

Special Issue Reprint

Plant Essential Oil with Biological Activity

3rd Edition

Edited by
Hazem Salaheldin Elshafie and Ippolito Camele

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Plant Essential Oil with Biological Activity: 3rd Edition

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Guest Editors

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About the Editors

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Editorial

From Chemotaxonomy to Green Biocides: An Overview of New Studies on the Composition and Functional Properties of Some Plant Essential Oils

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Abstract

In recent years, the search for sustainable, bio-based alternatives to synthetic chemicals has intensified, positioning plant essential oils (EOs) at the forefront of applied phytochemical research. The following collection of ten articles from different geographical regions, published in *Plants* as part of the Special Issue “Plant Essential Oil with Biological Activity: 3rd Edition,” covers various aspects of recent scientific research on plant EOs, ranging from chemotaxonomy to green biocides, with particular emphasis on chemical composition and functional properties. Further attention is given to specific predominant single constituents and their bio-selectivity, modes of action, and innovative applications in the medical and pharmaceutical sectors, particularly against major diseases such as cancer and Alzheimer’s.

Keywords: essential oils; chemotaxonomy; green biocides; plant disease; pharmacological properties; sustainable agriculture

1. Introduction

In recent years, the demand for sustainable, bio-based substitutes for synthetic compounds has accelerated, establishing plant essential oils as central subjects in applied phytochemistry. Historically, EOs originate from ancient ethnobotanical practices, including Egyptian embalming (c. 3000 BCE) and traditional Ayurvedic and Greco-Roman medicine [1]. Recently, the application of plant essential oils has expanded into critical domains, including medicine, food technology, and plant protection, employing their antimicrobial, antioxidant, and biocidal properties [2,3].

Plant EOs exert their biological activity through a set of mechanisms that may target cellular integrity and metabolic function in microorganisms and pests [4,5]. The hydrophobic nature of EOs enables them to penetrate the cells and disrupt the cell membranes by increasing their permeability and causing leaks of some vital constituents such as proteins and nucleic acids [4]. This membrane disruption may be accompanied by interference with enzymatic systems involved in energy production including ATP synthesis and cell wall biosynthesis [4]. In addition, many EO constituents such as terpenes and phenolic compounds are also able to induce oxidative stress by generating reactive oxygen species, which can later damage the cellular components and harm microbial viability [6]. On the other hand, some EOs can inhibit microbial communication and biofilm formation by altering gene expression and interfering with quorum sensing process [7].

These different mode of action and possible mechanisms make EOs effective against a broad spectrum of harmful phyto- and human pathogens, whether bacteria or fungi. In

addition, plant EOs prevent different pathogens from developing resistance towards the synthetic chemical and pharmaceutical agents. Moreover, the different modes of action explain their wide variety of uses in different fields including agriculture, food systems, and medicine.

2. Diversity, Bioactivity, and Applications of Plant EOs

The ten articles in this collection, published in *Plants*, explore the vast chemical diversity and biological potential of EOs derived from various botanical families, including Hypericaceae, Asteraceae, Rutaceae, Lamiaceae, and Apiaceae.

This Special Issue includes recent studies on plant EOs that span three main research areas: (i) chemical characterization and its link to bioactivity; (ii) agricultural applications, especially antifungal control; and (iii) biomedical and pharmacological development. The first group emphasizes chromatographic profiling to identify bioactive compounds, including selective butyrylcholinesterase (BuChE and AChE) inhibitors, as well as antimicrobial and antioxidant properties and chemical variability. The second group highlights agricultural uses, showing EO effectiveness against some serious fungal diseases and insect pests. The third group focuses on biomedical potential, including green biocide applications and notable cytotoxic activity against cancer cell lines. Overall, these studies demonstrate the multifunctional value of plant essential oils across diverse scientific fields.

2.1. Group A: Chemical Characterization and Bioactivity Screening

Calva et al. [8] investigated the EO from *Hypericum aciculare* (Hypericaceae) collected in southern Ecuador and chemically characterized its constituents using GC-FID and GC-MS analysis, revealing the presence of 53 constituents including hydrocarbon and aldehyde n-decanal as the predominant compounds. The study showed significant and selective inhibition against butyrylcholinesterase (BuChE) compared to acetylcholinesterase (AChE) enzymes. The authors concluded that *H. aciculare* EO is a promising candidate for further research into the mechanism of selective cholinesterase inhibition and potential natural therapeutic remedies for dementia.

Valarezo et al. [9] characterized the EO from *Ageratina dendroides* (Asteraceae), evaluated its biological activities and also determined its chemical composition through GC-MS and GC-FID. The obtained results demonstrated that this EO has notable antioxidant activity and moderate anticholinesterase activity. The findings highlight the importance of *A. dendroides* EO as a source of bioactive compounds with multiple promising pharmacological applications.

Zhigzhitzhapova et al. [10] investigated the EO of *Artemisia frigida* (Asteraceae), focusing on the stability and variability of its chemical profile, as EO components contribute to antioxidant activity, a key plant response to stress. Although EO composition responds to biotic interactions, general climatic changes have little effect on overall component ratios, and significant variations occur mainly under extreme conditions, such as high altitudes, exposed slopes, or human disturbance. The EO profile consists of a stable “core” of 24 compounds, likely under genetic control, and more variable components influenced by environmental and epigenetic factors. Its composition also changes across developmental stages and seasonal cycles.

Flores et al. [11], who provided a comprehensive analysis of the EO from the aerial parts of *Lasiocephalus ovatus* (Asteraceae), investigated its chemical composition, enantioselective profile, and preliminary biological activities. Using GC-FID/MS, the authors identified 71 compounds, predominantly sesquiterpene hydrocarbons and oxygenated sesquiterpenes. The main constituents included β -cyclogermacrene and spathulenol. The EO showed antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, as well as

moderate antioxidant and anti-inflammatory effects, biological properties that are mainly attributed to its rich terpenoid profile, particularly oxygenated sesquiterpenes.

2.2. Group B: Antifungal and Agricultural Applications

Et-tazy et al. [2] investigated the in vitro antioxidant and antifungal activities of four EOs (*Rosmarinus officinalis*, *Myrtus communis*, *Origanum compactum*, and *Eugenia aromatica*) and their major chemical constituents against four post-harvest fungi affecting chickpea during storage (*Fusarium culmorum*, *Rhizopus oryzae*, *Penicillium italicum*, and *Aspergillus niger*). Results showed that all tested EOs exhibited varying levels of antifungal activity. In addition, they all displayed antioxidant properties, which may contribute to preserving seed quality by reducing oxidative stress during storage. This study highlights the potential of plant-derived EOs to act as dual-function bio-preservatives, combining antifungal and antioxidant effects. It also supports the use of natural products such as plant EOs as eco-friendly post-harvest protection agents in agricultural storage systems.

Moura et al. [3] evaluated the antifungal activity of citrus essential oil against sour rot in Tahiti acid lime caused by *Geotrichum citri-aurantii*. The results showed that citrus EO significantly reduced disease symptoms and inhibited fungal growth responsible for post-harvest decay. Its effectiveness is attributed to multiple mechanisms, including direct antifungal action and disruption of pathogen development processes. Treated fruits maintained better quality and exhibited an extended shelf life compared with untreated controls. Overall, the study demonstrates the potential of this essential oil as a natural alternative to synthetic fungicides and highlights its relevance for sustainable post-harvest citrus preservation and disease management.

Zhou et al. [12] investigated essential oils from patchouli, catnip, lavender, and mint (family Lamiaceae) for their bioactivity against *Thrips flavus* and their effects on crops and weeds. The oils exhibited strong insecticidal activity against the pest, as well as herbicidal effects on selected weed species. Additionally, they showed phytotoxic or growth-modulating effects on crop plants, indicating selective biological activity depending on plant type. These findings suggest that Lamiaceae essential oils have strong potential as eco-friendly agricultural agents for integrated pest and weed management, reducing dependence on synthetic agrochemicals while supporting more sustainable farming practices.

Elshafie et al. [4] undertook a broad investigation into the antifungal properties of plant essential oils against phytopathogenic fungi involved in plant diseases and post-harvest decay, reporting strong inhibitory effects across multiple fungal species, including reduced spore germination, impaired mycelial growth, and decreased pathogen viability. Proposed mechanisms of action include disruption of fungal cell membranes and interference with key metabolic processes. The authors emphasize the potential application of essential oils as natural fungicides in both pre- and post-harvest contexts, as their use could significantly reduce reliance on chemical fungicides, enhance crop protection, and contribute to environmentally sustainable plant disease management strategies.

2.3. Group C: Biomedical and Pharmacological Applications

In the review study carried out by Tanasă et al. [1], the authors highlighted the potential of EOs as natural, eco-friendly alternatives to synthetic biocides. They reported that EOs have significant antimicrobial, antibiofilm, and anti-quorum sensing activities. The authors focus on the role of EOs in preventing biofilms, which are responsible for the majority of microbial infections and the development of pathogen resistance. The study also addressed the mechanisms of action, such as disrupting cell membranes and regulating virulence factors, as well as the synergistic effect with antibiotics and the limitations of stability and selectivity.

For the first time, Vaglica et al. [13] investigated the chemical composition and cytotoxic properties of the EO from *Bifora testiculata*. GC-MS analysis revealed that it is rich in aliphatic aldehydes, with trans-2-dodecenal as the major component. When tested on human cancer cell lines (breast, melanoma, and colon), both the EO and trans-2-dodecenal exhibited remarkable cytotoxic activity, inducing apoptosis, with IC₅₀ values ranging between 7.93 and 14.41 µg/mL for the EO and between 1.88 and 5.29 µg/mL for trans-2-dodecenal. These results indicated that the single compound was more potent than the crude EO. The use of the antioxidant N-acetyl-L-cysteine reversed this effect, indicating that the mechanism involves the generation of reactive oxygen species (ROS). The authors conclude that the EO's activity is primarily due to its main aldehyde component, suggesting potential for further development as an anti-cancer agent.

3. Conclusions

In conclusion, the studies in this Special Issue highlight the chemical diversity of EOs from families such as Hypericaceae, Asteraceae, Rutaceae, Lamiaceae, and Apiaceae, demonstrating their strong biological activities, including antifungal, agricultural, and biomedical applications. Overall, this collection emphasizes the potential of EOs as versatile natural resources for sustainable agriculture and novel therapeutic development. The systematic screening approaches used also provide a reliable framework for identifying promising EO candidates for targeted applications in agriculture, the food industry, and medicine.

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Article

Chemical Composition, Enantioselective Profile, and Preliminary Screening of Biological Activities of the Essential Oil from Aerial Parts from *Lasiocephalus ovatus* Schltdl.

