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Essential Oil of *Origanum vulgare* var. *aureum* L. from Western Romania: Chemical Analysis, In Vitro and In Silico Screening of Its Antioxidant Activity

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Abstract: This investigation aims to assess the chemical composition and antioxidant properties of *Origanum vulgare* var. *aureum* L. essential oil (OEO). The oil was obtained with a 0.34% (*v*/*w* dried weight) yield and investigated by gas chromatography-mass spectrometry (GC–MS) analysis. The main compounds of the OEO were found to be gamma-terpinene (22.96%), para-cymene (14.72%), germacrene (11.64%), beta-trans-ocimene (9.81%), and cis-beta-ocimene (7.65%). Furthermore, individual antioxidant assays 1,1-diphenyl-2-picrylhydrazyl (DPPH) and [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium] (ABTS) radical scavenging activities and beta-carotene/linoleic acid bleaching were carried out. OEO demonstrated better scavenging effects on the DPPH (IC_{50} 93.12 ± 0.03 µg/mL) and ABTS (IC_{50} 27.63 ± 0.01 µg/mL) assays (significantly lower IC_{50} values; $p \leq 0.001$) than ascorbic acid (IC_{50} 127.39 ± 0.45 µg/mL). In the beta-carotene/linoleic acid bleaching assay, the OEO exhibited a higher Relative antioxidant activity (RAA %) (82.36 ± 0.14%) but lower compared with butylated hydroxyanisole (BHA) (100%), with no significant differences ($p > 0.05$) observed. According to molecular docking results, the first two main compounds of the OEO, para-cymene, and gamma-terpinene, may potentially contribute to the biological antioxidant activity of the oil by inhibiting ROS (reactive oxygen species)-producing enzymes such as lipoxygenase and xanthin oxidase. These experimental data suggest that OEO could represent a valuable new natural antioxidant source with functional properties in the food or pharmaceutical industries.

Keywords: antioxidant activity; essential oil; golden oregano; molecular docking; *Origanum vulgare* var. *aureum* L.; steam distillation

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1. Introduction

Food deterioration during storage is a major environmental issue and concern of the food industry that generates over a billion tons of food waste and USD 940 billion in economic losses annually [1]. One of the leading causes of food degradation is lipid oxidation which generates off-flavors and loss of nutrients in fat-containing foods. Several

techniques can be adopted to decrease auto-oxidation, such as preventing oxygen access by employing suitable packaging materials, storing food products at lower temperatures, or inactivating enzymes catalyzing oxidation [2]. However, these methods are not always applicable or economic from nutritional and technological points of view [3]. Therefore, using food antioxidants, especially synthetic ones, has become indispensable in prolonging the shelf-life of foodstuffs.

The modern food consumer does not perceive food as basic nutrition alone but also for the health benefits it can generate. Furthermore, the suspicions regarding the harmful effects on human health of synthetic antioxidants such as gallic acid esters (e.g., propyl gallate, octyl gallate, and dodecyl gallate), butylhydroxytoluene (BHT) or butylated hydroxyanisole (BHA) [4–6] have led to increased consumer demands for minimally processed foods with clean, easy-to-read labels and long shelf lives. Consequently, in the last few decades, the food industry companies were urged to access natural extracts as a source of food additives to preserve food safety without harming consumers' health. Through their extracts, especially essential oils (EO), aromatic and medicinal plants are a valuable source of biologically active compounds [7–9]. Moreover, recent investigations have revealed that EOs possess promising biological properties, such as antioxidant, antimicrobial, and antiviral effects [8,10–12], recommending them as an alternative to synthetic food additives.

The *Origanum* (Lamiaceae) genus is an annual, perennial, and shrubby herb extensively found in the Mediterranean region [13]. *Origanum* is classified into three groups, ten sections and thirty-eight species (six subspecies and seventeen hybrids) [14]. Two commercially important varieties, *Origanum vulgare* subsp. *hirtum* (Greek oregano) and *Origanum onites* (Turkish oregano) are also among the most marketed and used spice plants [14–16]. The flavoring properties of oregano are mainly associated with its aromatic substances, especially its EO [14,17]. Carvacrol is the main volatile oil compound in oregano herbs, responsible for the characteristic "oregano" scent. Other compounds that dominate the EOs of genus members are thymol, para-cymene, and gamma-terpinene. Several investigations reported that oregano oil has a powerful antimicrobial effect against bacteria, yeast, and fungi. Furthermore, EOs extracted from *Origanum* sp. are known to possess cytotoxic activities against cancer cells, to protect cells from reactive oxygen species (ROS) degradation, and to generate an antioxidant and anti-inflammatory biological effect via key enzymes (lipoxygenase) inhibition [18,19]. Also, oregano oil possesses antioxidant properties effective in slowing color loss and lipid oxidation in fatty foods and scavenging free radicals [20–22].

However, some genus members remain insufficiently studied, such as *Origanum vulgare* var. *aureum* L., for which scientific literature provides insufficient data about its EO antioxidant properties. Furthermore, the probable mechanism of action against lipid oxidation of *Origanum vulgare* var. *aureum* essential oil (OEO) has not been investigated previously. Considering the background, we aimed to investigate the chemical composition and evaluate the antioxidant properties of OEO. Moreover, we aimed to analyze in silico the antioxidant biological effects based on the inhibitory capacity of OEO components against key enzymes involved in producing reactive oxygen species utilizing a molecular docking-based approach.

