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Evaluation of Enzyme Inhibition and Kinetic Profiles of *Origanum* Essential Oils on Metabolic and Central Nervous System Targets

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ABSTRACT: Noncommunicable diseases (NCDs) are now the leading cause of death globally, accounting for around 70% of annual mortality, with a particularly severe impact in low-income countries. The genus *Origanum* L. (Lamiaceae), comprising 43 species distributed worldwide, includes several species traditionally used in ethnomedicine. This study aimed to: (I) identify the chemical composition of essential oils (EOs) from the aerial parts of *Origanum vulgare*, *Origanum majorana*, and *Origanum heracleoticum* by gas chromatography–mass spectrometry (GC–MS); (II) evaluate their antioxidant activity via 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 2,20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and FRAP assays; (III) assess their inhibitory effects on enzymes involved in metabolic and central nervous system (CNS) functions; and (IV) determine the inhibition mechanisms of the most active EOs and their main constituents using Michaelis–Menten kinetics. *O. vulgare* EO, rich in carvacrol and *p*-cymene, exhibited the highest antioxidant activity and acted as an uncompetitive inhibitor of α-glucosidase. *O. majorana* EO, dominated by γ-terpinene and terpinen-4-ol, showed the strongest tyrosinase (TYR) inhibition (monophenolase and diphenolase reactions), acting as a mixed-type inhibitor. The EO of *O. heracleoticum*, characterized by carvacrol, thymol, and γ-terpinene, displayed significant inhibitory activity against acetylcholinesterase (mixed inhibition) and butyrylcholinesterase (competitive inhibition). To date, no studies have reported the effects of *O. majorana* EO on TYR, α-amylase, or α-glucosidase, nor have comparative studies explored the inhibition of lipase by all three species' EOs. These findings suggest that *Origanum* EOs hold promise as multifunctional agents for managing metabolic and CNS disorders, supporting their potential use in pharmaceutical and nutraceutical applications.

1 | Introduction

Noncommunicable diseases (NCDs) are now a prominent in causing of death worldwide, with the approximately range of 70% of deaths yearly, with a particularly significant impact in less-income countries [1]. The most common NCDs include cardiovascular diseases, neurological diseases, diabetes, cancer, and chronic respiratory diseases, all associated with risk factors

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Highlights

1. This is the first study to evaluate kinetic inhibition of metabolic and CNS enzymes by *Origanum* EOs.
2. EOs showed uncompetitive, competitive, and mixed-type inhibition depending on the enzyme.

such as excessive alcohol consumption and smoking as well as physical inactivity in connection to unbalanced diet [2]. These risk factors are often linked to lifestyle and dietary changes that have emerged especially from the late 20th century, when the global rise in these diseases has been noted [3]. To combat this health emergency, it is essential to promote healthy diets, with a special emphasis on plant-based products, rich in bioactive compounds (BACs). BACs are "extra-nutritional" molecules present in plants in minimal quantities, such as antioxidants, phytosterols, flavonoids, and carotenoids [4]. Mentioned compounds have a fundamental position in the prevention of NCDs thanks to their anti-inflammatory, antioxidant, and antimicrobial properties, offering valid support to reduce the risk of developing these diseases [5, 6]. Antioxidants are substances that slow or prevent oxidative damage to specific molecules within living organisms. To protect vulnerable substrates from oxidation, plants produce a wide range of antioxidants, including alkaloids, phenolic compounds, and vitamins such as C and E [7]. As the human body cannot generate enough antioxidants on its own to counteract the constant action of reactive oxygen species (ROS), dietary antioxidants, especially those of plant origin, are considered essential for maintaining health. These compounds act as scavengers that protect cells and tissues against damage made [8] by free radicals. While preventive processes, repair mechanisms, and physical defenses contribute to cellular protection, the role of antioxidants is essential to balance the excess of free radicals [9]. A regular intake of antioxidants can help reduce the presence of these free radicals in the body, thereby improving overall well-being and reducing the hazard of NCDs, which usually are cancer, diabetes, cataracts, kidney and liver problems, renal toxicity, and coronary artery disease [10, 11]. Oxidative stress, a condition resulting from an excessive production of free radicals or insufficient antioxidants, generates an imbalance between these two components. Free radicals, being highly reactive and not stable, can harm the body vital processes if present in high concentrations; however, at low levels, they play a role in preventing infections [12]. The accumulation of free radicals can damage proteins, lipids, and DNA. It can cause DNA strand breakage, degradation of nitrogenous bases, and the interaction between DNA and proteins, thus altering cellular function. This damage can accelerate aging and contribute to the development of neurodegenerative disorders, autoimmune diseases, cardiovascular conditions, cancer, and other illnesses. Consequently, oxidative stress is closely linked to the pathogenesis of several diseases [13, 14]. Essential oils (EOs) such as those from oregano and clove have demonstrated potent antioxidant properties, credited to their high phenol amount. The level of antioxidants in plants can vary depending on intrinsic and extrinsic factors, which influence their production and accumulation [15, 16]. At the same time, the growing interest of human population for natural sources has prompted the food industry to innovate to enhance product quality, safety, and nutritional value. Nevertheless, the number of BACs in plant products can vary significantly depending on several factors, including genetics, environmental conditions, agronomic practices, storage methods, and industrial processing [17]. For example, antioxidant levels such as beta-carotene and vitamin C in fruits and vegetables depend on factors like climate conditions and specific varieties grown as well as postharvest treatments. These variations not only affect potential health benefits but also influence the organoleptic characteristics of foods [18, 19]. The genus *Origanum* L. (Lamiaceae) includes 43 species distributed globally. Several species of *Origanum* are used both as remedies in ethno medicine and in culinary or ingredients for herbal teas [20]. Among these, *Origanum vulgare* L. has a diuretic effect and can be used for treatment of intestinal pain also for colds and stomach aches; O. *vulgare* L. subsp. *viride* (Boiss.) is utilized for dyspepsia and stomach aches; O. *vulgare* L. subsp. *vulgare* promotes wound healing; *Origanum majorana* L. is used for treatment of colds and catarrh; *Origanum onites* L. treats cold, stomach and abdominal pain, shoulder stiffness, rheumatism, backache, and gastric disorders [21]. Previous studies on *Origanum* species composition have shown a rich and diverse phytochemicals that include various classes of terpenes, phenols, and flavonoids [22]. In vitro and in vivo studies have also shown that many *Origanum* species are active in different biological activities, for example, antidiabetic, anti-obesity, antihyperlipidemic, antimicrobial, antioxidant, antiproliferative, and antinociceptive effects [23].