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Abstract

Traditionally, *Lasiocephalus ovatus* Schltdl. (Asteraceae) has been used as an aromatic medicinal plant, particularly in the treatment of kidney-related ailments. However, scientific evidence validating its chemical composition and bioactivity remains limited. According to our literature search, there are no previous studies on the in vitro antibacterial, antioxidant, or anti-inflammatory activities of the essential oil from the aerial parts of *Lasiocephalus ovatus*; therefore, this study provides the first experimental evidence of these biological activities for this species. An essential oil (EO) was steam-distilled from the aerial parts of *L. ovatus*, grown at 4410 m above sea level in the paramos of Chimborazo Province (Ecuador), and subsequently analyzed. The distillation yield was 0.21% (*w/w*) based on dry plant material. Gas chromatography was employed for qualitative (GC-MS) and quantitative (GC-FID) analyses, using two different capillary columns, coated with 5% phenyl methyl polysiloxane (non-polar) and polyethylene glycol (polar) stationary phases. Dual stationary phases were required to provide complementary selectivity, which reinforced the identification and quantification of compounds. The major components of the EO were silphinene (3.4–3.5%), δ -selinene (3.6–3.1%), β -cyclogermacrene (18.7–18.1%), kessane (4.5–4.2%), spathulenol (13.3–13.3%), viridiflorol (3.1–3.0%) and neophytadiene (4.8–4.4%), values referred to the non-polar and polar phase respectively. The enantioselective analysis revealed that (1S,5S)-(–)- α -pinene, (1S,5S)-(+)- β -pinene and (R)-(–)- α -phellandrene were enantiomerically pure, whereas germacrene D was present as a scalemic mixture. The essential oil of *L. ovatus* exhibited a minimum inhibitory concentration (MIC) of 250 μ g/mL against *Staphylococcus aureus* and 500 μ g/mL against *Escherichia coli*. Its antibacterial activity is likely associated with the presence of bioactive sesquiterpenes such as silphinene, δ -selinene, and spathulenol, which are known for their membrane-disruptive properties. Regarding its antioxidant potential, the observed moderate radical scavenging activity (SC_{50} = of 375.7 μ g/mL) can be attributed to its complex mixture, particularly to oxygenated terpenoids like viridiflorol and spathulenol, which are recognized for their radical-neutralizing capacity. In the anti-inflammatory assay, the EO's moderate potency (IC_{50} = 165.29 $\pm$ 4.75 μ g/mL) is also consistent with the anti-inflammatory profile reported

for several of its major constituents, including spathulenol and viridiflorol. While significantly lower than that of aspirin ($28.85 \pm 7.66 \mu\text{g/mL}$), this bioactivity is considerable within the context of a plant extract. Overall, the antibacterial, antioxidant, and anti-inflammatory effects are consistent with the EO's terpene-rich composition, particularly oxygenated sesquiterpenes, while the enantiomeric distribution of chiral monoterpenes may further modulate bioactivity; consequently, future studies should include enantioselective quantification, broader antioxidant assays (e.g., ABTS, FRAP, ORAC, CUPRAC), cytotoxicity at active concentrations, and mechanistic and in vivo validation.

Keywords: *Lasiocephalus ovatus*; Asteraceae; essential oil; enantioselective analysis; anti-inflammatory activity; antimicrobial activity; radical scavenging activity; mass spectrometry

1. Introduction

Ecuador flora is one of the most diverse among countries worldwide, due to the fact that its territory intersects the tropical Andes and the Tumbes-Chocó-Magdalena corridor, which are considered global biodiversity hotspots [1]. Approximately 26% of the total flora is considered endemic [2], where a 17,748 species have been confirmed to be native [3]. This condition has attracted the attention of several research groups, as most of these species remain chemically unexplored [4]. Furthermore, plants and natural products often exhibit important biological activities and have been used for centuries as vegetal drugs or pharmaceutical active principles. Knowledge of these uses is frequently transmitted from generation to generation [5].

In recent years, the study of secondary metabolites has increased in line with the use of traditional, complementary, and integrative medicine worldwide. The World Health Organization reports that most of the world population (80%) uses some form of traditional, complementary, and integrative medicine (T&CM) [6], including essential oils, as an alternative therapeutic approach [7–9]. For this reason and considering that Ecuador is a “megadiverse” country [10], the authors have been studying natural products for many years, with a special focus on essential oils [11–13].

The European Pharmacopoeia (Ph. Eur.) defines essential oils (EOs) as oily products, obtained by distillation techniques from plant material or by mechanical techniques from the pericarp of *Citrus* sp. fruits [14]. The EOs are produced and accumulated in specific areas of plants, such as oil cells, glandular trichomes or secretory ducts [15]. Plants synthesize EOs as a protection mechanism against pests and insects [16]. Their chemical composition is complex and diverse. Biosynthetically, EO constituents mainly fall into three categories: terpenoids, phenylpropanoids, and acetate derivatives. These mixtures consist of esters, aldehydes, alcohols, phenols, oxides, ketones and acids [11,12,17]. This chemical diversity supports a broad range of biological effects. It can contribute to antimicrobial activity through membrane disruption, to antioxidant activity through radical scavenging and modulation of oxidative stress, and to anti-inflammatory effects through inhibition of pro-inflammatory enzymes and signaling pathways [18–20].

The chemical composition of EOs depends on several factors, including the geographical location of the species, time of collection, and cultivation practices, among others [21,22]. Therefore, the description of new EOs represents a major objective of the research group's work, with the aim of contributing to the preservation of the Ecuadorian biodiversity through the development of new bioeconomically relevant products. Among the most promising families, Asteraceae must be mentioned, where unprecedented EOs can easily be discovered. This family is generally found in arid and semi-arid lands of tropical areas,

widely distributed all around the world [23]. The EOs obtained from Asteraceae are often characterized by promising biological activities, including antioxidant, antimicrobial, anti-inflammatory, hepato-protective, and antidiabetic effects [24–28]. According to the Catalogue of Vascular Plants of Ecuador, this family has 863 species, of which 370 are considered endemic, and widely distributed in the four climatic regions of Ecuador [29].

Many studies have reported the presence of 23 species of the genus *Lasiocephalus* distributed in the highlands of montane forests and paramos of Perú, Colombia, Venezuela, Bolivia, and Ecuador [30]. In particular, certain species of the genus *Aetheolaena* also contribute to this classification, because they have been reclassified within the genus *Lasiocephalus* for presenting key morphological similarities, such as floral structure and leaf arrangement [31–33]. Furthermore, the catalog of Vascular Plants of Ecuador shows species of the genus *Aetheolaena* as synonyms of the genus *Lasiocephalus* [29]. This genus is characterized by the fact that the species can grow in two forms: as shrubs with small, elongated leaves and as vines with broad leaves [32]. For the present study, the aromatic species *Lasiocephalus ovatus* Schltdl. (Asteraceae) has been selected, whose volatile and non-volatile fractions have only been partially studied or described in outdated reports [34–36]. In Ecuador, *L. ovatus* is traditionally known as “Arquitectura” or “Arquitecto”, to which medicinal properties are attributed in the relief of kidney ailments or to disinfect liver and gallbladder [37,38]. It is considered a native Andean species, growing at elevations between 2500 and 4500 m above sea level, and distributed in the provinces of Cañar, Chimborazo, Tungurahua, Cotopaxi, and Pichincha [29]. According to the Missouri Botanical Garden (Table 1), the listed names are recognized as synonyms of *L. ovatus*.

Table 1. Synonymy of *L. ovatus* according to the Missouri Botanical Garden (Tropicos) [39].

Name	Status
<i>Lasiocephalus ovatus</i> Schltdl.	Accepted name
<i>Culcitium ovatum</i> (Schltdl.) S.F. Blake	Synonym
<i>Culcitium reflexum</i> Kunth	Synonym
<i>Culcitium sodiroi</i> Hieron	Synonym
<i>Culcitium uniflorum</i> Hieron	Synonym
<i>Gnaphalium uniflorum</i> Lam	Synonym
<i>Lasiocephalus sodiroi</i> (Hieron.) Cuatrec	Synonym
<i>Senecio reflexus</i> (Kunth) Cuatrec	Synonym
<i>Senecio superandinus</i> Cuatrec	Synonym

A review of the literature indicates that research on the genus *Lasiocephalus* has mainly focused on taxonomic descriptions, revealing an important knowledge gap regarding its secondary metabolites. Likewise, studies on its essential oils remain scarce, being limited to qualitative descriptions of their composition (GC-MS) with occasional antibacterial evaluations [40,41], often without chromatographic profiling using stationary phases of different polarity, quantitative GC-FID data, enantioselective analyses, or broader biological analyses, particularly for *L. ovatus*.

To the best of our knowledge, there are no previous studies reporting the in vitro antibacterial, antioxidant, or anti-inflammatory activities of the essential oil obtained from the aerial parts of *Lasiocephalus ovatus*. Therefore, the present work addresses this scientific gap by providing a comprehensive chemical and enantioselective characterization and by experimentally evaluating (and thereby validating) these biological activities.

2. Results

2.1. Chemical Composition of the EO

The aerial parts of *L. ovatus* were subjected to steam distillation, yielding an essential oil (EO) with a yield of 0.21% (*w/w*) based on dry plant material. A total of seventy-one compounds were identified and quantified by GC-MS and GC-FID analyses using two capillary columns of different polarity. These compounds accounted for 98.0% and 91.6% of the total EO composition on the nonpolar and polar columns, respectively. The main compounds (>3% on both columns) identified in the volatile fraction were silphinene (19) (3.4–3.5%), δ -selinene (45) (3.6–3.1%), β -cyclogermacrene (46) (18.7–18.1%), kessane (51) (4.5–4.2%), spathulenol (55) (13.3–13.3%), viridiflorol (57) (3.1–3.0%) and neophytadiene (65) (4.8–4.4%).

The composition of the essential oil of *L. ovatus* was dominated by the presence of sesquiterpenes hydrocarbons (45.5–42.6%), followed by oxygenated sesquiterpenoids (32.0–31.2%). Oxygenated monoterpenes (0.9–0.5%) were present in the lowest proportion on both columns. The chromatograms for the non-polar and the polar phase are shown in Figures 1 and 2, respectively. Figure 3 shows the chemical structures of the EO main constituents.

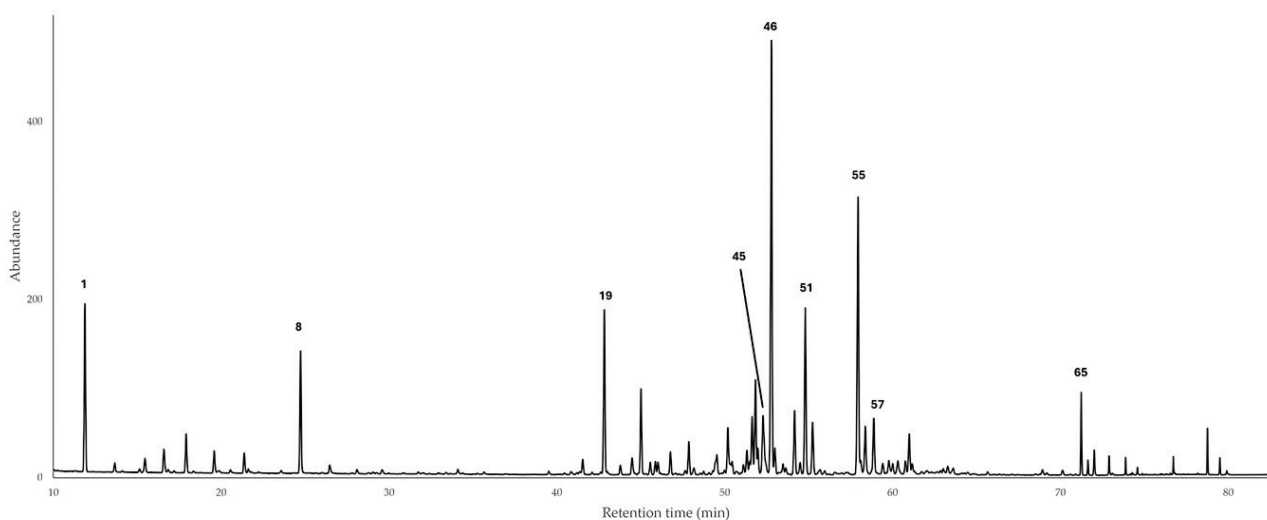


Figure 1. GC–MS chromatogram of *L. ovatus* EO in a 5% phenyl-methylpolysiloxane-based column (non-polar). The main components are presented in Table 2, according to the number corresponding to each peak.

Table 2. Chemical analysis of *L. ovatus* EO on two stationary phases of different polarity (5%-Phenyl methyl polysiloxane and Polyethylene glycol).