2. Materials and Methods

2.1. Raw Material and Chemicals

O. vulgare var. *aureum* L. stems, leaves, and flowers were harvested at full blossom from the village of Ludești de Jos, Hunedoara County (Romania) (45°05′ N 22°19′ E) in July 2021. A voucher specimen (VSNH.BUASTM-93/2) was taken in the Herbarium of the Faculty of Agronomy, University of Life Sciences "King Michael I" from Timișoara. Anhydrous sodium sulphate (Na_2SO_4), potassium persulfate ($K_2S_2O_8$), hexane, methanol, chloroform, ethanol, and ascorbic acid were obtained from Sigma-Aldrich (Germany). In addition, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), β-carotene, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic

acid) diammonium salt (ABTS), C_8-C_{20} alkane standard mixture and butylated hydroxyanisole (BHA) were obtained from Merck Company (Darmstadt, Germany).

2.2. Extraction of OEO

The collected specimens were air dried in the dark at laboratory temperature (end of 2021/beginning of 2022) and cut into ca. 1.5 cm long parts before the distillation. OEO was obtained by steam distillation operating a modified Craveiro-type apparatus described previously by Jianu et al. [23]. After extraction, the OEO was collected, dried over anhydrous Na_2SO_4, and kept in amber-sealed vials (−18 °C) for future analysis (yielding 0.34% *v*/*w*).

2.3. Gas Chromatography-Mass Spectrometry Analysis

This assay was performed on a gas chromatograph (HP6890 Gas-Chromatograph) coupled with a mass spectrometer (HP5973 Mass Spectrometer). The oil sample was diluted 1:1000 in hexane before 1 µL was injected in a splitless mode. The sample passed through a capillary Br-5MS column with a 5% Phenyl-arylene-95% Dimethylpolysiloxane phase; the column was 30 m long, with a 0.25 mm internal diameter, 0.25 µm film thickness (Bruker, Billerica, MA, USA), with a helium flow rate of 1 mL/min. The column was heated at a program starting from 50 °C to 300 °C at 6 °C/min, where the final hold was 5 min. The MS Quad temperature was set at 150 °C, and the ionization energy was 70 eV. After 3 min, solvent delay started scanning compounds between 50 to 550 amu. The resulting peaks represented compounds found in the oil sample and were identified by comparing their mass spectra with the ones from the NIST2.0 library (USA National Institute of Science and Technology software, Version NIST 2.0 library). The percentage area of each compound was calculated by dividing its area by the total area and multiplying by 100. Furthermore, their retention indices (RIs) were calculated using the linear equation obtained from a calibration curve of a standard alkane C_8-C_{20} and then compared to previously published values in the literature to confirm the identity of the identified components [24].

2.4. Antioxidant Activity by 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Radical Scavenging Assay

A DPPH radical scavenging assay was conducted using the stable radical DPPH [25]. A methanolic solution of DPPH radical at different concentrations, from 1.5 mg/mL to 2.93 µg/mL, was prepared. A ratio of 1:10 DPPH/sample (*v*/*v*) was plated in triplicates on 96 well plates and incubated in the dark at room temperature. After a 30 min reaction period, absorbances were registered at 515 nm on a spectrophotometer Tecan i-control, 1.10.4.0 infinite 200Pro. BHA and ascorbic acid were used as positive controls. The following equation computed the DPPH scavenging activity: % DPPH scavenging activity = (A_{blank} − A_{OEO})·100/A_{blank}, where: A_{blank} and A_{OEO} are the absorbances of the control and the tested oil. IC_{50} (µg/L) value, defined as the concentration of oil required to scavenge the formation of 50% of free radicals, was calculated using the BioDataFit 1.02 program (Chang Broscience Inc, Castro Valley, CA, USA) and expressed as means ± standard deviation (SD) of three independent experiments.

2.5. Antioxidant Activity by [2,2′-Azinobis(3-Ethylbenzothiazoline-6-Sulfonic Acid) Diammonium] (ABTS) Radical Scavenging Assay

ABTS radical scavenging activity was conducted using the method previously reported by Rădulescul et al. (2021) [26] with a slight modification. Briefly, ABTS (7.0 mM) and $K_2S_2O_8$ (2.5 mM) were added to an amber-colored bottle for the preparation of ABTS cation ($ABTS^+$) and kept in the dark for 14 h at 21 °C. First, the $ABTS^+$ solution was diluted in ethanol (approximately 1:80) to an absorbance of 0.700 ± 0.035 at 734 nm [27]. Subsequently, 100 µL of the OEO in methanol, with various concentrations (from 1.5 mg/mL to 9.3 µg/mL), were mixed with 1 mL of $ABTS^+$ solution. The absorbances were measured at 734 nm after 30 min (at 21 °C in the dark). BHA and ascorbic acid served as positive controls. Finally, the ABTS scavenging activity was analyzed by IC_{50} (µg/L) value as the

inhibitory concentration of the OEO required to scavenge the formation of 50% of ABTS, expressed as means ± standard deviation (SD) of three independent experiments.

2.6. Beta-Carotene/Linoleic Acid Bleaching Assay

The *β*-Carotene/Linoleic acid bleaching test was assessed employing the method described by Rădulescu et al. (2021) [26]. First, a stock solution of *β*-carotene was obtained by dissolving 0.5 mg β-carotene in 1 mL of $CHCl_3$, 25 µL linoleic acid, and 200 mg Tween 40. A vacuum rotary evaporator removed chloroform under a vacuum (at 40 °C for 5 min), and then 100 mL of distilled water was added to the residue to form a transparent yellowish emulsion. Next, 350 µL of oil in methanol (2 mg/mL) was stirred exhaustively with 2.5 mL of *β*-carotene stock solution and incubated for 48 h at 21 °C. The same methodology was repeated for the synthetic antioxidant (BHA) serving as positive control and a blank sample (350 µL of methanol). After this incubation period, the absorbance of the samples was read at 490 nm in three independent experiments. The following equation computed the Relative Antioxidant Activity (RAA %): $RAA = A_{OEO}/A_{BHA}$, where A_{OEO} and A_{BHA} are the absorption of OEO and the absorbance of the BHA, respectively.