The aim of the presented scientific research was (I) to study the volatile fractions of *O. vulgare* L., *O. majorana* L., and *Origanum heracleoticum* L. at the EOs; (II) to test the antioxidant activity with three different assays (1,1-diphenyl-2-picrylhydrazyl radical [DPPH], FRAP, and 2,20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) [ABTS]); (III) to test the potential of EOs by evaluating the potential inhibitory activities against enzymes participate in neurodegenerative diseases (acetyl- and butyrylcholinesterase [BChE], tyrosinase [TYR]) as well as in metabolic disorders (amylase, glucosidase, and lipase); and (IV) to clarify the mechanism of action of EOs against the abovementioned enzymes by studying their kinetics.

2 | Material and Methods

2.1 | Reagents

All used reagents were purchased from Sigma Aldrich (Milan, Italy).

2.2 | Plant Material

The aerial parts of three species (*O. vulgare*; *O. majorana*; and *O. heracleoticum*) were collected at the flowering stage in South of Italy (Salerno-Italy) and were subsequently dried in the absence of light at room temperature. The species was identified by Prof. V. De Feo based on Flora d'Italia [24] and a voucher specimen (DF/740/2025; DF/741/2025; DF/742/2025) was deposited in the herbarium of the Chair of Medical Botany of the University of Salerno.

2.3 | Extraction and Analysis of EO

Three hundred 45 g of dried aerial parts of the sample were subjected to hydrodistillation for 3 h, following the procedure described in the European Pharmacopoeia [25]. The oils were solubilized in *n*-hexane, filtered over anhydrous sodium sulfate, and stored under N_2 at +4°C in the dark until tested and analyzed.

Gas chromatography–mass spectrometry (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 voltage 70 eV; electron multiplier voltage energy 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 mode, splitless (1 μL of a 1:1000 *n*-hexane solution); injector and detector temperatures 250°C and 290°C, respectively. Helium was used as the carrier gas (1.0 mL/min). The majority of constituents were identified by comparing their Kovats retention indices (Ri), which were determined relative to the retention times (Rt) of *n*-alkanes (C_{10}–C_{35}), to either those in the literature [26, 27] and mass spectra on the column or those of authentic compounds available in our laboratories via the NIST 02 and Wiley 275 libraries [28]. Peak area normalization was used to get the component relative concentrations. No response factors were calculated.

2.4 | Analysis of Total Phenol Content (TPC)

TPC in the EO was measured spectrophotometrically according to the Folin–Ciocalteu method [29]. First, 10 μL of EO or gallic acid standard solution diluted in MeOH (1:10), 790 μL of deionized water, and 50 μL of the Folin–Ciocalteu reagent were put in the cuvettes; after 8 min, 150 μL of Na_2CO_3 was also added. After 2 h incubation, at room temperature, the absorption value of the mixture was detected at 765 nm, using a Thermo Scientific Multiskan GO spectrophotometer (Thermo Fischer Scientific, Vantaa, Finland). Gallic acid was used as a standard to determine the calibration curve. The TPC was expressed as mg gallic acid equivalent (mg GAE)/g of EO. All experiments were carried out in triplicate.

2.5 | Antioxidant Activity

2.5.1 | DPPH Assay

The antiradical activity of the stable DPPH was measured using the protocol of Xiang et al. [30] with slight modifications. In its radical state, DPPH possesses an absorption band at 515 nm that vanishes in the presence of antiradical chemicals. To produce final concentrations ranging from 10 to 1000 μg/mL, aliquots of the EO were dissolved in methanol. In a straight-sided cuvette, an aliquot of methanol solution containing different concentrations of either methanolic or sample was added to a DPPH solution (60 μM) to a final volume of 1 mL. As a control, an identical quantity of DPPH solution was applied to the cuvette, and methanol alone was used as the blank. After 45 min, the absorbance at 515 nm was measured with a Multiskan GO spectrophotometer (Thermo Fisher Scientific, Vantaa, Finland). The result was expressed as the IC_{50} value, which is the sample concentration required to lower DPPH absorbance by 50% Trolox (6-hydroxy 2,5,7,8-tetramethylchroman-2-carboxylic acid). All experiments were carried out in triplicate.

2.5.2 | FRAP Assay

The FRAP test was carried out in accordance to Zhang et al. [31] with some modifications. At an acidic pH, antioxidant chemicals were tested for their capacity to decrease the complex of Fe(III)-2,4,6-tripyridyl-*s*-triazine (also known as [Fe (III)-(TPTZ)2]$^{3+}$) to Fe (II), [Fe(II)-(TPTZ)2]$^{2+}$. The resulting complex is colored (navy blue). At a wavelength of 593 nm, the reaction may be spectrophotometrically examined. The FRAP reagent was made up of 23 mM acetate buffer (pH 3.6), 10 mM TPTZ in 400 mM HCl, and 20 mM $FeCl_3$. To generate the calibration curve, several quantities of ferrous sulfate heptahydrate, $FeSO_4 \cdot 7H_2O$, ranging from 27.801 to 278.010 mg/L, were used. The reaction took place in a well with a final volume of 272 μL. For 30 min, the reaction mixture was incubated in a dark environment at 37°C. The absorbance of the FRAP alone blank was subtracted from the absorbance of the FRAP with the samples. The $FeSO_4 \cdot 7H_2O$ values were reported as the mg Fe^{2+}/g of sample. The reference standard was Trolox. All experiments were carried out in triplicate.