N.	Compounds	5% Phenyl Methyl Polysiloxane					Ref.	Polyethylene Glycol					Ref.
		LRI ^a	LRI ^b	%	σ	RSD (%) ^c		LRI ^a	LRI ^b	%	σ	RSD (%) ^c	
1	α -pinene	932	932	2.9	0.32	0.08	[42]	1019	1017	2.7	0.04	0.17	[43]
2	β -pinene	977	974	0.3	0.06	0.06	[42]	1106	1108	0.2	0.01	0.11	[44]
3	<i>trans</i> -3-decene	992	993	0.5	0.10	0.12	[42]	1141	-	0.3	0.02	0.15	[45]
4	α -phellandrene	1008	1002	0.8	0.09	0.11	[42]	1161	1160	0.7	0.01	0.23	[46]
5	<i>p</i> -cymene	1029	1020	0.5	0.03	0.05	[42]	1269	1270	0.4	0.01	0.05	[47]
6	<i>cis</i> - β -ocimene	1041	1032	0.1	0.01	0.05	[42]	1239	1240	0.1	0.01	0.06	[48]
7	<i>trans</i> - β -ocimene	1051	1044	0.5	0.03	0.05	[42]	1255	1250	0.4	0.01	0.05	[49]
8	1-undecene	1092	1090	2.4	0.31	0.08	[48]	1141	1139	2.2	0.17	0.09	[50]
9	Undecane	1106	1100	0.3	0.01	0.04	[42]	1403	-	Trace	-	0.10	[51]
10	<i>trans</i> -limonene oxide	1136	1137	0.1	0.01	0.00	[42]	1488	1482	Trace	-	0.09	[52]
11	7-methyl-3-methylenecyclohex-2-enal	1144	1140	0.1	0.01	0.03	[42]	1101	-	Trace	-	0.11	[53]
12	(<i>Z</i>)-isocitral	1155	1160	0.2	0.05	0.07	[42]	1594	-	0.1	0.01	0.05	[54]
13	<i>p</i> -menth-8-en-2-one	1186	1191	0.1	0.02	0.03	[42]	1195	-	Trace	-	0.13	[55]
14	Myrtenol	1205	1212	0.1	0.03	0.03	[42]	1792	1792	Trace	-	0.02	[56]
15	1-tridecene	1292	1290	0.2	0.01	0.05	[42]	1344	1343	0.1	0.01	0.04	[57]
16	6-hydroxycarvotanacetone	1305	1309	0.3	0.02	0.07	[42]	1410	-	0.2	0.01	0.03	[58]

Table 2. Cont.

N.	Compounds	5% Phenyl Methyl Polysiloxane					Ref.	Polyethylene Glycol					Ref.
		LRI ^a	LRI ^b	%	σ	RSD (%) ^c		LRI ^a	LRI ^b	%	σ	RSD (%) ^c	
17	undec-9-en-1-al	1316	1322	0.1	0.01	0.05	[42]	1818	-	0.3	0.01	0.06	§
18	silphiperfol-5-ene	1322	1326	0.5	0.04	0.02	[42]	1413	1403	0.3	0.02	0.03	[53]
19	Silphinene	1341	1344	3.4	0.35	0.05	[42]	1457	-	3.5	0.07	0.03	§
20	7- <i>epi</i> -silphiperfol-5-ene	1344	1345	0.1	0.01	0.05	[42]	1450	-	0.1	-	0.03	§
21	silphiperfol-4,7(14)-diene	1356	1358	0.4	0.05	0.05	[42]	1846	-	0.3	0.02	0.04	§
22	Cyclosativene	1366	1369	0.8	0.16	0.04	[42]	1465	-	0.5	0.04	0.03	§
23	Ylangene	1374	1373	2.2	0.34	0.04	[42]	1478	1483	2.1	0.14	0.03	[54]
24	unidentified (MW = 204)	1383	-	0.6	0.04	0.04		1491	-	0.6	0.02	0.03	-
25	β -cubebene	1387	1387	0.5	0.04	0.04	[42]	1530	1532	0.3	0.01	0.03	[55]
26	β -elemene	1390	1389	0.4	0.03	0.07	[42]	1560	1560	0.2	0.01	0.03	[56]
27	α -gurgujene	1401	1409	0.9	0.35	0.04	[42]	1509	1511	0.7	0.02	0.03	[57]
28	α -cedrene	1415	1410	0.2	0.01	0.04	[42]	1560	1566	Trace	-	0.03	[58]
29	(<i>E</i>)- β -caryophyllene	1419	1417	1.1	0.15	0.04	[42]	1584	1585	1.0	0.03	0.02	[59]
30	2,5-dimethoxy- <i>p</i> -cymene	1423	1424	0.1	0.01	0.06	[42]	1878	1876	0.1	0.02	0.02	[60]
31	β -copaene	1430	1430	0.1	0.01	0.06	[42]	1529	-	0.3	0.01	0.03	§
32	α -guaiene	1438	1437	0.1	0.01	0.06	[42]	1652	1659	Trace	-	0.02	[55]
33	<i>cis</i> - β -farnesene	1440	1439	0.2	0.01	0.04	[42]	1639	1639	0.1	0.03	0.02	[61]
34	myltayl-4(12)-ene	1444	1445	0.7	0.11	0.04	[42]	1605	-	0.7	0.02	0.02	§
35	amorpha-4,11-diene	1445	1449	0.8	0.13	0.04	[42]	1577	-	1.0	0.03	0.07	§
36	unidentified (MW = 218)	1453	-	0.3	0.03	0.06		1602	-	0.1	0.01	0.02	-
37	Humulene	1456	1452	2.4	0.13	0.04	[42]	1655	1656	2.1	0.06	0.07	[62]
38	Alloaromadendrene	1458	1458	0.3	0.03	0.06	[42]	1631	1631	0.4	0.02	0.02	[63]
39	9- <i>epi</i> - β -caryophyllene	1460	1464	0.7	0.10	0.04	[42]	1630	1631	0.5	0.02	0.05	[64]
40	4,5-di- <i>epi</i> -aristolochene	1471	1471	0.2	0.04	0.04	[42]	1668	1665	0.3	0.02	0.02	[65]
41	β -chamigrene	1474	1476	0.6	0.13	0.04	[42]	1709	1702	0.9	0.04	0.02	[66]
42	γ -muurolene	1477	1478	0.4	0.02	0.04	[42]	1679	1681	0.5	0.02	0.02	[67]
43	γ -curcumene	1479	1481	1.0	0.13	0.06	[42]	1686	1683	0.7	0.10	0.04	[68]
44	germacrene D	1482	1480	2.8	0.19	0.04	[42]	1696	1694	3.1	0.13	0.06	[62]
45	δ -selinene	1490	1492	3.6	0.44	0.04	[42]	1699	1707	3.1	0.13	0.02	[60]
46	β -cyclogermacrene	1498	1500	18.7	0.48	0.06	[42]	1721	1720	18.1	0.47	0.06	[62]
47	α -muurolene	1501	1500	0.6	0.03	0.04	[42]	1716	1716	0.8	0.05	0.02	[69]
48	germacrene A	1509	1508	0.3	0.02	0.04	[42]	1705	1700	0.7	0.04	0.02	[70]
49	δ -cadinene	1521	1522	0.7	0.16	0.04	[42]	1679	-	0.5	0.02	0.02	§
50	<i>cis</i> -calamenene	1527	1528	0.3	0.06	0.04	[42]	1825	-	0.2	0.01	0.02	§
51	Kessane	1532	1529	4.5	0.29	0.04	[42]	1769	1779	4.2	0.53	0.02	[71]
52	Isokessane	1539	1530	2.0	0.29	0.05	[72]	1769	1777	2.1	0.05	0.04	[73]
53	α -calacorene	1547	1544	0.4	0.02	0.09	[42]	1907	-	0.2	0.01	0.02	§
54	β -vetivenene	1551	1554	0.4	0.02	0.04	[42]	1847	1836	0.3	0.02	0.04	[74]
55	spathulenol	1585	1577	13.3	0.49	0.02	[42]	2129	2132	13.3	0.15	0.01	[46]
56	Hedycaryl	1592	1589	2.3	0.11	0.05	[42]	2072	2077	2.0	0.08	0.01	[75]
57	Viridiflorol	1601	1592	3.1	0.14	0.05	[42]	2081	2084	3.0	0.42	0.03	[47]
58	<i>cis</i> -isolongifolanone	1611	1612	0.6	0.12	0.03	[42]	2022	-	0.4	0.03	0.02	§
59	humulene II epoxide	1617	1624	0.7	0.05	0.05	[42]	2025	2025	0.5	0.05	0.02	[76]
60	Eremoligenol	1621	1629	0.7	0.13	0.05	[42]	2178	2182	0.8	0.07	0.03	[77]
61	unidentified (MW = 218)	1627	-	0.7	0.06	0.05		2091	-	0.6	0.05	0.04	-
62	<i>epi</i> - α -muurol	1635	1640	0.9	0.14	0.05	[42]	2150	2151	0.8	0.07	0.01	[44]
63	Agarospirol	1639	1646	2.0	0.10	0.03	[42]	2183	-	2.1	0.17	0.01	§
64	Khusinol	1675	1679	0.1	0.02	0.03	[42]	2251	-	0.1	0.01	0.01	§
65	Neophytadiene	1844	1843	4.8	0.37	0.03	[42]	1927	1922	4.4	0.21	0.02	[78]
66	hexahydrofarnesyl acetone	1856	1856	0.6	0.04	0.03	[42]	2132	2131	0.3	0.01	0.01	[79]
67	unidentified (MW = 212)	1866	-	1.8	0.13	0.03		2559	-	1.4	0.12	0.01	-
68	oplopanonyl acetate	1892	1895	0.6	0.05	0.01	[42]	2770	-	0.5	0.04	0.01	§
69	Cyclohexadecanolide	1937	1940	0.3	0.02	0.01	[42]	2239	-	0.3	0.01	0.01	§
70	unidentified (MW = 231)	2234	-	1.8	0.14	0.01		2236	-	1.9	0.05	0.01	-
71	unidentified (MW = 214)	2274	-	0.9	0.07	0.01		3208	-	0.9	0.07	0.01	-
	monoterpene hydrocarbons			5.6						4.8			
	oxygenated monoterpenes			0.9						0.5			
	sesquiterpene hydrocarbons			45.5						42.6			
	oxygenated sesquiterpenes			32.0						31.2			
	diterpene			4.8						4.4			
	Others			9.2						8.1			
	Total			98.0						91.6			

^a Calculated linear retention index; ^b Linear retention index according to Ref.; Trace < 0.1%; MW = molecular weight; § = identification based on MS only. ^c RSD = Relative standard deviation of retention times.

2.2. Enantioselective Analysis

An enantioselective GC-MS analysis was carried out on the essential oil of *L. ovatus* (Table 2). The biological relevance of stereochemistry lies in the fact that many microbial targets and inflammatory pathways involve chiral recognition. Enzymes, membrane-associated proteins, and other chiral binding environments can distinguish between mirror-image molecules. As a result, enantiomers may differ markedly in binding affinity and in the downstream cellular effects they trigger [80].