2.7. In Silico Prediction of Bioactivity and Molecular Docking Studies

Molecular docking simulations were performed using a method previously described [23]. In brief, docking targets were optimized using the 3D crystallographic structure of proteins available from the RCSB Protein Data Bank [28] (Table 1). The 38 OEO components were downloaded from the Pubchem repository [29] as SDF files and were later converted to PDBQT files using Autodocktools [30]. The PyRx v0.8 virtual screening software (The Scripps Research Institute, La Jolla, CA, USA) and Vina's embedded scoring function [31] were used for molecular docking. Each input molecular structure's target protein was docked with the default number of conformers (eight per each ligand structure). The structure of each protein native ligand (NL) (Table 1) was retrieved from their respective PDB file and converted to the PDBQT format as described above. In order to validate our protocol, the calculated root means square deviation (RMSD) between predicted and actual native ligand docking conformation for every case could not exceed a 2 Å threshold. The coordinates and size of the docking grid box were selected to match the active binding domain (Table 1) perfectly. The software generated ΔG binding energy values (kcal/mol) as docking scores for each docked molecule. Protein-ligand binding interactions were also investigated using Accelrys Discovery Studio 4.1. (Dassault Systems BIOVIA, San Diego, CA, USA).

Table 1. Docking parameters used for the in silico evaluation of the 38 OEO components.

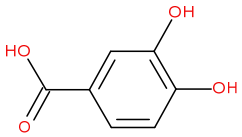
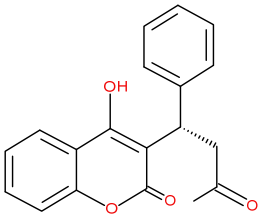
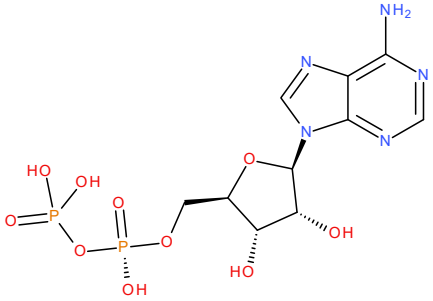
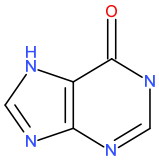
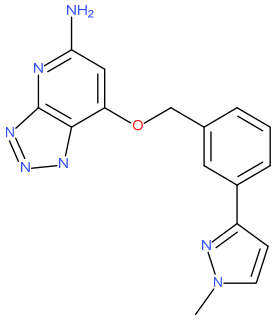
Protein	PDB ID	Native Ligand	Grid Box Center Coordinates and Size (Å)
Lipoxygenase	1N8Q	 Protocatechuic acid	center_x = 20.6778 center_y = 2.2697 center_z = 19.5423 size_x = 8.5358 size_y = 9.1096 size_z = 9.9453

Table 1. *Cont.*

Protein	PDB ID	Native Ligand	Grid Box Center Coordinates and Size (Å)
CYP2C9	1OG5	 S-Warfarin	center_x = −20.4200 center_y = 85.2723 center_z = 38.2181 size_x = 9.6524 size_y = 10.6625 size_z = 11.5535
NADPH-oxidase	2CDU	 Adenosine-5′-diphosphate	center_x = 18.4683 center_y = −5.1659 center_z = −0.0901 size_x = 12.3286 size_y = 15.3831 size_z = 15.1287
Xanthine oxidase	3NRZ	 Hypoxanthine	center_x = 37.6450 center_y = 19.2898 center_z = 17.5578 size_x = 11.6388 size_y = 9.8519 size_z = 10.1251
Myeloperoxidase	5QJ2	 7-{[3-(1-methyl-1H-pyrazol-3-yl) phenyl]methoxy}-1H-[1,2,3]triazolo[4,5-b]pyridin-5-amine	center_x = −20.1951 center_y = 11.9649 center_z = 32.9278 size_x = 9.7712 size_y = 12.5445 size_z = 11.9903

2.8. Statistical Analysis

Conventional statistical methods were employed to compute the mean ± standard deviation (SD) of three independent experiments carried out separately for the antioxidant activity assays. In addition, a post hoc test (Tukey) was applied to compare the significant differences between the mean values obtained from the antioxidant activity measurements, with a significance of 0.05 ($p < 0.05$). Analysis was performed using the IBM SPSS 25.0 package (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. OEO Yield and Chemical Composition

A greenish-yellow oil with a pungent smell was obtained from dry *O. vulgare* var. *aureum* L. (stems, leaves, and flowers), with a 0.34% (*v*/*w*) yield. Our results agree with

those mentioned in the literature, which record yields of obtaining EOs from different oregano species as lower than 1% [32].

Table 2 summarizes the chemical compositions of the EO isolated from *O. vulgare* var. *aureum* L. by steam distillation. The GC-MS analysis identified 38 compounds in the analyzed sample (Figure 1), with gamma-terpinene (22.96%), para-cymene (14.72%), germacrene (11.64%), beta-trans-ocimene (9.81%), and cis-beta-ocimene (7.65%) as main compounds of the oil (Table 2). In addition, beta-linalool (3.61%), isothymol methyl ether (3.34%), beta-phellandrene (2.44%), beta-caryophyllene (2.35%), alpha-terpinene (1.84%), beta-cadinene (1.78%), alpha-farnesene (1.55%), (-)-spathulenol (1.35%), beta-myrcene (1.31%) and alpha-himalachene (1.29%) were identified in smaller quantities.