2.5.3 | ABTS Assay

The ABTS test was performed using the method described by Xiang et al. [30]. In ultrapure water, made a solution of ABTS diammonium salt (7 mM) and ammonium persulfate (2.45 mM) was prepared. 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). Trolox (2.5 mM) was utilized as an antioxidant standard. The working standards were generated and diluted with methanol. 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 as a control. The results are reported as mg of Trolox equivalent (TE) per gram of EO. All experiments were carried out in triplicate.

2.6 | Enzymatic Activities

2.6.1 | Cholinesterases Inhibition Assay

Cholinesterase activity was assessed by the colorimetric assay of Ellman et al. [32] with some modifications. Briefly, 10 μL of the EOs or their main constituents (carvacrol, thymol, and γ-terpinene) dissolved in MeOH, at various concentrations (100, 10, 1, and 0.1 μg/mL for EOs; 200, 150, 100, and 50 μg/mL for carvacrol, thymol, and γ-terpinene), and 25 μL of 0.28 U/mL acetyl cholinesterase acetylcholinesterase (AChE) (or BChE) solution were added to 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 and the reaction was put for 30 min 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 was used as a reference drug.

The enzyme activity was calculated as a percentage using the following formula:

$$\% = [(A_C - A_E)/A_c] \times 100 \quad (1)$$

where A_C represented the absorbance of enzyme solution without EO, A_E 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.

2.6.2 | TYR Inhibition Assay

The inhibitory effects on TYR were determined by evaluating both the monophenolase and diphenolase activities of the enzyme, using the method of Khatib et al. [33], with minor modifications. The reaction mixture was prepared in a 96 multiwell plate: In each well, aliquots 8 µL of EO (0.1–1 mg/mL) were mixed with 400 µL of substrate, 120 µL of TYR (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.

$$\% = \frac{[A_0 - A_1]}{A_0} \times 100 \quad (2)$$

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.

2.6.3 | α-Amylase Inhibition Assay

Jaradat's approach, with minor modifications, was used to assess the amylase activity [34]. The mixture, composed by 100 µL of different concentrations of the EO (previously dissolved in methanol 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), was 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). All experiments were carried out in triplicate, and the results were expressed as IC_{50} value.

2.6.4 | α-Glucosidase Inhibition Assay

α-Glucosidase inhibitory activity was assessed following the method proposed by Si et al. [35] 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 EOs or their main constituents (carvacrol, thymol, and γ-terpinene) dissolved in MeOH to obtain various concentrations (5–1000 µg/mL for EOs and 100, 50, 25, and 10 for carvacrol, thymol, and γ-terpinene). 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; after, 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 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 following formula:

$$\% = \left[\frac{A_C - A_E}{A_c}\right] \times 100$$

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

2.6.5 | Lipase Inhibition Assay

The assay was performed according to Bendicho et al. [36] 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 EOs or their main constituents (carvacrol, thymol, and γ-terpinene) at different concentrations (5–1000 µg/mL, obtained by dissolving the EO in methanol; 100, 50, 25, 10, and 1 µg/mL obtained by dissolving carvacrol, thymol, and γ-terpinene in methanol), and 20 µL of the enzyme solution. The mixture was preincubated 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 was used as positive control. All experiments were carried out in triplicate, and the results were expressed as IC_{50} value.

2.7 | Enzyme Kinetics

Enzyme kinetic studies were performed for EOs and their main compounds that showed the lowest IC_{50} value.

Lineweaver–Burk plots were used to linearize the substrate-velocity data and to investigate the kinetic parameters determining the type of inhibition, the Michaelis–Menten constant (K_m), and maximum velocity (V_{max}) values. Additionally, the inhibition constant (K_i) was determined to express the affinity of the EO for binding to enzymes [37]. 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, whereas various concentrations of AChI or BChI were increased from 0.250 to 2.5 mM, in the absence (control) and in the presence of 5 μg/mL of EO or 10 μg/mL of carvacrol. 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 TYR, 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 or 150 μg/mL of thymol. 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, whereas various concentrations of NPD (0.5–50 mM) in the absence (control) and in the presence of 30 μg/mL of EO or 10 μg/mL of carvacrol 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.

2.8 | Statistical Analysis

GraphPad Prism version 6.0 (GraphPad Software Inc., SanDiego, CA, USA) for Windows was used to do analyses of data obtained with *t*-test analysis (three times for each determination, at p level < 0.05).

3 | Results

3.1 | Chemical Composition of Three EOs

GC–MS analysis allowed the identification of a total of 91 compounds. As reported in Table 1, the three EO samples of *O. vulgare*, *O. majorana*, and *O. 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 (12.44%), as well as that of hydrocarbon sesquiterpenes (2.40%) and oxygenated sesquiterpenes (0.40%).

In contrast, the EO of *O. 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 *O. 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 (25.09%) and sesquiterpenes (2.42%). Among the hydrocarbon monoterpenes, γ-terpinene (14.20%) was the most abundant, whereas among the sesquiterpenes, germacrene D (9.44%) and caryophyllene (7.33%) were the most represented in the EO.

3.2 | TPC and Antioxidant Activities

The TPC and antioxidant activity were evaluated. The antioxidant activity was assessed using DPPH, ABTS, and FRAP assays, each targeting different ROS. 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 extracts' antioxidant potential. The results obtained are reported in Table 2.