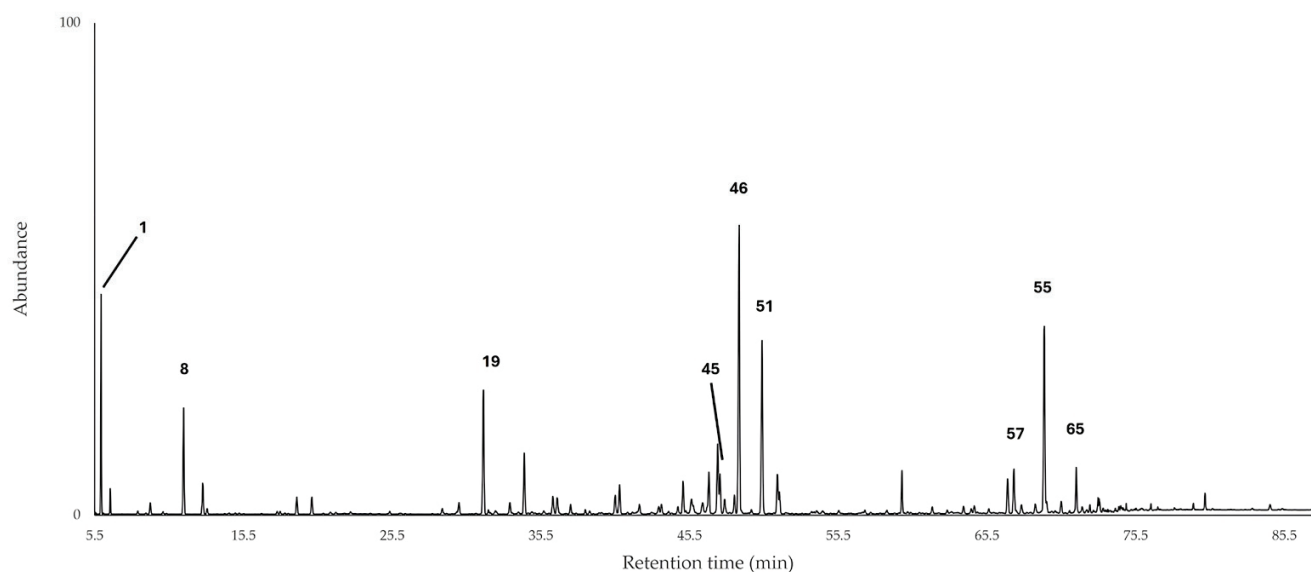


Figure 2. GC-MS chromatogram of *L. ovatus* EO in a polyethylene glycol-based column (polar). The main components are presented in Table 2, according to the number corresponding to each peak.

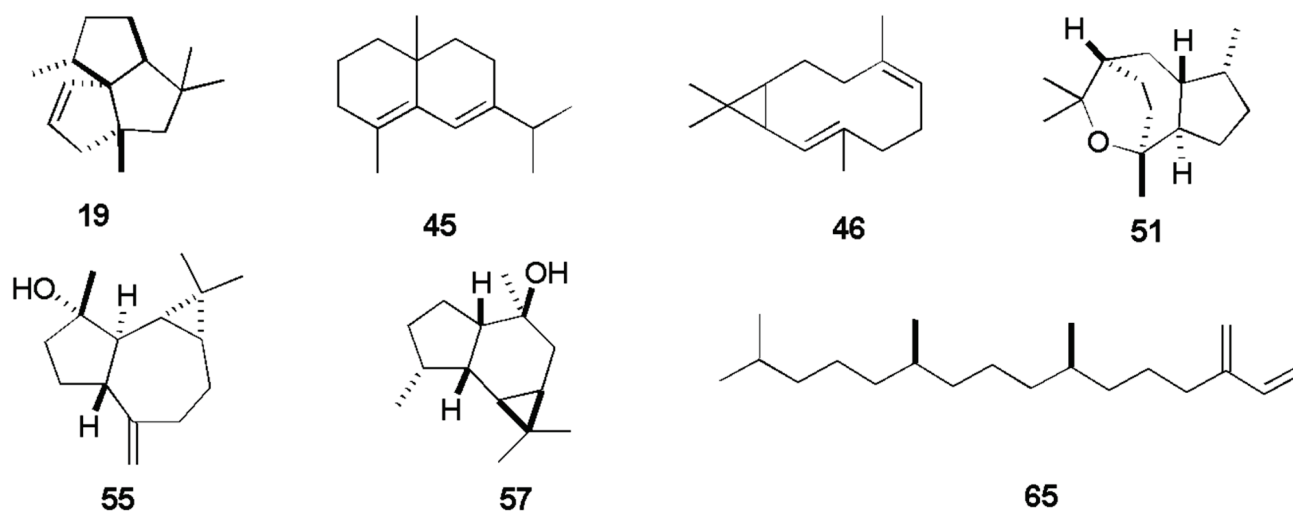


Figure 3. Major constituents (>3% on both columns) of *L. ovatus* EO according to Table 2: silphinene (19), δ -selinene (45), β -cyclogermacrene (46), kessane (51), spathulenol (55), viridiflorol (57) and neophytadiene (65).

Three compounds, (1*S*,5*S*)-(–)- α -pinene, (1*S*,5*S*)-(+)- β -pinene and (*R*)-(–)- α -phellandrene, were enantiomerically pure, whereas germacrene D was present as both the enantiomeric forms in a scalemic mixture. The enantiomeric purity of α -pinene was determined on a 2,3-diacetyl-6-*tert*-butyldimethylsilyl- β -cyclodextrin-based column, because its enantiomers are inseparable on the other chiral selector. For all the others chiral compounds, a 2,3-diethyl-6-*tert*-butyldimethylsilyl- β -cyclodextrin-based column was used, carrying out a better separation. The enantiomeric peak corresponding to germacrene D enantiomers showed good separation, with a resolution of $R_s = 1.43$. The complete results are detailed in Table 3.

Table 3. Enantiomeric analysis of the components of *L. ovatus* EO.

Chiral Selector	Enantiomer	LRI ^a	LRI ^b	Enantiomeric Distribution %	e.e. %	Rs ^c
DAC	(1 <i>S</i> ,5 <i>S</i>)-(–)- α -pinene	915	915	100.0	100.0	-
DAC	(1 <i>S</i> ,5 <i>S</i>)-(+)- α -pinene	917	918	-		
DET	(1 <i>S</i> ,5 <i>S</i>)-(–)- β -pinene	950	950	-	100.0	-
DET	(1 <i>S</i> ,5 <i>S</i>)-(+)- β -pinene	965	965	100.0		
DET	(<i>R</i>)-(–)- α -phellandrene	1020	1021	100.0	100.0	-
DET	(<i>S</i>)-(+)- α -phellandrene	1023	1025	-		
DET	(<i>R</i>)-(+)-germacrene D	1460	1460	32.9	34.3	1.43
DET	(<i>S</i>)-(–)-germacrene D	1466	1467	67.1		

DAC = 2,3-diacetyl-6-*tert*-butyldimethylsilyl- β -cyclodextrin; DET = 2,3-diethyl-6-*tert*-butyldimethylsilyl- β -cyclodextrin; LRI = linear retention index; ^a values from pure standards [81]; ^b calculated linear retention index; e.e. = enantiomeric excess; ^c Resolution between enantiomeric peaks was calculated as $R_s = 2(t_{R2} - t_{R1}) / (w_1 + w_2)$, where t_R is the retention time and w is the peak width at baseline.

2.3. Antimicrobial Activity

The antibacterial activity of the EO from the aerial parts from *L. ovatus* was evaluated using the broth microdilution method. Table 4 shows the tested microorganisms and minimum inhibitory concentration (MIC) values of both the EO and positive control. The values of the negative control are also shown. Ampicillin was used as a positive control for *Staphylococcus aureus*, and ciprofloxacin was used as a positive control for *Escherichia coli*. The *L. ovatus* EO reported MIC values of 250 μ g/mL against *S. aureus* and 500 μ g/mL against *E. coli*. This preliminary screening, employing two well-characterized reference strains (*S. aureus* ATCC 25923 and *E. coli* ATCC 25922) as standard Gram-positive and Gram-negative models, provides an initial assessment of the EO's antibacterial potential and establishes a benchmark for its spectrum of activity.

Table 4. Antibacterial activity of the EO from *L. ovatus*.

Microorganism Strains	Essential Oil	Positive Control	Negative Control OD600
	MIC (μ g/mL)		
<i>Staphylococcus aureus</i> ATCC 25923	250	0.35	2.377
<i>Escherichia coli</i> ATCC 25922	500	1.62	2.512

MIC: Minimum Inhibitory Concentration. Positive controls: ampicillin for *S. aureus*, ciprofloxacin for *E. coli*. Negative control: Sterile growth medium inoculated with the bacterial suspension, showing normal growth (optical density at 600 nm, OD600, >2.0 after 24 h incubation).

2.4. Radical Scavenging Capacity

The free radical scavenging capacity of the EO was assessed using the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). Table 5 shows the scavenging capacity (SC₅₀) of EO and positive control. The maximum evaluated concentration was 1000 μ g/mL. *L. ovatus* EO showed a SC₅₀ of 375.7 μ g/mL.

Table 5. Antioxidant activity of the EO from *L. ovatus*.

Sample	DPPH
	SC ₅₀ (μ g/mL) $\pm$ SD
<i>L. ovatus</i> essential oil	375.7 $\pm$ 2.1
Trolox	33.0 $\pm$ 1.5

SC₅₀: Half-maximal scavenging concentration. Values represent the mean $\pm$ standard deviation (SD) of $n = 3$ independent experiments.

2.5. Anti-Inflammatory Activity

The inhibition of superoxide production by neutrophils (oxidative burst) by *L. ovatus* is summarized in Table 6: Superoxide generation was measured using the water tetrazolium salt (WST-1) assay.

Table 6. Anti-inflammatory effect of the EO from *L. ovatus*.

Concentration (µg/mL)	<i>L. ovatus</i> Essential Oil	Aspirin
3.1	ND	13.26 ± 2.21
6.2	2.77 ± 0.43	24.74 ± 3.63 **
12.5	5.63 ± 1.07	33.28 ± 3.09 **
25.0	19.94 ± 1.76	47.62 ± 5.11 *
50.0	27.88 ± 1.28	58.39 ± 2.44 **
100.0	34.61 ± 1.05	70.84 ± 4.38 **
200.0	49.12 ± 0.81	86.35 ± 5.67 **

The values represent the mean ± SD, n = 3. ND: Not Detected (inhibition below the limit of quantification). Significant values, ** $p < 0.01$, * $p < 0.05$, using Student's *t*-test; *L. ovatus* essential oil versus aspirin.

A comparative analysis of the IC₅₀ values revealed that the anti-inflammatory potency of the EO (165.29 ± 4.75 µg/mL) was significantly inferior to that of the reference drug aspirin (28.85 ± 7.66 µg/mL). Despite the difference in efficacy, the essential oil showed a clear dose–response relationship across concentration of 6.2–200 µg/mL, and its anti-inflammatory activity is reported here for the first time.

3. Discussion

3.1. Chemical Composition and Main Components

The essential oil yield obtained in this study (0.21% *w/w*) is within the average values reported for the Asteraceae family (0.1–2.0%) [17,82,83], which agrees with the values observed in congeneric species [36].

In 1990, De Bernardi et al. studied for the first time the volatile fraction obtained from the stems of *L. ovatus* by hexane extraction and subsequent GC-MS analysis, identifying only sesquiterpene compounds: copaene, alloaromadendrene, humulene, eremophilene, α -curcumene [35]. Although the technique used to obtain the volatile fraction of *L. ovatus* differs from the technique used in our work, the qualitative analysis of the chemical profile shows that the common components are: alloaromadendrene, humulene, β -cubebene, β -elemene, δ -selinene, α -muurolene.

Years later, Araujo et al. obtained the essential oil from the aerial parts of *L. ovatus* collected in Sangay National Park. Chemical analysis identified a total of 27 compounds, with 1,2,5,5-tetramethyl-1,3-cyclopentadiene and camphor as the major constituents, representing 11.90% and 40.48% of the oil, respectively. However, this study was conducted using only a single chromatographic separation column, and the percentage composition was not determined using a quantitative method. In addition, oxygenated monoterpene compounds predominated in percentage over sesquiterpene hydrocarbons [36].