Table 2. Chemical composition of OEO as determined by GC-MS analysis.

No.	Compound Name	MW (g/mol)	$RI_{exp.}$	Area %
1	Alpha-thujene	136.23	912	0.83
2	Alpha-pinene	136.23	919	0.35
3	Camphene	136.23	934	0.16
4	Beta-phellandrene	136.23	955	2.44
5	Beta-pinene	136.23	960	0.26
6	Beta-myrcene	136.23	970	1.31
7	Alpha-phellandrene	136.23	987	0.15
8	3-Carene	136.23	990	0.05
9	Alpha-terpinene	136.23	998	1.84
10	Para-cymene	134.21	1007	14.72
11	Beta-terpinyl acetate	196.29	1011	0.35
12	Beta-trans-ocimene	136.23	1018	9.81
13	Cis-beta-ocimene	136.23	1030	7.65
14	Gamma-terpinene	136.23	1044	22.96
15	Beta-linalool	154.25	1087	3.61
16	Isothymol methyl ether	164.24	1243	3.34
17	Dihydroedulan	194.31	1299	1.01
18	Carvacrol	150.22	1309	0.62
19	Alpha-cubebene	204.35	1363	0.06
20	Alpha-copaene	204.35	1394	0.27
21	Beta-bourbonene	204.35	1402	0.97
22	Beta-elemene	204.35	1408	0.71
23	Beta-caryophyllene	204.35	1439	2.35
24	Beta-cubebene	204.35	1450	0.45
25	Alpha-caryophyllene	204.35	1476	0.46
26	Alloaromadendrene	204.35	1481	0.75
27	Germacrene D	204.35	1503	11.64
28	Gamma-elemene	204.35	1517	0.98
29	Alpha-muurolene	204.35	1520	0.52
30	Alpha-farnesene	204.35	1525	1.55

Table 2. *Cont.*

No.	Compound Name	MW (g/mol)	$RI_{exp.}$	Area %
31	Alpha-himalachene	222.37	1529	1.29
32	Gamma-cadinene	206.37	1534	0.51
33	Beta-cadinene	206.37	1540	1.78
34	(-)-Spathulenol	220.35	1595	1.35
35	Caryophyllene oxide	220.35	1600	0.35
36	Alpha-cadinol	222.37	1669	0.49
37	Chamazulene	184.28	1735	0.14
38	Isoaromadendrene epoxide	220.35	1806	0.51
			Total identified (%):	**98.59%**

The retention experimental ($RI_{exp.}$) was determined on a Br-5MS column using a homologous series of n-alkanes (C_8-C_{20}); MW—molecular weight.

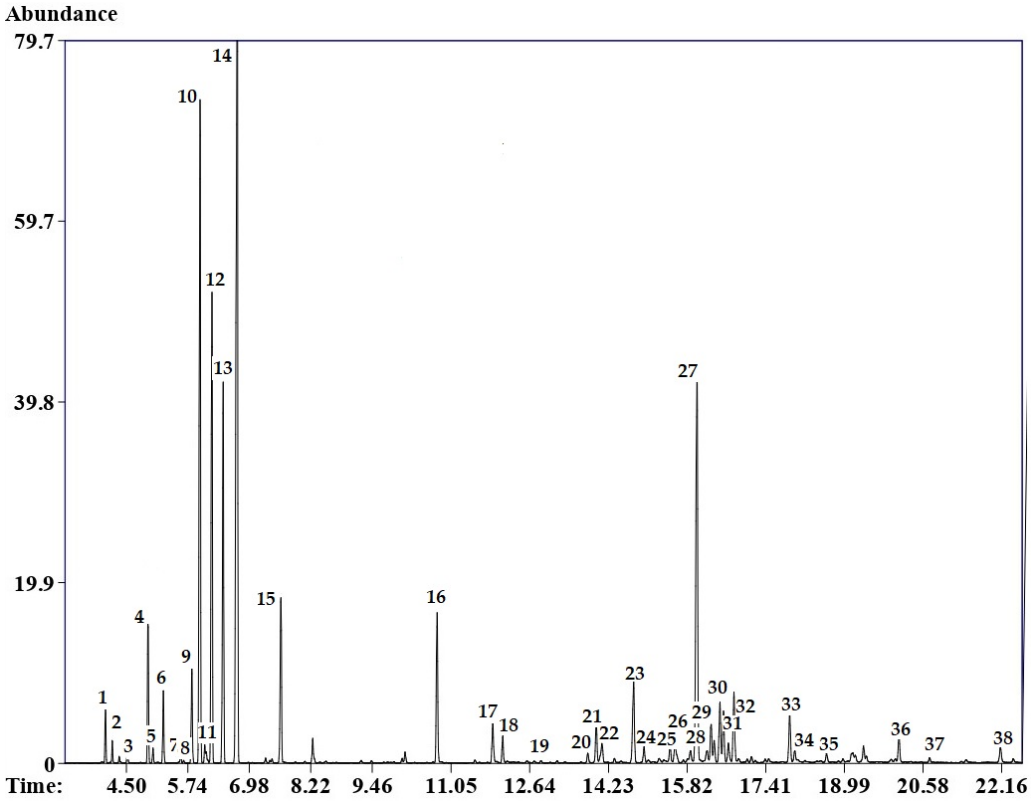


Figure 1. Gas chromatogram of OEO from western Romania.