As shown in Table 2, *O. 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, 0.4 TE eq. mg/g in the ABTS test, and 3.84 mg Fe^{2+} eq./g in the FRAP test. These results were consistent with the findings of the TPC assay, where the EO of *O. vulgare* displayed the highest total phenol content (201.22 mg GAE/mL).

TABLE 1 | Chemical composition of the essential oils (Eos) of *Origanum vulgare, Origanum majorana*, and *Origanum heracleoticum*.

	RT	Compound	*Origanum vulgare*	*Origanum majorana*	*Origanum heracleoticum*	KI[a]	KI[b]	Identification
1	12.8	α-Thujene	0.85 ± 0.1	2.26 ± 0.25	1.01 ± 0.14	927	1110	1.2.3
2	13.1	α-Pinene	1.53 ± 0.5	0.73 ± 0.10	0.41 ± 0.05	932	932	1.2.3
3	14.0	Camphene	0.43 ± 0.2	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	0.6 ± 0.07	—	0.7 ± 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	1.6 ± 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	1.4 ± 0.04	0.2 ± 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 ± 0.7	5.3 ± 0.21	1023	1023	1.2.3
15	19.9	Limonene	0.67 ± 0.04	4.91 ± 0.25	0.4 ± 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	4.7 ± 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	0.1 ± 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	(4*E*,6*Z*)-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

(Continues)

TABLE 1 | (Continued)

	RT	Compound	*Origanum vulgare*	*Origanum majorana*	*Origanum heracleoticum*	KI[a]	KI[b]	Identification
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	0.1 ± 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	0.1 ± 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

(Continues)

TABLE 1 | (Continued)

	RT	Compound	*Origanum vulgare*	*Origanum majorana*	*Origanum heracleoticum*	KI[a]	KI[b]	Identification
84	55.4	Caryophyllene 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 (%)	2.1	3.83	25.09			
		Oxygenated sesquiterpenes (%)	0.4	0.69	2.42			
		Yield (%)	0.52	0.29	0.15			

Note: Identification method: 1 = Kovats index; 2 = comparison of mass spectra; 3 = co-injection with standard compounds. T = traces <0.01%; — = absent.
[a]Kovats index on a DB-5MS column.
[b]Kovats index from literature.

TABLE 2 | Antioxidant potential and total phenolic content of the essential oils.

	DPPH (IC_{50} mg/mL)	ABTS (Trolox eq. mg/g)	FRAP (mg Fe^{2+} eq./g EO)	TPC (mg GAE/mL)
Origanum vulgare	0.28^{b} ± 0.05	0.4^{c} ± 0.04	3.84^{c} ± 0.8	201.22^{c} ± 1.2
Origanum majorana	3.59^{d} ± 0.08	0.04^{a} ± 0.01	0.25^{a} ± 0.05	85.58^{a} ± 0.9
Origanum heracleoticum	0.54^{c} ± 0.04	0.19^{b} ± 0.03	0.94^{b} ± 0.07	111.01^{b} ± 1.4
Trolox	0.03^{a} ± 0.01	—	10.30^{d} ± 0.08	—

Note: 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.
Abbreviations: ABTS, 2,20-azinobis (3-ethylbenzothiazoline-6-sulfonic acid); DPPH, 1,1-diphenyl-2-picrylhydrazyl radical; TPC, total phenolic content.

3.3 | Enzymatic Activities

Table 3 shows the enzymatic activities of the three species analyzed and of their main compounds. As can be seen, the samples and the constituents showed different activities depending on the enzyme studied. Regarding cholinesterases, the samples showed an inhibitory activity towards both AChE and BChE.

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 the inhibition of TYR, 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. About monophenolasic reaction *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). Moreover, for the diphenolase reaction, *O. majorana* was the most active sample with an IC_{50} value of 610.32 μg/mL.

TABLE 3 | Enzymatic activities of essential oils (Eos) and of their main compounds.

IC_{50} μg/mL							
	AChE	**BChE**	**Tyrosine**	**Dopa**	**α-amylase**	**α-glucosidase**	**Lipase**
Origanum vulgare	28.22 ± 1.2^{c}	390.23 ± 3.5^{c}	650.00 ± 2.5^{c}	na	na	331.50 ± 2.1^{a}	67.35 ± 1.2^{f}
Origanum majorana	56.40 ± 3.6^{e}	330.54 ± 2.8^{c}	466.90 ± 1.6^{b}	610.32 ± 3.1^{b}	na	395.20 ± 3.7^{b}	72.74 ± 0.9^{g}
Origanum heracleoticum	5.46 ± 0.5^{b}	201.28 ± 1.4^{b}	680.00 ± 4.8^{c}	1260.4 ± 4.2^{c}	na	451.90 ± 4.2^{c}	14.94 ± 0.7^{b}
Carvacrol	48.5 ± 0.7^{d}	nt	nt	nt	nt	na	20.1 ± 1.2^{c}
Thymol	97.1 ± 2.3^{f}	nt	nt	nt	nt	396.9 ± 3.8^{b}	30.5 ± 2.1^{d}
γ-Terpinene	102.9 ± 1.6^{g}	nt	nt	nt	nt	765.2 ± 7.6^{d}	53.9 ± 4.2^{e}
Galantamine	0.70 ± 0.3^{a}	10.00 ± 1.3^{a}	—	—	—	—	—
Kojic acid	—	—	13.40 ± 2.2^{a}	66.70 ± 1.5^{a}	—	—	—
Acarbose	—	—	—	—	1.20 ± 0.2	950.20 ± 3.6^{e}	
Orlistat	—	—	—	—	—	—	11.00 ± 0.8^{a}

Note: Results are the mean ± SD of three experiments. na = not active (IC_{50} > 1000 μg/mL); nt = not tested. Different letters indicate mean values significantly different at $p < 0.05$, according to a one-way ANOVA followed by Tukey's post hoc test.
Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase.