The two studies distinct chemical profiles. In our study, the essential oil was dominated by sesquiterpene hydrocarbons, whereas the only compound common to both studies was a α -pinene, present at 1.9% in the previous report compared with 2.9% in our oil.

Regarding other studies on *Lasiocephalus* species, the essential oil obtained from the aerial parts of *L. longipenicillatus*, collected in Piñago, Mérida State, Venezuela (2300 m above sea level), was reported to contain only 11 compounds. The oil was predominantly composed of α -pinene, α -humulene, and germacrene D, accounting for 48.3%, 15.8% and 15.5% of the total composition, respectively. Monoterpenes were the predominant class of compounds, whereas sesquiterpene hydrocarbons were present in lower proportions [41]. These three major constituents were also detected in our EO; however, they occurred at

much lower levels (2.9%, 2.4%, and 2.8%, respectively). The collection of this species in two different seasons of the year showed that the chemical profile of the essential oil changes evidencing that the sesquiterpene compounds are in higher percentage when *L. longipenicillatus* is collected at the beginning of the year, while the monoterpenes are present in higher percentage when the plant is collected in the middle of the year [40]. A comparison among the major constituents (>3.0% in at least one oil) of *L. ovatus* (Sangay Park), *L. ovatus* (Chimborazo Province) and *L. longipenicillatus* is represented in Figure 4.

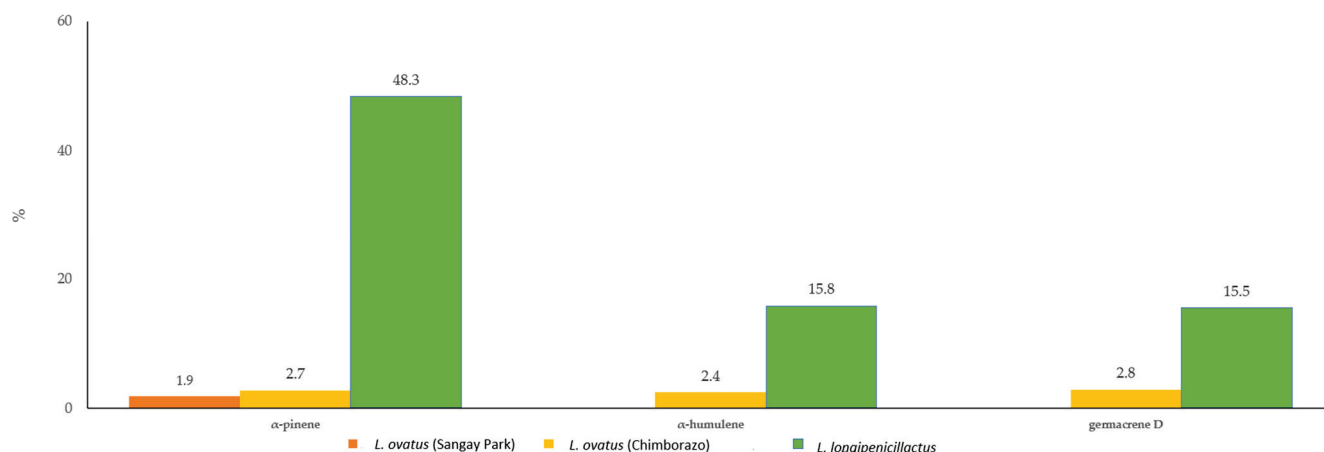


Figure 4. Compared abundance of major compounds (>3.0% in at least one oil) in the EOs of *L. ovatus* (Sangay Park), *L. ovatus* (Chimborazo) and *L. longipenicillatus*.

Although some species of the genus *Senecio* are classified in *Lasiocephalus* Willd. ex Schltldl, and most are endemic to Ecuador; the volatile fraction of this group has not been studied [84].

For the first time, a comprehensive qualitative and quantitative analysis of the essential oil obtained from aerial parts of *L. ovatus* was conducted using GC-MS and GC-FID with different stationary phases (a nonpolar 5% phenylmethylpolysiloxane phase and polar polyethylene glycol phase), ensuring effective separation of all components present in the oil. The resulting chemical profile revealed the presence of compounds distinct from those reported in previous studies. Moreover, the lack of standardized studies on the genus *Lasiocephalus* highlights the importance of these findings, which provide a valuable reference for future investigations of unexplored species within this taxon.

In this study, two major (>1%) peaks could not be reliably identified and were therefore reported as unidentified constituents. The compound at RI 1866 (**67**) showed prominent EI ions at m/z 212 and 197 (base peak), with fragments at m/z 182, 169, 152, 141, 115, and 91, suggesting a low-volatility terpenoid-like molecule, potentially an oxygenated sesquiterpene or a related high-boiling derivative. The second unidentified compound (**71**) at RI 2234 exhibited a spectrum dominated by aliphatic fragments (base peak m/z 83, intense m/z 55, and ions at m/z 172, 185, 199, and 214), which is more consistent with a high-boiling, non-polar long-chain aliphatic or lipid-like structure than with typical mono- or sesquiterpenes. In both cases, library matching was inconclusive, and no authentic standards were available, so no tentative structures were assigned. Future work should confirm their identities using improved chromatographic separation, high-resolution MS to determine elemental composition, and, where feasible, isolation followed by NMR, together with solvent and procedural blanks to rule out exogenous contamination for compound (**71**).

3.2. Chiral GC-MS Analysis for Enantiomeric Distribution

Enantiomeric analysis of essential oils serves as a critical determinant for validating their authenticity, quality and biological properties, as the characteristic presence of specific enantiomers can reveal both botanical origin and potential adulteration [85–87]. Advanced chromatographic techniques, in particular chiral column gas chromatography-mass spectrometry (chiral GC-MS), allow the separation, identification and quantification of enantiomers through diastereomeric interactions with specialized stationary phases such as modified cyclodextrins [13,88,89].

The enantioselective analysis of the EO of *L. ovatus* (Table 3) revealed that (–)- α -pinene, (+)- β -pinene, and (–)- α -phellandrene were present as enantiomerically pure compounds. This result is consistent with their biosynthetic origin since both terpenes α -pinene and β -pinene share a common chiral precursor: the pinyl cation. It is known that the configuration of their stereogenic centers is inherited from this precursor, which explains the high stereospecificity observed in their synthesis [81]. On the other hand, germacrene D showed a scalemic mixture, with a slight enantiomeric excess of 34.3% in favor of the (S)- (–)-germacrene D enantiomer. In particular, this unsaturated sesquiterpene could undergo protonation under aqueous and potentially acidic conditions (e.g., due to organic acids present in the plant material) at the high temperatures of the process [90].

3.3. Antimicrobial Activity

The need to identify novel antimicrobial agents is highlighted by the global rise in antimicrobial resistance. Natural products, especially EOs, are considered valuable resources because they often exhibit mechanisms of action that differ from conventional antibiotics and may help combat resistant strains [91]. By providing preliminary evidence on the antibacterial activity of the EO extracted from the aerial parts of *L. ovatus*, our findings contribute to this field.

Data from the broth microdilution assay demonstrated measurable antibacterial activity for *L. ovatus* EO. As shown in Table 4, the oil inhibited the Gram-positive reference strain *Staphylococcus aureus* ATCC 25923 at 250 $\mu\text{g/mL}$, whereas a higher MIC (500 $\mu\text{g/mL}$) was required to inhibit the Gram-negative bacterium *Escherichia coli* ATCC 25922. As expected, the MICs of the reference antibiotics (ampicillin and ciprofloxacin) were considerably lower than those observed by *L. ovatus* EO. Nevertheless, the relevance of EOs lies in their chemical complexity. This complexity provides a starting point for future work to identify the constituents driving the observed activity and to test, in a targeted way, whether synergistic or antagonistic interactions occur among components. In this study, synergy is only hypothesized and should be confirmed in future assays.

The difference in susceptibility between the two tested microorganisms is consistent with the well-documented trend that Gram-negative bacteria are generally less susceptible to EO's. The outer membrane of Gram-negative organisms, rich in lipopolysaccharides (LPS), acts as a hydrophilic barrier, limiting the diffusion of the hydrophobic constituents typical of EOs [92]. In contrast, the more accessible peptidoglycan layer in the cell wall of Gram-positive bacteria like *S. aureus* allows for easier penetration of these bioactive compounds, leading to membrane disruption and cell lysis [93].

The MIC values presented here are in line with those of numerous other EOs that have been identified as having antimicrobial potential, locating *L. ovatus* in a category that shows promise for additional research [94]. Rather than serving as a direct replacement for conventional drugs, the practical application of this EO may be found in synergy. A compelling area of future research would be to investigate whether *L. ovatus* oil can act synergistically with traditional antibiotics, potentially lowering required doses and mitigating resistance development [95]. Furthermore, because of its antimicrobial profile, it

can be used in situations where pure antibiotics are not needed, like in natural disinfectants, food packaging materials, or natural cosmetic preservatives, where its moderate activity is adequate and frequently preferred [96].

The biological activity of an essential oil is largely determined by chemical composition. The profile of *L. ovatus* EO, detailed in a preceding section, is dominated by compounds such as silphinene, δ -selinene, β -cyclogermacrene, kessane, spathulenol, viridiflorol and neophytadiene, which have been reported to exhibit antibacterial properties in the scientific literature [97]. It is most probable that the overall effect is not due to a single molecule but develops from the complex synergistic or additive interactions between its major and minor chemical constituents. This multifaceted nature is a hallmark of essential oils and is thought to be a key reason behind the low incidence of resistance reported against them [98].

3.4. Radical Scavenging Capacity

The search for natural antioxidants has increased, driven by their critical role in mitigating oxidative stress—a key contributor to chronic diseases and food spoilage. While synthetic antioxidants like BHT and BHA are effective, their use is increasingly analyzed due to potential health concerns, creating a significant market for natural alternatives. Within this context, our study provides a first evaluation of the radical scavenging potential of the EO derived from *L. ovatus*.

The results from the DPPH assay, a typical method for assessing antioxidant activity, reveal that *L. ovatus* EO possesses a measurable ability to neutralize free radicals. The oil achieved a 50% scavenging capacity (SC_{50}) at a concentration of 375.7 $\mu\text{g/mL}$. The antioxidant Trolox, a water-soluble analog of vitamin E, exhibited a much lower SC_{50} of 33 $\mu\text{g/mL}$, confirming its superior efficacy.

Although the oil's activity is modest compared to the pure standard, the significance of an SC_{50} in the hundreds of $\mu\text{g/mL}$ range becomes clear when contextualized within the extensive literature on EOs. Numerous EOs derived from well-known aromatic plants have SC_{50} values that fall into the same or even higher range (e.g., 300–1000 $\mu\text{g/mL}$) [99]. Therefore, the activity demonstrated by *L. ovatus* EO is comparable to that of numerous plant-derived EOs, aligning it as a moderately active natural antioxidant.

In the DPPH assay, radical-scavenging is largely driven by hydrogen atom transfer and or single-electron transfer processes. Compounds bearing oxygenated functionalities often contribute more strongly than terpene hydrocarbons because they can more readily participate in these reactions. In this context, the oxygenated sesquiterpenes detected in our EO, particularly spathulenol and viridiflorol, provide a plausible chemical basis for the observed DPPH activity. Spathulenol has been experimentally evaluated as an isolated constituent and showed relevant radical-scavenging capacity in DPPH, and other oxidative systems (do Nascimento et al., 2018) [100]. Likewise, viridiflorol, reported as a major constituent in *Allophylus edulis* essential oil, displayed measurable antioxidant activity in both DPPH and ABTS assays (Trevizan et al., 2016) [101].