Popa et al. [33] reported linalool (26.54%), para-cymene (20.81%), and gamma-terpinene (13.73%) as the major compounds for the oil isolated from Romanian *O. aureum*. Furthermore, high contents of linalool (25.5%), para-cymene (20.7%), and gamma-terpinene (15.66%) were also documented for the golden oregano oil by Moisa et al. [34]. In contrast, the oil obtained from Polish *O. aureum* contained mainly cis-sabinene hydrate (18.7%), germacrene D (16.5%), and thymol (12.2%), respectively [35]. The variability of oil content and the chemical composition of the oregano herbs EOs is strongly dependent on the environment and local conditions of the plants, climatic conditions, and geographical distribution of the plant collections, as previously documented [35–38].

3.2. Assessment of Antioxidant Activity

It is well-known that free radicals initiate the process of lipid peroxidation and the propagation of the chain of radical structures. Therefore, several methods have been developed to determine compounds' antioxidant properties [39–41]. For example, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical (ABTS) assays are two spectrophotometer techniques widely employed for assessing the antioxidant properties of natural extracts. Briefly, the DPPH method is based on reducing the purple DPPH radical to 1,1-diphenyl-2-picryl-hydrazine (DPPH-H). In contrast, the ABTS method implicates the reduction or radical scavenging of a blue/green ABTS radical to a colorless sulfonic acid [42]. Butylated hydroxyanisole (BHA) and ascorbic acid were used as the positive control. The IC_{50} values were computed employing BioDataFit 1.02 program (Chang Broscience Inc, Castro Valley, CA, USA). In our study, OEO reduced the stable free radical DPPH with an IC_{50} value of 93.12 ± 0.03 µg/mL (Table 3). Tukey's test shows that the OEO's DPPH values are significantly lower than ascorbic acid ($p < 0.01$). Previously, Popa et al. documented low antioxidant capacity for Romanian *O. vulgare* var. *aureum* volatile oil, with a 58.18 ± 0.07% inhibition [33]. Comparable effects were reported by Moisa et al. for oils isolated from *O. vulgare* var. *aureum* leaves and flowers with 63.1 ± 0.7% and 66.4 ± 2.23% inhibitions [34]. Better results were documented for oils extracted from *O. vulgare* var. *aureum* stems and whole plant, respectively, with inhibitions ranging between 80.3 ± 0.01% to 88.6 ± 0.1% [34]. However, because of the various modes of result expression used in different studies, directly comparing our findings with those reported in the literature is difficult. Nevertheless, the DPPH radical scavenging ability registered for the studied OEO is in accord with those previously described for *O. vulgare* (46.66–97.61 µg/mL), *Poliomintha longiflora* (83.70 ± 4.12 µg/mL), *O. onites* L. (116.74–132.93 µg/mL) and *O. syriacum* (91.45 ± 2.30 µg/mL) EOs [43–46].

Table 3. The antioxidant activity of essential oil extracted from *O. vulgare* var. *aureum*.

Samples Tested	Parameters		
	DPPH, IC_{50} (µg/mL)	ABTS, IC_{50} (µg/L)	β-Carotene/Linoleic Acid (% Inhibition Rate)
OEO	93.12 ± 0.03	27.63 ± 0.01	82.36 ± 0.14
BHA	10.11 ± 0.01	8.71 ± 0.01	100
Ascorbic acid	127.39 ± 0.45	35.89 ± 0.05	n.d.

n.d.—not determined.

In the ABTS assay, OEO exhibited a good ability to scavenge the ABTS radical (IC_{50} 27.63 ± 0.01 µg/mL) (Table 3), significantly more potent ($p < 0.01$) than standard ascorbic acid (IC_{50} 35.89 ± 0.05 µg/mL) according to the Tukey's test. In contrast, BHA revealed significantly ($p < 0.001$) higher antioxidant capability (IC_{50} 8.71 ± 0.01 µg/mL) compared to OEO and ascorbic acid. Nevertheless, our results reveal that OEO was more effective in scavenging ABTS radicals than other oregano species: *O. tyttanthum*, *O. compactum*, *O. vulgare*, and *L. graveolens* [47–50].

Previously published research investigating the DPPH and reactive oxygen species scavenging capability of different extracts discovered that these extracts have similar scavenging performance in both cases [51,52]. This is to be expected, given that it is dependent on the extract components' capacity to reduce free radicals. Based on this, OEO could be a good source of antioxidants that can also quench endogenous reactive oxygen species.

The beta-carotene/linoleic acid bleaching assay is based on the discoloration of beta-carotene determined by its reaction with the radicals resulting from the oxidation of linoleic acid in an emulsion. The presence of an antioxidant can delay the rate of beta-carotene discoloration. Table 3 displays the inhibition of beta-carotene bleaching by the OEO and the

positive control used (BHA). In the case of the beta-carotene-linoleic acid bleaching assay, OEO was able to inhibit the linoleic acid oxidation (82.36 ± 0.14%) effectively but lower than BHA (100%) ($p > 0.05$). No previous investigations were documented concerning OEO activity in *the* beta-carotene/linoleic acid system to allow us to make direct comparisons.

3.3. In Silico Prediction of the Mechanism by Molecular Docking Analysis

In recent years, computer simulation methods such as molecular docking and molecular dynamics simulation have been widely used to study structure-function relationships and interaction mechanisms [53,54].

The OEO's potential protein-targeted antioxidant effect was evaluated using molecular docking. The obtained docking scores for the 38 OEO components are depicted in Table 4.

Table 4. Docking scores for compounds **1–38** (binding energy, kcal/mol); compounds with better docking scores than the target native ligand score is highlighted.