All three samples were not found to be active in inhibiting α-amylase. On the contrary, 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). Finally, *O. heracleoticum* was found to be the most active with regard to lipase inhibition (14.94 μg/mL), with IC_{50} comparable to orlistat (11.00 μg/mL), used as a control. *O. vulgare* (67.35 μg/mL) and *O. majorana* (72.74 μg/mL) showed inhibitory activity with IC_{50} lower than 100 μg/mL.

Moreover, the main compounds of the EOs (carvacrol, thymol, and γ-terpinene) were tested only in cases where the EOs IC_{50} value was found to be close to that of the reference standard. Specifically, the inhibitory activity against AChE, α-glucosidase, and lipase was evaluated.

Carvacrol was the most active towards AChE and lipase with an IC_{50} value of 48.5 μg/mL and 20.1 μg/mL, respectively.

Instead, thymol showed an IC_{50} value of 396.9 μg/mL lower respect to carvacrol and γ-terpinene against lipase.

3.4 | Kinetic Studies

For each enzyme, a kinetic study was conducted considering the EO with the strongest inhibitory activity.

A kinetic study was also performed on the enzymes (AChE, lipase, or glucosidase) for which carvacrol, thymol, or γ-terpinene exhibited the highest inhibitory activity.

Tables 4 and 5 report the maximum rate of enzymatic reaction (V_{max}), the substrate concentration where the corresponding reaction rate is 0.5 of V_{max} (K_m) and the inhibition constant (K_i). This constant is independent from experimental conditions and is a measure of the affinity of the inhibitor for the enzyme [38].

Overall, the analysis of Lineweaver–Burk plots (Figures 1–6) showed that *O. heracleoticum* EO inhibits AChE and lipase in a mixed manner but acts competitively on BChE. *O. majorana* EO inhibits TYR in mixed manner in both monophenolase and diphenolase reactions, and *O. vulgare* was an uncompetitive inhibitor of α-glucosidase.

Instead, the analysis of Lineweaver–Burk plots (Figures 7–9) showed that carvacrol inhibits AChE and lipase in a mixed manner; instead, thymol acts on α-glucosidase in uncompetitive manner.

The Lineweaver–Burk plot (Figure 1) indicated that for AChE, the lines intercepted both the *Y*- and the *X*-axes at different points and were not parallel, showing a mixed-type inhibition for *O. heracleoticum* EO.

Instead for BChE, the LB plot (Figure 2) showed that the lines intersected at same point on the *Y*-axes indicating a competitive-type inhibition for *O. heracleoticum* EO.

O. heracleoticum EO exhibited a mixed-type inhibition against AChE, differing from those competitive reported for galantamine, the reference standard for cholinesterases [39], while showed the same type of inhibition of galantamine against BChE.

In Figures 3 and 4, Lineweaver–Burk plots showed that the two lines, in the presence and in the absence of *O. majorana* EO, intercepted both the *Y*- and the *X*-axes at different points and were not parallel, indicating a mixed-type inhibition of this EO for TYR in monophenolase and diphenolase reactions.

Instead, kojic acid, the reference standard as TYR inhibitor, acts in competitive manner [40].

The Lineweaver–Burk plot analysis (Figure 5) showed that the two lines, in the presence and in the absence of *O. vulgare* EO, intercepted both the *Y*- and the *X*-axes at different points and were parallel, indicating an uncompetitive inhibition of this EO

TABLE 4 | V_{max}, K_m, and K_i values for cholinesterases, tyrosinase, α-glucosidase, and lipase inhibition by *Origanum vulgare, Origanum majorana*, or *Origanum heracleoticum* essential oils (EOs).

Enzyme	Essential oil	V_{max} (μmol/min)	K_m (μM/mL)	K_i (μg/mL)	Type of inhibition
AChE	*Origanum heracleoticum*	2162 ± 0.023	4356 ± 0.14	1.7 ± 0.6	Mixed
BChE	*O. heracleoticum*	1435 ± 0.0137	2952 ± 0.23	80.18 ± 5.6	Competitive
Tyrosinase (monophenolase reaction)	*Origanum majorana*	0.054 ± 0.007	1393 ± 0.12	5414 ± 113	Mixed
Tyrosinase (diphenolase reaction)	*O. majorana*	0.039 ± 0.005	0.693 ± 0.35	2031 ± 142	Mixed
α-Glucosidase	*Origanum vulgare*	0.064 ± 0.004	1.245 ± 0.15	143.4 ± 32.7	Uncompetitive
Lipase	*O. heracleoticum*	0.0196 ± 0.004	1.585 ± 0.13	90.90 ± 13.6	Mixed

Note: V_{max}: maximum rate of enzymatic reaction, K_m: substrate concentration where the corresponding reaction rate is 0.5 of V_{max}, K_i: inhibition constant. Data are expressed as mean ± standard deviation ($n = 3$).
Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase.

TABLE 5 | V_{max}, K_m, and K_i values for acetylcholinesterase, α-glucosidase, and lipase inhibition by carvacrol, thymol, and γ-terpinene.

Enzyme	Compound	V_{max} (μmol/min)	K_m (μM/mL)	K_i (μg/mL)	Type of inhibition
AChE	Carvacrol	3.362 ± 0.567	1.086 ± 0.034	4.794 ± 1.342	Mixed
Lipase	Carvacrol	0.0365 ± 0.0013	1.053 ± 0.005	9.048 ± 0.126	Mixed
α-Glucosidase	Thymol	2.928 ± 0.157	1.069 ± 0.285	614.8 ± 27.6	Uncompetitive

Note: V_{max}: maximum rate of enzymatic reaction, K_m: substrate concentration where the corresponding reaction rate is 0.5 of V_{max}, K_i: inhibition constant. Data are expressed as mean ± standard deviation ($n = 3$).
Abbreviation: AChE, acetylcholinesterase.