This finding has a variety of practical ramifications that go beyond its numerical value. The oil's moderate activity is highly relevant for certain industries, even though it might not be appropriate for applications requiring strong antioxidant power at very low concentrations. For instance, in the food industry, this oil could be explored as a natural preservative in products like meats or oils, where its combined antioxidant and previously demonstrated antibacterial properties could work in tandem to extend shelf life [102]. In a similar vein, such an oil could be used in cosmetic formulations not only for its fragrance but also to prevent rancidity and possibly provide mild skin protection [103].

3.5. Anti-Inflammatory Activity

The oxidative burst, the rapid release of reactive oxygen species (ROS) from activated neutrophils, serves as a critical driver in the pathogenesis of numerous chronic inflammatory conditions [104]. Consequently, therapeutic strategies aimed at tempering excessive neutrophil hold promise. Although conventional non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin, provide clinical efficacy, their prolonged administration is linked to considerable adverse effects [105]. This has increased interest in safer, naturally derived agents. In this context, and to the best of our knowledge, we report the first investigation the ability of *L. ovatus* EO to inhibit the neutrophil oxidative burst, assessed by superoxide anion production, in isolated human neutrophils.

The results show that *L. ovatus* EO has a dose-dependent inhibitory effect on superoxide anion production. A measurable suppression was observed starting at 6.2 µg/mL, with inhibition increasing to nearly 50% at the highest tested concentration (200 µg/mL). The calculated IC₅₀ value for the essential oil was 165.29 µg/mL. As expected, the reference drug aspirin exhibited a clearly lower IC₅₀ of 28.85 µg/mL, consistent with its established anti-inflammatory activity.

Although aspirin was more potent, the biological relevance of the EO lies in its consistent, dose-dependent suppression of a key ROS-mediated inflammatory process by a complex natural mixture. This activity is not isolated; the potency of *L. ovatus* EO is comparable to that reported for other medicinal plant Eos in similar neutrophil-based models [106]. Thus, its effect falls within a recognized spectrum of natural anti-inflammatory candidates.

The underlying mechanism likely differs from that of aspirin. Whereas NSAIDs as aspirin directly targets COX enzymes, the anti-inflammatory effects of essential oils are often multifactorial and may involve interference with signaling pathways that activate neutrophils (e.g., NF-κB or MAPK pathways), or direct scavenging of already-formed ROS [107]. The EOs composition, rich in terpenoid constituents such as δ-selinene, β-cyclogermacrene, kessane, spathulenol, viridiflorol and neophytadiene, offers a plausible chemical basis, as several of these compound classes have been associated with an anti-inflammatory activity [108]. The observed effect may reflect of synergistic interactions among various constituents, a commonly reported feature of complex natural extracts [109].

The practical implications of this moderate activity should not be underestimated. Not all therapeutic applications require the high potency of a pharmaceutical drug. There is a growing market for natural products that offer gentle modulation of the immune response. The concentration range at which *L. ovatus* EO is active is of interest for potential topical applications. For instance, it could be explored as a candidate for formulations such as creams, gels, or balms aimed at mitigating oxidative stress and inflammation in conditions like arthritis, muscle soreness, or mild skin irritations [110]. Future studies assessing its cytotoxicity and skin irritation potential are necessary to confirm its safety profile for dermal use. In such applications, a balanced, multi-mechanistic approach with a favorable safety profile is often more desirable than extreme potency alone.

4. Materials and Methods

4.1. Plant Material

The leaves of *L. ovatus* were collected on 12 April 2025 in the Sendero de los Hieleros area of San Andrés (Guano Canton, Chimborazo Province, Ecuador) at 4410 m above sea level. Plant material was obtained from numerous shrubs scattered around the coordinates 01°30'04'' S and 78°47'22'' W, within an approximate 200 m radius; a composite average sample was obtained and mixed for analysis. Samples were immediately transported in sterile containers to the Natural Products Laboratory at the Escuela Superior Politécnica de Chimborazo (ESPOCH) for processing. Botanical identification was carried out by

Professor Jorge Caranqui, head of the ESPOCH Herbarium, who compared the collected sample with samples stored in the Herbarium, finding they matched in morphological aspects. The leaves were dried in an oven at 35 °C for 48 h to minimize evaporation, reduce moisture, and prevent the thermal degradation of volatile components prior to distillation.

A voucher specimen was deposited under the code Ofc.No.027.CHEP.2025 and deposited in the ESPOCH Herbarium. This study was conducted under research permit No MAATE-ARSFC-2025-0167 granted by the Ministerio de Ambiente, Agua y Transición Ecológica del Ecuador (MAATE). The botanical sample was transported to the laboratories of the Universidad Técnica Particular de Loja under the mobilization guide 02964 issued by the Ministerio de Ambiente, Agua y Transición Ecológica del Ecuador (MAATE).

4.2. EO Distillation and Sample Preparation

Steam distillation process was performed using a modified Dean-Stark apparatus [111]. Briefly, the entire dried leaves of *L. ovatus* (700 g) were distilled for 5 h according to the literature [112,113]. This process was carried out only once using all the collected plant material, due to the difficult access to the collection site. In order to remove excess water, the essential oil was subjected to treatment with anhydrous sodium sulfate (Sigma-Aldrich, St. Louis, MO, USA) and permanently stored at a temperature of −15 °C until use. For chromatographic analyses (GC), four samples were prepared by weighing 10 µL aliquots and diluting them with 1 mL of cyclohexane, which contained n-nonane as an internal standard at a concentration of 0.70 mg/mL.

4.3. Qualitative Chemical Analysis

The chemical profile of the volatile components of *L. ovatus* EO was qualitatively characterized by gas chromatography-mass spectrometry (GC-MS) using a system composed of a Trace 1310 chromatograph coupled to an ISQ 7000 single-quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). In order to guarantee sufficient separation and identification of the compounds. A volume of 1 µL of cyclohexane solution in split mode (40:1 ratio), with helium (Indura S.A., Guayaquil, Ecuador) serving as the carrier gas, while maintaining a constant flow rate of 1 mL/min. The injector was maintained at 250 °C, and finally, ionization was carried out by electron impact at 70 eV, with a mass range of 40–400 *m/z* in SCAN mode.

Separations were performed on two columns of different polarity: a non-polar DB-5ms (5%phenylmethylpolysiloxane) and a polar TR-WAX (polyethylene glycol), both 30 m × 0.25 mm i.d., with a film thickness of 0.25 µm (Thermo Fisher Scientific, Waltham, MA, USA). The oven temperature program for the DB-5ms column was: 60 °C (5 min), ramp to 100 °C, then 3 °C/min to 150 °C, followed by 5 °C/min to 200 °C, and finally 15 °C/min to 250 °C (15 min hold). The same program was applied to the TR-WAX column, except that the final temperature was 230 °C (15 min hold).

The identification of compounds present in the essential oil of *L. ovatus* was based on the comparison of their mass spectra and linear retention indices (LRI) with reference libraries. Linear retention indices (LRIs) were calculated using a series of *n*-alkanes (C₉–C₂₅) according to the method of Van Den Dool and Kratz [114]. The *n*-alkanes C₉–C₂₅ were purchased from Sigma-Aldrich, St. Louis, MO, USA. For each identified compound, the mean retention time was calculated, and the relative standard deviation (RSD) of the retention times was determined.

4.4. GC-FID Quantitative Analyses

The quantification of the constituents of the essential oil of *L. ovatus* was performed by gas chromatography with an ionizable flame detector (GC-FID), using the same chromatographic equipment used in the qualitative analysis. Identical instrumental conditions were

maintained in terms of thermal programming and the same stationary phases of different polarity (DB-5ms and TR-WAX), carrier gas flow (He, 1 mL/min) and injector temperature (250 °C), and the split was reduced to 10:1 (versus 40:1 used in the qualitative analysis) to adjust the sensitivity of the minor compounds in their quantification. Furthermore, the relative response factor (RRF) of each compound was calculated according to the enthalpy of combustion [115], the integration areas were correlated with the RRFs, and two calibration curves (one for each column) were constructed using isopropyl caproate as the calibration standard (synthesized and purified in this work, it presented a purity of 98.8%, confirmed by gas chromatography analysis) and nonane as the internal standard (Sigma-Aldrich, St. Louis, MO, USA). The correlation coefficient was >0.999 in both curves, as the solutions with the standards were prepared according to the literature [13].

4.5. Enantioselective Analysis

An enantioselective analysis of the four pairs of enantiomers presented by the essential oil of *L. ovatus* was carried out using gas chromatography coupled to mass spectrometry (GC-MS). The analysis was performed using enantioselective capillary columns of 25 m length, 0.25 mm internal diameter and 0.25 µm in phase thickness, with stationary phases based on β-cyclodextrin derivatives: 2,3-diacetyl-6-*tert*-butyldimethylsilyl-β-cyclodextrin (DAC) and 2,3-diethyl-6-*tert*-butyldimethylsilyl-β-cyclodextrin (DET) (Mega, Milan, Italy). The instrumental conditions were as follows: a split ratio of 50:1, a constant helium carrier gas pressure of 70 kPa, and a temperature program commencing at 60 °C for 2 min, increasing at 2 °C/min until reaching 220 °C, and maintaining this final temperature for 2 min. Mass spectra and linear retention indices (LRI), calculated using a series of C₉–C₂₅ *n*-alkanes (Sigma-Aldrich, St. Louis, MO, USA), were used to compare the detected enantiomers with data from the injection of enantiomerically pure standards. This was done according to the method of Van den Dool and Kratz. The selection of the DAC and DET columns was based on their observed efficiency in resolving the specific enantiomeric pairs present in the sample.

4.6. Antimicrobial Activity Assay

4.6.1. Bacterial Strains and Culture Conditions

Staphylococcus aureus (ATCC 25923; Gram-positive) and *Escherichia coli* (ATCC 25922; Gram-negative) were used in this study. Strains were cultured overnight at 37 °C in Mueller-Hinton broth (MHB) under aerobic conditions. Prior to testing, bacterial suspensions were adjusted to 0.5 McFarland (approximately $1\text{--}2 \times 10^8$ CFU/mL) using sterile saline solution (0.85% NaCl).

4.6.2. Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) was determined by the broth microdilution method in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [116] with minor modifications. Briefly, a two-fold serial dilution of the EO was prepared in MHB (Mueller–Hinton broth (MHB) was purchased from TM Media (Delhi, India), code TM 325) in a 96-well microtiter plate, resulting in a concentration range from 1000 to 0.98 µg/mL. Each well was then inoculated with the standardized bacterial suspension to a final concentration of approximately 5×10^5 CFU/mL. Wells containing only broth and inoculum (growth control) and broth with inoculum and a known antibiotic (ampicillin or ciprofloxacin, obtained from Sigma-Aldrich /Merck, St. Louis, MO, USA, as a positive control) were included. The negative control consisted of sterile growth medium inoculated with the bacterial suspension, which showed normal growth (OD₆₀₀ > 2.0 after 24 h of incubation).

The plates were incubated at 37 °C for 24 h. After incubation, 40 µL of a 0.2 mg/mL nitrobluetetrazolium chloride (NBT, ca. 98% TLC, Merck Sigma-Aldrich, St. Louis, MO, USA) solution was added to each well and incubated for a further 30 min. The MIC was defined as the lowest concentration of the extract that prevented the visible bacterial growth and the color change in NBT (from yellow to blue).