Target PDB ID	1N8Q	1OG5	2CDU	3NRZ	5QJ2
Docked OEO Component ID	**Binding Free Energy ΔG (kcal/mol)**				
Native ligand	−5.7	−9.8	−9.3	−6.7	−8.5
1	−6.4	−5.7	−5.5	−5	−6.3
2	−5.3	−5.6	−5.1	−0.9	−5.6
3	−4	−5.6	−5.2	0.1	−5.7
4	−5.7	−6.2	−5.8	−6.5	−6
5	−5	−5.6	−5.2	−0.9	−5.8
6	−5.5	−5.4	−5	−5.8	−5.5
7	−5.7	−6.2	−5.6	−6.6	−6.3
8	−6.4	−5.7	−5.5	−2.8	−6.1
9	−5.9	−6.1	−5.6	−6.7	−6.4
10	−5.9	−6.2	−5.7	−7	−6.5
11	−3.1	−6.6	−5.8	−2.7	−6.5
12	−5.2	−5.5	−5.2	−6.2	−5.7
13	−4.9	−5.5	−5	−5.8	−5.6
14	−5.7	−6.2	−5.6	−6.8	−6.5
15	−4.9	−5.3	−5.2	−5	−5.8
16	−4.6	−6.3	−6.1	−6.9	−6.4
17	−2.2	−7.1	−6.3	1.4	−7.1
18	−6	−6.2	−6	−7.1	−7
19	−1.7	−7.3	−6.6	2.7	−7.7
20	−2.4	−7.5	−6.3	2.1	−7.3
21	−3.9	−7	−7	−1.5	−7.7
22	−2.3	−7.1	−6.4	−1.2	−6.9
23	−2.9	−7.4	−6.9	1.6	−7
24	−1.7	−7.5	−6.5	2.9	−7.7
25	−1.2	−7.1	−5.9	3.4	−6.8
26	−1.2	−7.4	−6.5	5.5	−7.3
27	−2.2	−7.4	−6.5	−1.4	−7.6
28	−1.1	−7	−5.8	−0.1	−7.2
29	−2.6	−7.2	−6.3	6.2	−7.3
30	−4.1	−6.8	−6.2	−7.3	−6.5
31	0.2	−7.3	−6.5	9.9	−7.3
32	−3.5	−7.2	−7.2	−1.6	−7.3
33	−3	−7.5	−7.4	−1.3	−7.6
34	0.5	−7.1	−6.5	5.9	−6.9
35	−0.9	−7.4	−6.5	5.1	−6.5
36	−2.3	−7.1	−6.9	−0.3	−6.9
37	−4.9	−7.9	−7.5	−6.1	−7.8
38	−0.2	−7.2	−7.1	6.8	−7

Background color explicitly presents the results obtained.

Our in silico-based method aimed to identify protein targets that can be inhibited by multiple components of our OEO or, at the very least, by the EO's major constituents. In order to achieve this goal, all 38 OEO components were investigated in silico for their inhibitory potential against proteins involved in producing reactive oxygen species (ROS) [55]. The proteins selected for the docking-based virtual screening of the OEO components were Lipoxygenase, CYP2C9, NADPH-oxidase, Xanthine oxidase, and Myeloperoxidase. Docking scores show that of the five investigated proteins, two cases stand out (lipoxygenase—1N8Q and xanthine oxidase—3NRZ), where several components recorded the same or better docking scores than the respective protein's NL score. However, in potentially assessing a biological effect of a multicomponent mixture such as the present OEO, one must consider the quantity of each molecule in that mixture.

Figuring out correlations between docking scores is challenging since every protein has distinct binding site features, and native ligands yield different docking scores, resulting in varying control values for each score set. To compensate for these drawbacks, we determined each docking score as a percentage of its corresponding native ligand's score (considered 100%). These percentages were illustrated as a radar chart, with the scores for every compound being indicated by plot lines while the protein targets formed the chart's corners. Compounds with a calculated percentage of 10% or below were all assigned a 10% value because positive ΔG values would give negative percentages, and the centre of the chart would become crowded and unintelligible. When a chart plot line stretches toward a specific protein target corner, this would indicate that the oil components scored close to or higher than that protein's NL. The final result should reveal the most likely targeted protein by the EO's constituents.

Given that a particular compound may have excellent docking scores, but only a small quantity of that molecule is contained in the volatile oil, we first plotted the components that exceed 1% of the oil content (Figure 2A). Aside from a few compounds, the same trend can be seen in this figure, where most constituents show higher affinity towards the same two proteins (lipoxygenase—1N8Q and xanthine oxidase—3NRZ) where compounds registered higher docking scores than the native ligands used as positive controls in this setting. If further compounds are removed from the same chart so that only major components that exceed a 5% weight content of the OEO remain, the trend, as mentioned earlier, becomes more visible. Aside from compound 27 (Germacrene D), which performed poorly against all five proteins, compounds 10 (para-cymene) and 14 (gamma-terpinene) stand out as the most abundant OEO components that also performed better than the native ligands when docked in both lipoxygenase (1N8Q) and xanthine oxidase (3NRZ). It was previously demonstrated that para-cymene has antinociceptive and anti-inflammatory activity associated with lipoxygenase inhibition [56]. Another study investigating the antioxidant activity of thyme oils found that the EO's lipoxygenase inhibition was primarily due to thymol, para-cymene, and linalool [57]. Similarly, gamma-terpinene was previously reported as an in vitro lipoxygenase inhibitor [58]. Given their highly similar structures, except for para-cymene having an aromatic ring and gamma-terpinene having a cyclohexadiene ring, both compounds exhibit a similar binding pattern within the lipoxygenase binding domain near the iron site, where both compounds interact through multiple hydrophobic interactions with the surrounding amino acids (Figure 3).