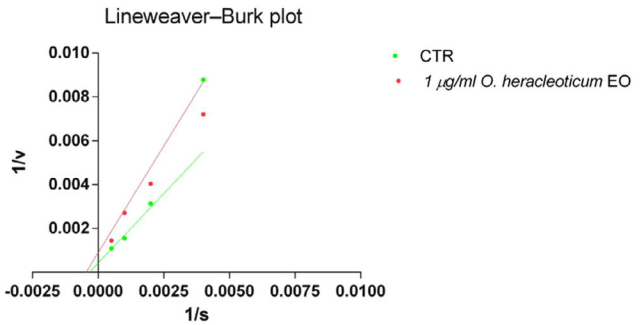


FIGURE 1 | Lineweaver–Burk plot of AChE inhibition in absence (CTR) or presence of *Origanum heracleoticum* EO. EO, essential oils.

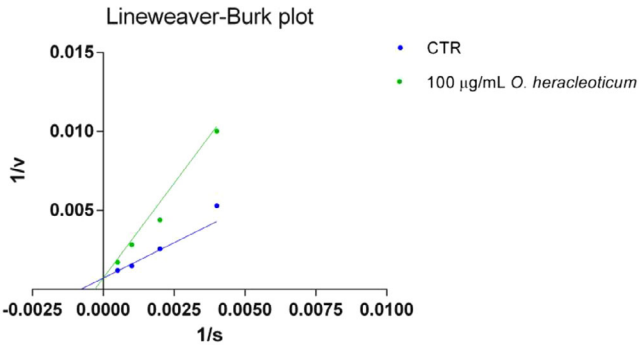


FIGURE 2 | Lineweaver–Burk plot of BChE inhibition in absence (CTR) or presence of *Origanum heracleoticum* EO.

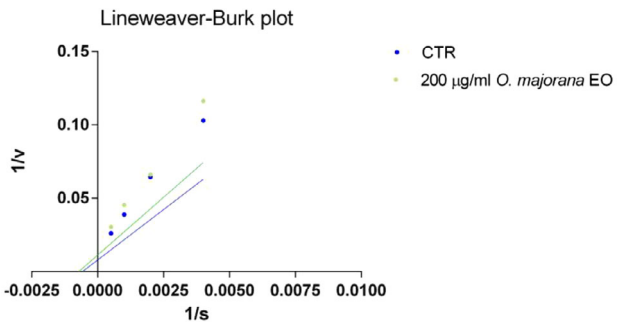


FIGURE 3 | Lineweaver–Burk plot of tyrosinase (monophenolase reaction) inhibition in absence (CTR) or presence of *Origanum majorana* EO. EO, essential oils.

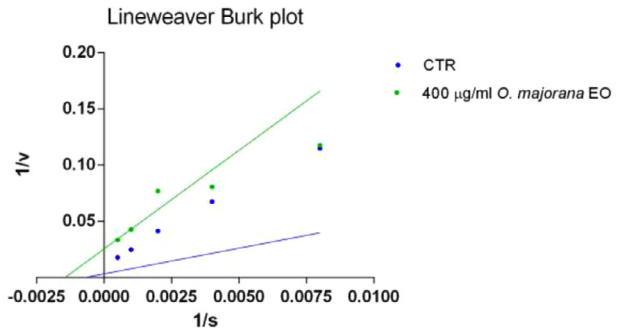


FIGURE 4 | Lineweaver–Burk plot of tyrosinase (diphenolase reaction) inhibition in absence (CTR) or presence of *Origanum majorana* EO. EO, essential oils.

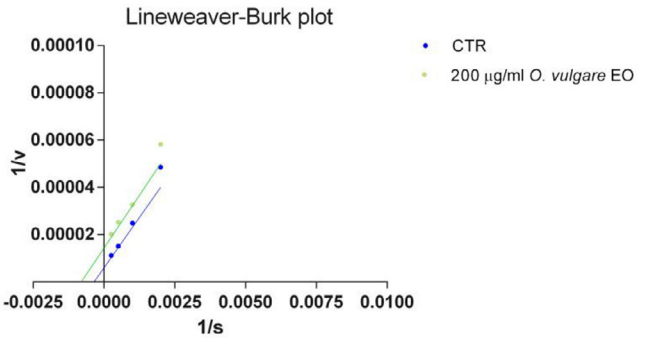


FIGURE 5 | Lineweaver–Burk plot of α-glucosidase inhibition in absence (CTR) or presence of *Origanum vulgare* EO. EO, essential oils.

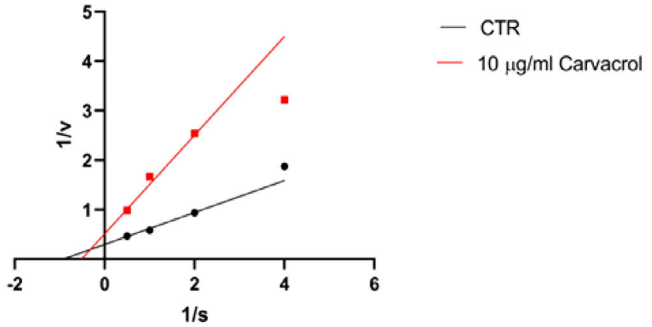


FIGURE 7 | Lineweaver–Burk plot of AChE inhibition in absence (CTR) or presence of carvacrol.

for α-glucosidase; unlike acarbose that is a competitive inhibitor [41].