4.7. Antioxidant Activity Assay (DPPH Free Radical Scavenging)

The free radical scavenging activity of the EO was evaluated using the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich, St. Louis, MO, USA) radical method, as described by Brand-Williams et al. (1995) [117] with minor modifications. Briefly, a 0.1 mM solution of DPPH in absolute methanol (obtained from Pharmco by Greenfield Global, Palo Alto, CA, USA) was prepared. The EO was diluted in methanol to obtain concentrations ranging from 10 to 1000 µg/mL. In a 96-well microplate, 50 µL of each dilution was mixed with 150 µg of the DPPH solution. The mixture was incubated for 30 min at room temperature in the dark. Absorbance was then recorded at 517 nm using a microplate reader. A negative control (DPPH solution with methanol instead of sample) and a blank (methanol without DPPH) were included for baseline correction. The radical scavenging activity was calculated as a percentage of inhibition using the following formula:

$$\%Inhibition = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of the test sample. The results were expressed as the half-maximal inhibitory concentration (IC₅₀), calculated from the dose–response curve using non-linear regression analysis. Trolox was used as a positive control.

4.8. Anti-Inflammatory Activity (Oxidative-Burst Assay)

The anti-inflammatory activity of *L. ovatus* essential oil was evaluated by the oxidative burst assay developed by Tan and Berridge [118], with modifications reported by Vinueza et al. [119]. Human neutrophils were isolated from heparinized venous blood obtained from one healthy adult donor (male; age 40 years) using density gradient centrifugation on Ficoll-Paque™ PLUS (from Cytiva, Uppsala, Sweden). The polymorphonuclear cell fraction was collected, and residual erythrocytes were lysed by a brief hypotonic shock (NH₄Cl from J.T. Baker, Mexico City, México, 0.83% *w/v*). The isolated neutrophils were then washed twice with Hank's Balanced Salt Solution from Sigma-Aldrich, St. Louis, MO, USA (HBSS, pH 7.4) and finally resuspended in HBSS at a density of 1 × 10⁷ cells/mL for immediate use in the assays.

Anti-inflammatory activity was determined as a function of the reduction in water-soluble tetrazolium salt (WST-1 obtained from Roche, Mannheim, Germany) in the presence of activated neutrophils. The assay was carried out in a total volume of 250 µL of HBSS (pH 7.4) containing 10⁷ neutrophils/mL, 500 µM WST-1, and various concentrations of essential oil (3.12, 6.25, 12.5, 25, 50, 100, 200 and 400 µg/mL) or aspirin (obtained from Sigma-Aldrich/Merck, St. Louis, MO, USA), which was used as the reference compound, both dissolved with HBSS enrichment with DMSO, such that the final concentration of DMSO in each well was 0.05%. The control contained HBSS, a neutrophil suspension, and WST-1. All compounds were equilibrated at 37 °C and the reaction was initiated by adding opsonized Zymosan A from Sigma-Aldrich, St. Louis, MO, USA (15 mg/mL), which was prepared by mixing it with human pooled serum, followed by centrifugation at 3000 rpm, and the pellet was suspended in HBSS. Absorbance was measured at 450 nm. DMSO Sigma-Aldrich/Merck, St. Louis, MO, USA (0.05% *v/v*) together with the neutrophil suspension

and opsonized Zymosan A aliquot were used as a blank, and the anti-inflammatory activity was expressed as the produced superoxide anion inhibition percent [13].

This test was conducted in compliance with ethical standards and upon approval by the Comité de Bioética en Investigación-CBIESPOCH, approval code CIBE-2025-0087.

5. Conclusions

The EO obtained from dried leaves of *L. ovatus* had a yield of 0.21%, the chemical profile showed that silphinene, δ -selinene, β -cyclogermacrene, kessane, spathulenol, viridiflorol and neophytadiene are the major constituents (>3.0%). The EO exhibited measurable biological activities, including antibacterial activity—being more effective against *Staphylococcus aureus* (MIC = 250 $\mu\text{g/mL}$) than against *Escherichia coli* (MIC = 500 $\mu\text{g/mL}$)—as well as antioxidant activity, with an SC_{50} of 375.7 $\mu\text{g/mL}$ in the DPPH assay. In addition, the EO showed anti-inflammatory potential in the oxidative burst model, reaching a maximum inhibition of 49.12% at the highest concentration tested (200 $\mu\text{g/mL}$), although it was markedly less potent than the positive control (aspirin).

Overall, the observed activities are consistent with the EO diverse terpene profile, particularly oxygenated sesquiterpenes that may support antibacterial and antioxidant effects. In parallel, monoterpene bioactivity can depend strongly on enantiomeric composition because chiral targets may respond differently to stereoisomers. Therefore, determining the enantiomeric distribution of key constituents would strengthen structure–activity interpretation and comparisons across essential oils.

In summary, these findings support the pharmacological potential of *L. ovatus* EO and warrant further studies to isolate and characterize the major constituents, evaluate component interactions, elucidate mechanisms through cytotoxicity testing and inflammatory marker analysis, and confirm efficacy in relevant in vivo models. A key limitation is the lack of cytotoxicity data at the active concentration ranges, which is necessary for a robust safety assessment in future applications, and antioxidant capacity was evaluated only by the DPPH assay without additional datasets, so complementary methods such as ABTS, FRAP, ORAC, or CUPRAC should be included in future work to strengthen and contextualize these findings.

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Article

Cytotoxic Effects of *Bifora testiculata* (L.) Spreng. Essential Oil and Its Main Component on Cancer Cell Lines

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Abstract

Bifora testiculata (L.) Spreng. (Apiaceae), an understudied species endemic to the Mediterranean and the only representative species of the genus *Bifora* in Sicily, was investigated for the first time for its essential oil (EO) chemical composition and cytotoxic properties. The EO was obtained via hydrodistillation and analyzed using GC-MS, revealing an aldehyde-rich profile (86.10%), dominated by *trans*-2-dodecenal (67.49%). Comparative analysis with previous studies on *B. testiculata* from Greece confirmed a similar aldehyde-rich profile, although minor compositional differences suggest potential chemotype variation. Given the biological relevance of *trans*-2-dodecenal and related aldehydes, further investigations into the cytotoxic properties of the EO of *B. testiculata* (**Bt**) and its main constituent against cancer cell lines were undertaken. Three human tumor cell lines (MDA-MB 231, A375, and CaCo2) and a human non-tumor cell line (HEK293) were subjected to viability tests using the MTT assay. The EO and *trans*-2-dodecenal exhibited remarkable cytotoxic activity against all cell lines, with IC₅₀ values ranging between 7.93 and 14.41 µg/mL for **Bt** and between 1.88 and 5.29 µg/mL for *trans*-2-dodecenal. AO/BE fluorescent staining and Hoechst nuclear staining showed the presence of apoptotic bodies in the treated cells. *N*-acetyl-L-cysteine was able to invert the effects of **Bt** and *trans*-2-dodecenal on cell lines, suggesting ROS involvement in cytotoxic activity. The results demonstrated that the **Bt** cytotoxic activity was mainly due to the presence of *trans*-2-dodecenal.

Keywords: *Bifora testiculata* (L.) Spreng; Apiaceae; aldehydes; anticancer agent; oxidative stress; apoptosis

1. Introduction

The genus *Bifora* Hoffm. is a member of the Apiaceae family and is classified under the tribe Coriandreae [1]. This widely distributed yet disjunct genus comprises three species: two species found across Eurasia, and one native to North America. *B. radians* M. Bieb. extends from Spain through southern and central Europe, reaching as far east as Iran. *B. testiculata* (L.) Spreng. has a broader and more southern range, spanning from Spain to Uzbekistan and extending to North Africa, the Levant, and Saudi Arabia. Meanwhile, *B.*

americana (DC.) A. Gray is native to the southern United States, predominantly Texas and Arkansas, with reports of introduction in Alabama [2].

B. radians has been traditionally used as a culinary spice and folk remedy in parts of Turkey, where its aerial parts are known as *yabani kişniş otu* or *kokarot* and are used for their stomachic and carminative effects [3–5]. In contrast, in Jordan, *B. testiculata*, known used under the name *كزبرة* (*kazbira*), is used under infusion together with coriander for its sedative and stomach-soothing properties [6]. While these ethnobotanical uses highlight the relevance of *Bifora* species in traditional medicine, scientific interest has predominantly focused on *B. radians*, especially for its insecticidal activity against agricultural pests [7–11] and for its antimicrobial effects against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Candida albicans* [12,13]. Moreover, its EO, rich in aliphatic aldehydes, has demonstrated antibacterial activity against *Paenibacillus larvae* and insecticidal properties against the aphid *Lipaphis pseudobrassicae* [14].

B. testiculata [syn.: *Anidrum testiculatum* (L.) Kuntze; *Atrema testiculatum* (L.) Miq.; *Coriandrum testiculare* Salisb.; *Coriandrum testiculatum* L.; *Corion testiculatum* (L.) Hoffmanns. & Link; *Sium testiculatum* (L.) Vest] (Figure 1) is an herbaceous annual plant that grows to a height of 20–40 cm. It is glabrous and typically exhibits freely branched stems. The leaves are one- to two-pinnate, oblong in outline, with upper leaves having linear lobes that may be flat, entire, or lobed. The rays can extend up to 10 mm, while the bracts are absent or reduced to a single bract with 2–3 subulate bracteoles. The flowers have nearly equal-sized petals, and the fruit is rugose, measuring approximately 2.5–3.5 mm long and 4.5–7 mm wide, with a short beak. The style, typically less than 0.2 mm long, is as long as the stylopodium [15].



Figure 1. Morphological aspects of *Bifora testiculata* collected in Sicily (Serre di Villalba, Caltanissetta, Italy), showing aerial parts used for EO extraction. Photo by Prof. Vincenzo Ilardi.

Despite extensive research on *B. radians*, *B. testiculata* remains largely unexplored, particularly regarding its biological and pharmacological properties. Given the increasing interest in plant-derived compounds for cancer treatment, the potential cytotoxic effects of *B. testiculata* EO (**Bt**) and its major components warrant investigation. Essential oils (EOs) from various Apiaceae species have demonstrated significant bioactivities, including cytotoxic effects on cancer cells [16]. The EO of plants rich in aldehydes has exhibited anti-cancer activity [17]. Cinnamon EO is endowed with a wide range of pharmacological functions, including antioxidant, antimicrobial, and anti-cancer activities. The main compound, cinnamaldehyde, has been characterized as an anti-cancer drug [18]. Citronellal, the main compound in the EO of *Cymbopogon* species, has been found to inhibit the growth of hypopharyngeal carcinoma cells, which indicates its potential as a drug for this type of cancer [19].

This study aimed to bridge this knowledge gap by evaluating the cytotoxic properties of **Bt** and providing new insights into its therapeutic applications. Therefore, **Bt** and its

main components were tested against human breast adenocarcinoma, human malignant melanoma, and human colon adenocarcinoma cell lines, three of the common solid tumor cancers today.

2. Results and Discussion

2.1. Chemical Composition of *Bt*

Hydro-distilled *Bt*, obtained from aerial parts collected in Sicily, Italy, was pale yellow. Overall, twenty-six different compounds were identified (Table 1).

Table 1. Chemical composition (%) of *B. testiculata* essential oil collected in Sicily, Italy, as determined by GC-MS.