No relevant literature is available regarding the direct inhibition activity of para-cymene against xanthine oxidase. However, in the case of gamma-terpinene, a study investigating the antioxidant and xanthine oxidase inhibitory activity of various sunflower essential oils discovered the monoterpene, as mentioned above, as one of the EO's major constituents. According to a Pearson correlation analysis, gamma-terpinene was one of the constituents associated with antioxidant and anti-xanthine oxidase activity [59]. Similar to the previous case, both compounds exhibit nearly the same conformation in the active domain of xanthine oxidase, interacting with the same amino acids via hydrophobic interactions. The binding patterns shown by both compounds are depicted in Figure 4. Given that six compounds scored higher than the co-crystallized ligand (the endogenous

hypoxanthine) in the case of xanthine oxidase, five of which are major components of OEO, accounting for approximately half of the OEO weight content, we can easily predict that the oil could have a significant inhibitory activity towards xanthine oxidase.

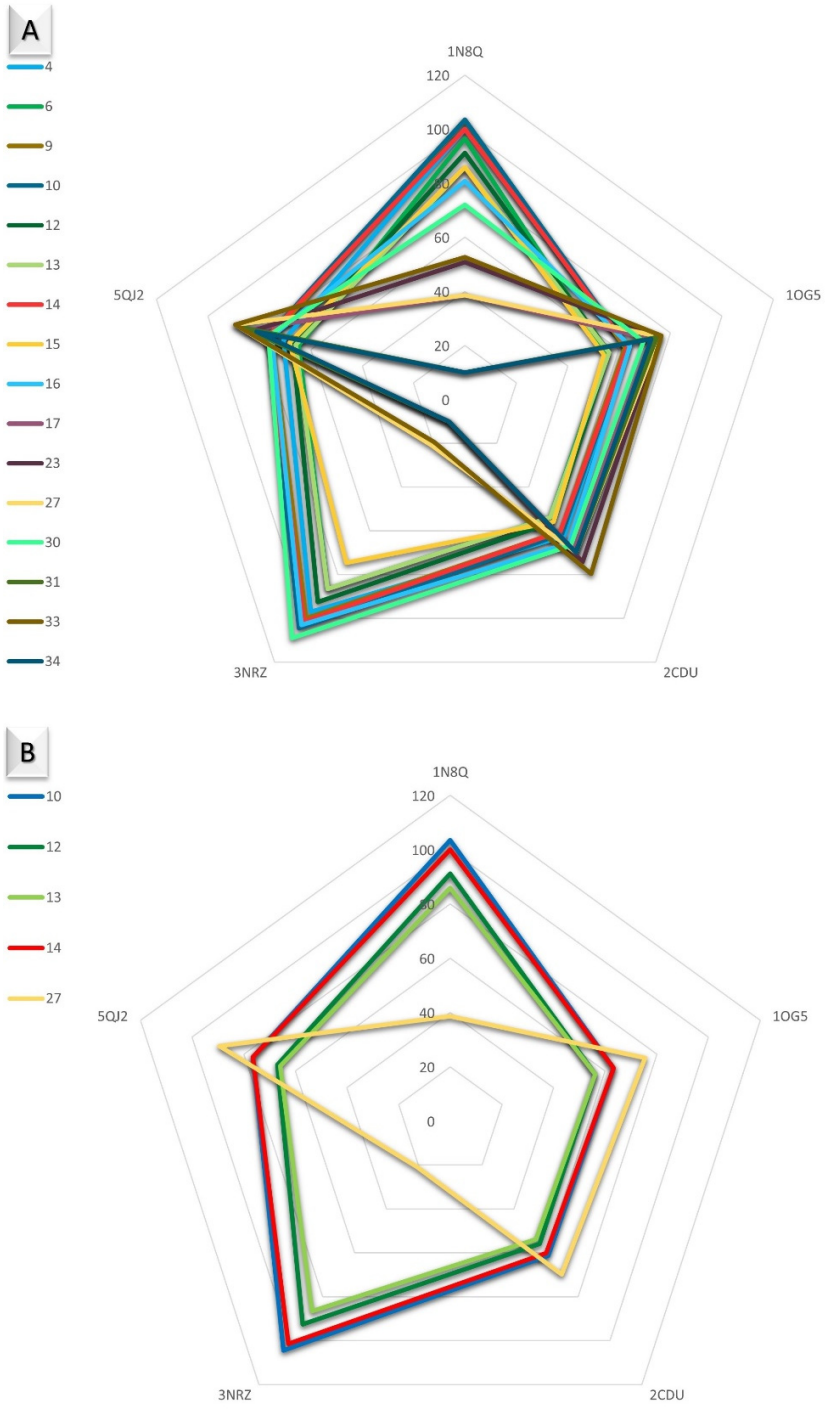


Figure 2. Graphical representation of docking scores related to antioxidant protein targets, corresponding to the components above 1% of the OEO content (**A**) and OEO constituents above 5% of the OEO content (**B**) (representing above 65% of the volatile oil); lines are generated for each compound based on the docking scores calculated for each target protein as a percentage of the NL's docking score used as the positive control (100%); the lines and are plotted as series in a radar chart, where the proteins represent the corners of the chart.