For lipase (Figure 6), the lines of control and *O. heracleoticum* EO coincided on a point on the right side of the *X*-axis and intercepted both the axes at different points. Also in this case, the inhibition was mixed; instead, orlistat the standard reference was reported to be a competitive inhibitor of lipase [42].

The Lineweaver–Burk plot (Figure 7) showed that for AChE, the lines intersect in the second quadrant of the plot, indicating a mixed-type inhibition mechanism for carvacrol.

In Figure 8, Lineweaver–Burk plot showed that the two lines, in the presence and in the absence of carvacrol, intercepted both the *Y*- and the *X*-axes at different points and were not parallel, indicating a mixed-type inhibition of this compound for lipase.

Instead in Figure 9, the two lines in Lineweaver–Burk plot, in presence and in absence of thymol, intercepted both the *Y*- and the *X*-axes at different points and were parallel, indicating an uncompetitive inhibition of this compound for α-glucosidase.

4 | Discussion

In the literature, there are studies both supporting the chemical composition observed in this work and highlighting differences. For instance, Simirgiotis et al. [43] analyzed an EO of *O. 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 et al. [43], 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. [44] 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. and Dragan et al., where carvacrol was also identified as the primary constituent, although the reported percentages varied between studies [45, 46]. About EO *O. majorana*, the composition obtained was in agreement with that present in the literature [47, 48], although in some cases there were differences in the percentages of the compounds. In fact, the work conducted by Raina et al. [49] analyzed an EO of *O. majorana* that presented a higher quantity of both terpinen-4-ol (31.15%) and sabinene hydrate (15.76%) compared to those analyzed in this work. The species *O. heracleoticum* is known to exhibit diverse chemotypes influenced by factors such as plant metabolism, climate, and the secretory activity of glandular hairs [50]. 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. [51] and Schillaci et al. [52]. 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.*

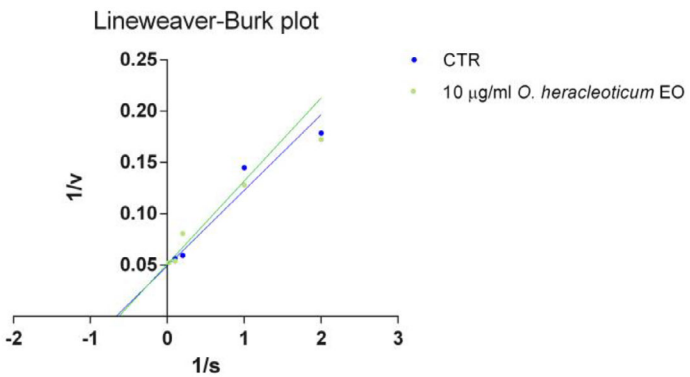


FIGURE 6 | Lineweaver–Burk plot of lipase inhibition in absence (CTR) or presence of *Origanum heracleoticum* EO. EO, essential oils.

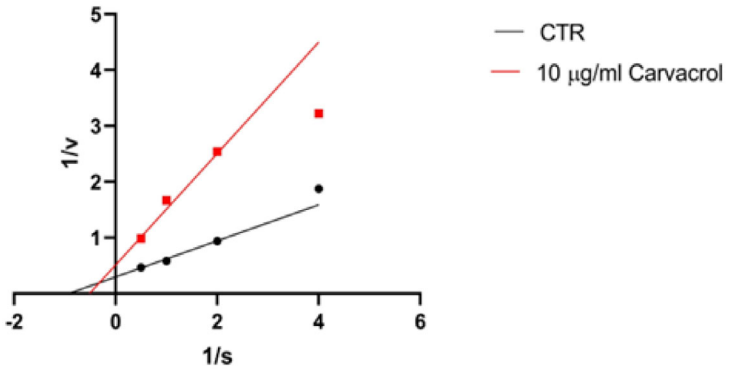


FIGURE 8 | Lineweaver–Burk plot of lipase inhibition in absence (CTR) or presence of carvacrol.

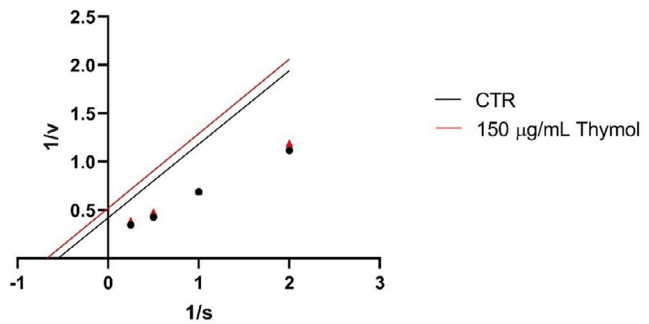


FIGURE 9 | Lineweaver–Burk plot of α-glucosidase inhibition in absence (CTR) or presence of thymol.

heracleoticum analyzed by Aytaç et al. [53] was more similar to our findings, as it also belonged to the carvacrol chemotype. However, their sample contained a significantly higher concentration of carvacrol (72.2%) compared to our results (15.79%).

Regarding antioxidant activity, 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 superior antioxidant activity compared to the results obtained by Simirgiotis et al. [43], with better values for both DPPH (4.7 mg/mL) and ABTS (1.25 TE eq. mg/g) assays. However, the TPC values in the two studies were comparable [24]. In the study conducted by Elansary et al., an EO of *O. vulgare* was analyzed, which exhibited a higher IC_{50} value (2.8 mg/mL) compared to the value obtained in our study [54].

In the study by Han et al. [55], the IC_{50} value obtained (0.33 mg/mL) was comparable to the value reported in this work. Regarding *O. majorana*, the values reported by Mossa et al. [56] differed from those obtained in this study. Specifically, the EO of *O. majorana* analyzed by Mossa exhibited a lower IC_{50} value (58.67 μg/mL) [56]. Different speech for *O. heracleoticum* where the values obtained were in agreement with those of Sarikurkcu et al. [51].

