighted the intrinsic variability of the *Origanum* genus. The EOs of the three species differ both in their main constituents and in the distribution of chemical classes, influencing the observed biological activities. Some species are more active against enzymes involved in

neurodegenerative disorders (*O. vulgare* for cholinesterase; *O. majorana* for tyrosinase), while others show greater efficacy against metabolic enzymes (*O. heracleoticum* for lipase).

The intraspecies study on *O. heracleoticum* revealed considerable phytochemical variability in both the volatile and non-volatile fractions. The eight EOs analysed present a composition dominated by phenolic monoterpenes, but with quantitative differences. Multivariate analyses allowed us to distinguish specific chemotypes, correlated with different biological activities. In fact, 'Marsico Nuovo' EO is more active against AChE, BChE (enzymes related to neurodegenerative diseases) and *Tenebrio molitor* (insecticidial activity). Instead, 'Sila' and 'Sicignano' EOs show greater potential to modulate metabolic enzymes such as lipase and α-glucosidase and demonstrate cytotoxic activity against the CACO2 tumor cell line. 'Nardò' and 'Sassari' EOs instead show greater antibiofilm activity against *L. monocytogenes, P. aeruginosa* and *A. baumannii*. The non-volatile fractions of the six *O. heracleoticum* samples, obtained by PLE, showed a high content of phenolic compounds and a chemical profile rich in secondary metabolites. Multivariate analysis of chemical data highlighted the presence of different sample clusters, reflecting chemical variability. PLE extracts showed good antioxidant potency and significant activity against enzymatic targets involved in metabolic disorders, particularly α-glucosidase and lipase.

Overall, the results suggest that species of the *Origanum* genus may represent an interesting source of bioactive compounds, potentially modulating biological processes involved in neurodegenerative and metabolic disorders, and biofilm infections. The variety of activities observed, combined with the natural variability between samples, indicates that these species could have promising applications in nutraceuticals, cosmetics, and phytotherapy.

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