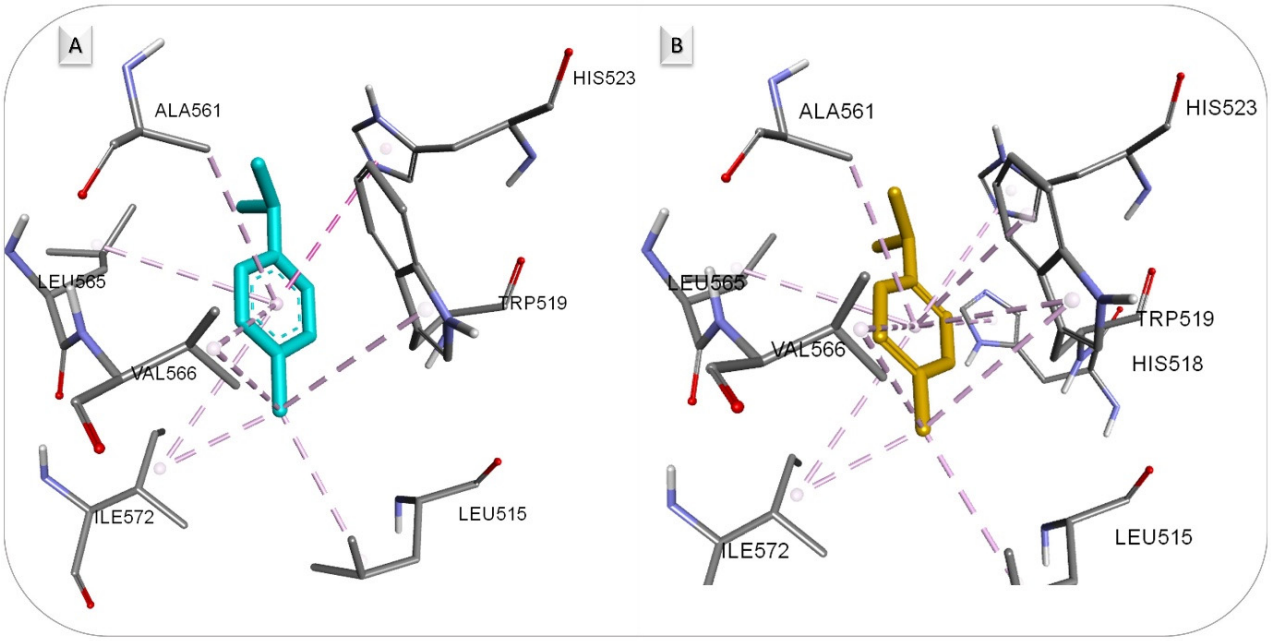


Figure 3. Lipoxygenase (1N8Q) structure in complex with docked compound (**A**) para-cymene (cyan) and (**B**) gamma-terpinene (gold) with interacting amino acids (grey sticks) via hydrophobic interactions (pink-dotted lines). Interatomic length between interacting atoms varied between 3.53–5.63 Å.

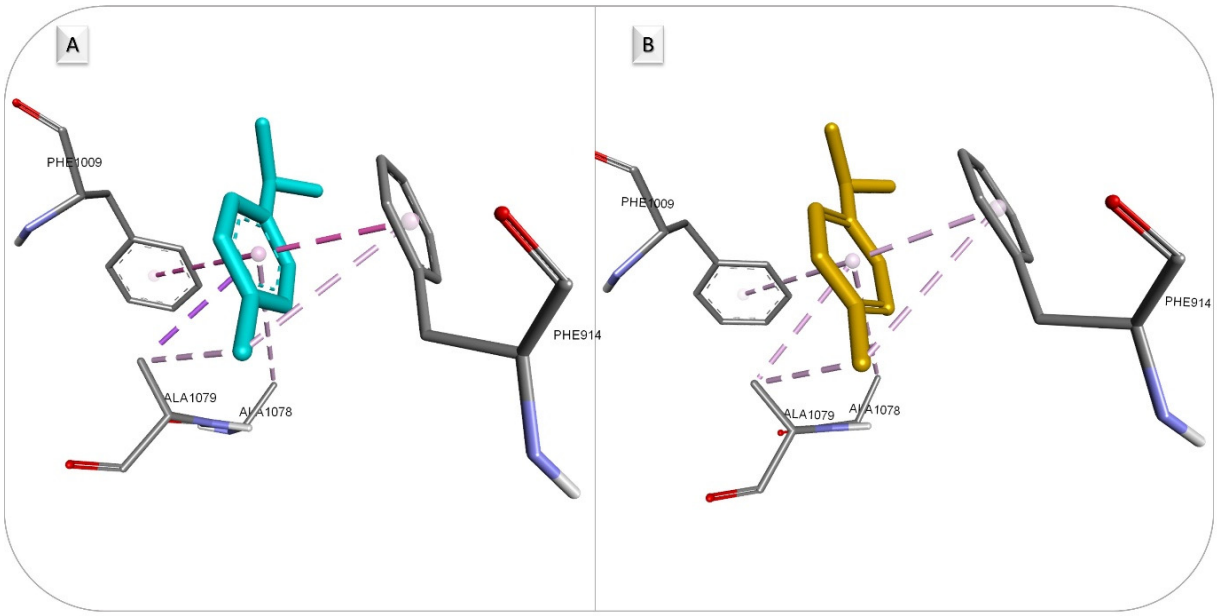


Figure 4. Xanthine oxidase (3NRZ) structure in complex with docked compound (**A**) para-cymene (cyan) and (**B**) gamma-terpinene (gold) with interacting amino acids (grey sticks) via hydrophobic interactions (pink/purple-dotted lines). Interatomic length between interacting atoms varied between 3.61–5.21 Å.

4. Conclusions

The present investigation demonstrated that OEO revealed better antioxidant properties (significantly lower IC50 values; $p \leq 0.001$) than ascorbic acid in vitro assays, such as DPPH and ABTS radical scavenging. Furthermore, according to molecular docking results, the first two major components of the volatile oil, para-cymene and gamma-terpinene,

may contribute to the EO's biological antioxidant activity by inhibiting ROS-producing enzymes such as lipoxygenase and xanthin oxidase. Due to the effective antioxidant activity recorded by OEO, it can represent a new natural source of antioxidants with potential applications in the food or pharmaceutical industry. However, further investigations are needed to elucidate the mechanism of action against oxidation reactions and to establish the safety of usage doses.

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