Enzyme inhibitors are an important group of drugs that are widely used in the management of various diseases. As a result, enzymes have become essential targets in drug discovery. For example, cholinesterase and TYR inhibitors are being studied for their potential in treating neurodegenerative disorders, whereas α-amylase, α-glucosidase, and lipase inhibitors are being explored for their ability to address metabolic conditions [57–60].

The cholinergic hypothesis of Alzheimer's disease (AD), initially based on studies of postmortem brain tissue [61], was supported by the development of approved drugs that target the inhibition of the enzyme AChE [62]. These drugs have been shown to significantly improve cognitive function [63] and may also slow disease progression [64]. However, combining AChE inhibitors with other therapeutic agents that mitigate neurodegeneration in AD may increase their efficacy [65]. Recent research indicates that AD is linked to inflammatory processes [66]. β-Amyloid peptides found in senile plaques characteristic of AD brains may trigger these inflammatory responses, which involve the release of ROS and other mediators [67]. ROS can damage cellular components and function as secondary messengers in inflammation. Antioxidants, by neutralizing ROS, can reduce oxidative stress and attenuate inflammatory pathways, offering potential benefits in the treatment of AD [68].

In addition, α-amylase and α-glucosidase are essential for carbohydrate digestion [57], and lipase plays a key role in the absorption of triacylglycerols [58]. Consequently, the inhibition of these enzymes could help prevent the development of NCDs. There is limited data in the literature about the enzymatic activities of EOs from *O. vulgare*, *O. majorana*, and *O. heracleoticum*, with studies primarily focusing on cholinesterases. The study conducted by Orhan and coworkers [69] analyzed EOs of *O. vulgare* and *O. majorana* evaluating their possible inhibition towards cholinesterases (AChE and BChE). In their findings, *O. vulgare* exhibited an inhibitory activity of 490 μg/mL for AChE and 520 μg/mL towards BChE. 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; similarly, our sample also showed lower IC_{50} values. The EO of *O. majorana* analyzed by Mossa et al. [56], however, was more active towards AChE compared to our findings (36.40 vs. 56.40 μg/mL). The study conducted by Sarikurkcu et al. [51] examined the inhibitory activity of EOs of *O. vulgare* and *O. heracleoticum* on different enzymes (AChE, BChE, TYR, α-amylase, and α-glucosidase) providing one of the few references regarding *O. heracleoticum* in the literature. Our data regarding α-amylase inhibition were inconsistent with those reported in this study; none of our

samples exhibited activity against this enzyme, whereas both *O. vulgare* and *O. heracleoticum* were able to inhibit the enzyme in scientific work [51]. This probably was due to different chemical composition of *O. vulgare* and *O. heracleoticum*, in fact these EOs were rich in thymol (58.33%) and linalool (96.31%) and for both compounds their antidiabetic activity was well known [70, 71].

As for cholinesterases, our samples displayed greater activity; however, for TYR inhibition both *O. vulgare* and *O. heracleoticum* samples analyzed by Sarikurkcu and coworkers [51] showed lower IC_{50} than ours. There are currently no studies in the literature regarding the EOs of *O. majorana* in relation to TYR, α-amylase, or α-glucosidase, nor there are studies involving all three species concerning the inhibition of lipase by EOs.

Regarding the inhibitory activity of the major compounds of the studied EOs, the results showed that carvacrol was the most active compound against AChE, with an IC_{50} value of 48.5 μg/mL, and against lipase, with an IC_{50} of 20.1 μg/mL, confirming its well-documented neuroprotective and antidiabetic properties reported in the literature [72–74].

These activities could be due to carvacrol high affinity for active sites of AChE and lipase as previously reported by Amato et al. [75].

In contrast, thymol exhibited good inhibitory activity against α-glucosidase, with an IC_{50} value less than half that of the reference standard, confirming its potential antidiabetic activity [53].

Interestingly, kinetic study demonstrated for the first time how carvacrol and thymol interact with these enzymes. The results revealed that their inhibition mechanisms are consistent with those observed for the corresponding EOs, suggesting that these compounds could be considered the main contributors to the enzymatic inhibition exerted by *O. heracleoticum* EO against AChE and lipase and *O. vulgare* EO against α-glucosidase.

5 | Conclusions

These findings suggest that these EOs may have therapeutic potential for the treatment of metabolic disorders such as diabetes and obesity, as well as neurological disease such as Alzheimer's and Parkinson's diseases. Moreover, to the best of our knowledge, these EOs have not been previously screened for their effects on enzymatic activities. The investigations of their mechanisms of action could enhance their potential use as adjuncts to conventional medications for Alzheimer's and Parkinson's diseases and/or diabetes, thereby minimizing side effects and improving the efficacy of standard treatments for these conditions.

Author Contributions

Conceptualization: Giuseppe Amato and Lucia Caputo. Methodology: Giuseppe Amato. Software: Lucia Caputo. Validation: Giuseppe Amato, Lucia Caputo, and Laura De Martino. Formal analysis: Laura De Martino, Barbara Borczak, and Joanna Kapusta-Duch. Investigation: Giuseppe Amato. Resources: Vincenzo De Feo. Data curation: Lucia Caputo. Writing – original draft preparation: Giuseppe Amato and Lucia Caputo. Writing – review and editing: Laura De Martino, Daniela Grulova, and Vincenzo De Feo. Visualization: Laura De Martino and Vincenzo De Feo. Supervision: Laura De Martino and Vincenzo De Feo. Project administration: Laura De Martino. Funding acquisition: Vincenzo De Feo. All authors have read and agreed to the published version of the manuscript.

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Ethics Statement

The authors have nothing to report

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

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

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