No.	Compounds ^a	KI ^b	KI ^c	Area (%) ^d
1	<i>trans</i> -2-Hexenal	852	855	0.13
2	Octanal	1002	1006	0.06
3	<i>p</i> -Cymene	1020	1022	0.01
4	Sylvestrene	1025	1028	0.01
5	(<i>E</i>)- β -Ocimene	1048	1050	0.02
6	<i>trans</i> -3-Undecene	1086	1088	0.04
7	Nonanal	1102	1102	0.07
8	<i>cis</i> -4-Decenal	1192	1193	0.03
9	<i>trans</i> -4-Decenal	1195	1197	0.08
10	Decanal	1205	1207	1.95
11	<i>cis</i> -2-Decenal	1253	1255	3.25
12	<i>trans</i> -2-Decenal	1257	1263	0.17
13	2-Octylfuran	1280	1284	0.11
14	<i>n</i> -Tridecane	1295	1300	0.14
15	Undecanal	1303	1310	0.14
16	<i>cis</i> -2-Undecenal	1347	1350	0.03
17	<i>trans</i> -2-Undecenal	1360	1361	0.42
18	<i>trans</i> -4-Undecenal	1396	-	3.38
19	Dodecanal	1408	1412	4.50
20	<i>cis</i> -2-Dodecenal	1460	1464	2.91
21	<i>trans</i> -2-Dodecenal	1465	1472	67.49
22	<i>trans</i> -2-Tridecenal	1550	1553	0.79
23	Dodecanoic acid	1564	1567	0.83
24	13-Tetradecenal	1602	1608	0.32
25	Tetradecanal	1604	1613	0.38
26	<i>trans</i> -2-Dodecenoic acid	1631	-	4.55
Monoterpene hydrocarbons				0.04
Hydrocarbons				0.18
Aldehydes				86.10
Aliphatic acids				5.38
Other				0.11
Total				91.81

^a Components listed in order of elution on an DB-5 MS apolar column; ^b Kovats indices on a DB-5 MS; ^c Kovats indices based on literature (<https://webbook.nist.gov>; accessed on 10 June 2025); ^d Relative percentage values of the separated compounds calculated from integration of the peaks.

Bt was particularly rich in aliphatic aldehydes, which accounted for 86.10% of its total composition. The predominant compound, *trans*-2-dodecenal (Figure 2), was the most abundant, representing 67.49% of the total EO. Other aldehydes present in noteworthy percentages included dodecanal (4.50%), *trans*-4-undecenal (3.38%), *cis*-2-decenal (3.25%), and *cis*-2-dodecenal (2.91%). Additionally, a moderate percentage of aliphatic acids (5.38%) was detected, with *trans*-2-dodecenoic acid (4.55%) being the most abundant. In contrast, monoterpenes, hydrocarbons, and other metabolites were found only in traces.

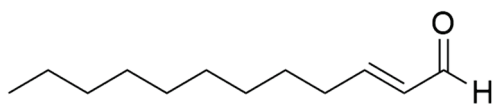


Figure 2. Chemical formula of *trans*-2-dodecenal.

Table 2 presents the major compounds (>3%) identified in the EOs of all *Bifora* taxa reported in the literature.

To date, only one study has reported the chemical composition of **Bt** based on plants collected in Greece [20]. Similar to our findings, that study showed a high percentage of aldehydes, with *trans*-2-dodecenal (56.3%) and dodecanal (4.0%) found in comparable proportions to those observed in **Bt**. However, Evergetis et al. [20] reported significant percentages of *trans*-2-tridecenal (16.1%) and heptadecanal (14.0%), which were virtually absent in the **Bt**. This suggests possible chemotype variations between different geographical populations of the species.

Studies on the EOs of other *Bifora* species, such as *B. radians*, have shown the presence of aldehydes as the principal constituents [18–20]. However, in these cases, the major constituents were *trans*-2-tridecenal (47.2–66.4%) and *trans*-2-tetradecenal (14.6–24.6%).

Despite the differences in specific aldehyde compositions, the consistently high aldehyde content observed in both *B. testiculata* and *B. radians* suggests that aldehydes may serve as taxonomic markers for the genus *Bifora*.

Table 2. Main constituents (>3%) of EOs from *Bifora* species reported in the literature.

Taxa	Origin, Parts	Compounds	Ref.
<i>B. radians</i> Bieb.	Turkey, ap	<i>trans</i> -2-tridecenal (47.2), <i>trans</i> -2-tetradecenal (23.4), tridecanal (5.9), <i>trans</i> -2-dodecenal (5.8)	[21]
<i>B. radians</i> Bieb.	Cultivated, ap	<i>trans</i> -2-tridecenal (66.4), <i>trans</i> -2-tetradecenal (14.6), <i>trans</i> -2-dodecenal (10.7)	[22]
<i>B. radians</i> Bieb.	Turkey, ap	<i>trans</i> -2-tridecenal (52.9), <i>trans</i> -2-tetradecenal (24.6)	[23]
<i>B. testiculata</i> (L.) Spreng.	Greece, ap	<i>trans</i> -2-dodecenal (56.3), <i>trans</i> -2-tridecenal (16.1), heptadecanal (14.0), dodecanal (4.0)	[20]

ap = aerial parts.

2.2. Effects of **Bt** on Tumor Cell Viability

To the best of our knowledge, no data are available on the cytotoxic activity of **Bt**. In this study, the cytotoxic activity of **Bt** and its main component, *trans*-2-dodecenal, was tested against a panel of three human tumor cell lines and one non-tumor cell line. Human breast cancer MDA-MB 231, human malignant melanoma A375, human colon adenocarcinoma CaCo2 cell lines, and non-tumor human embryonic kidney cell line HEK293 were used to determine the effects of **Bt** and its principal constituents on the viability of cells. All cell lines were exposed to treatments in the presence of different concentrations of **Bt** and *trans*-2-dodecenal for 72 h. The MTT assay was used as a relative measure of cell viability. The obtained data are presented in Figure 3 and Table 3.

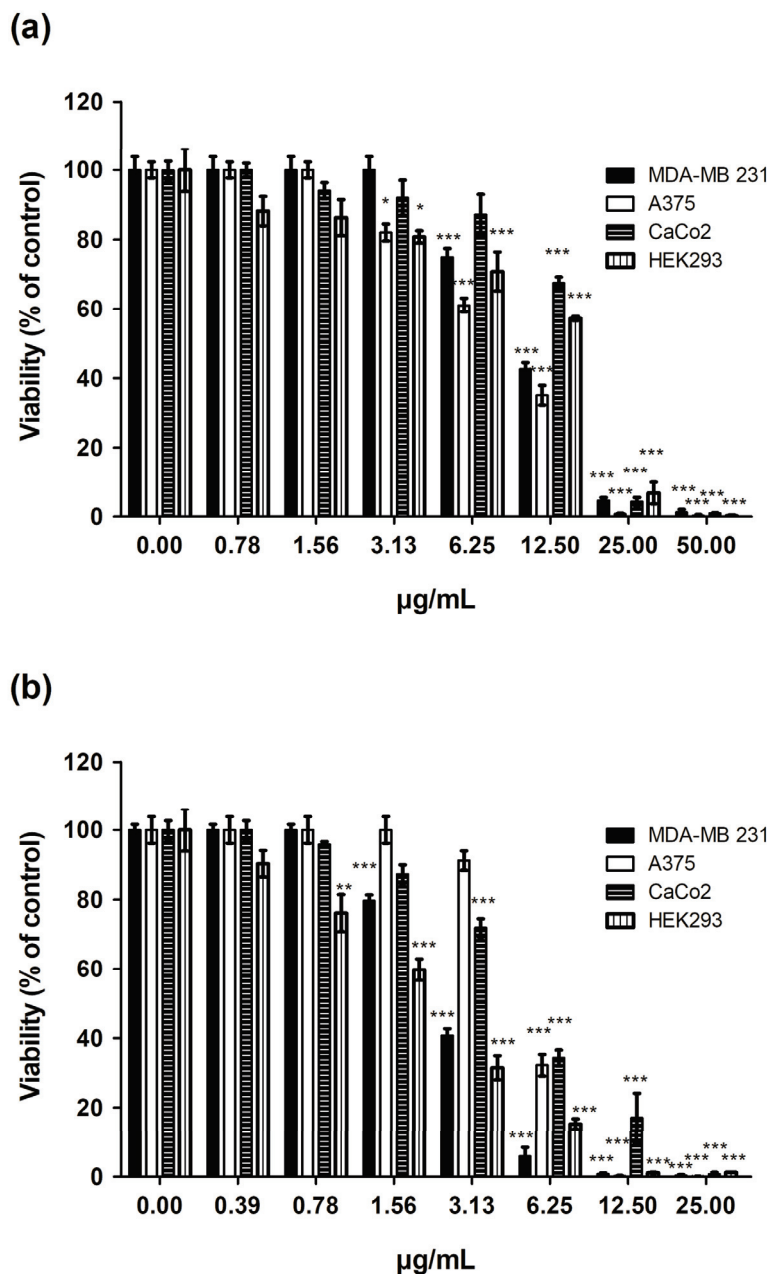


Figure 3. Dose-dependent cytotoxic effects of Bt (a) and *trans*-2-dodecenal (b) on human tumor (MDA-MB 231, A375, and CaCo2) and non-tumor cell lines (HEK293) after 72 h of treatment. Cell viability was measured using the MTT assay. Data are presented as mean $\pm$ SE of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (vs vehicle).

Table 3. In vitro cytotoxic activity of *B. testiculata* essential oil and *trans*-2-dodecenal on human cancer and non-cancer cell lines.

	Cell line (IC ₅₀ µg/mL) ^a			
	MDA-MB 231 ^b	A375 ^c	CaCo2 ^d	HEK293 ^e
EO	10.46	7.93	14.41	10.43
95% C.I. ^f	9.75–11.22	7.36–8.56	13.41–15.48	8.72–12.48
SI ^g	0.99	1.31	0.72	
<i>trans</i> -2-dodecenal	2.66 (14.59) ***	5.29 (29.02) ***	4.74 (26.00) ***	1.88 (10.31) ***
95% C.I.	2.56–2.76	5.02–5.57	4.36–5.16	1.69–2.08
SI	0.71	0.36	0.40	
Positive control				
Cisplatin	3.45 (11.50) ***	0.55 (1.83) ***	3.28 (10.93) ***	0.32 (1.07) ***

Table 3. Cont.

	Cell line (IC ₅₀ µg/mL) ^a			
	MDA-MB 231 ^b	A375 ^c	CaCo2 ^d	HEK293 ^e
95% C.I.	2.73–3.64	0.49–0.63	2.33–3.75	0.29–0.36
SI	0.09	0.58	0.097	

^a IC₅₀ = The concentration of compound that affords a 50% reduction in cell growth (after 72 h of incubation).

^b Human breast adenocarcinoma cell line. ^c Human malignant melanoma cell line. ^d Human colon adenocarcinoma cell line. ^e Human embryonic kidney cell line. ^f Confidence interval. ^g Selectivity index, calculated as the ratio of IC₅₀ for HEK293 cells/tumor cell lines; an SI value > 3 indicates high selectivity [24]. Values in brackets are in µM. ND: not determined. *** $p < 0.001$, compared with EO evaluated using the Tukey multiple comparison.

Bt showed remarkable cytotoxic activity, with IC₅₀ values ranging from 7.93 µg/mL for A375 cells to 14.41 µg/mL for the CaCo2 cell line. An intermediate IC₅₀ value for MDA-MB 231 (10.46 µg/mL) was also reported. Although the cytotoxic activity of **Bt** is significantly relevant to tumor cell lines, its selectivity index (SI: < 3) is not very high (Table 3). In fact, the IC₅₀ value of non-tumor embryonic kidney cells HEK293 (10.43 µg/mL) is close to that of tumor cell lines. **Bt** was found to be particularly rich in monoterpene aldehydes, of which *trans*-2-dodecenal was the predominant component, representing 67.49% of the total **Bt** composition. Although the anti-microbial activity of *trans*-2-dodecenal is known [25,26], no data on the cytotoxic activity of this compound have been reported. *trans*-2-Dodecenal, purified from **Bt**, was analyzed for its cytotoxic activity against tumor cell lines and its involvement in the biological activity of **Bt**. The results reported in Table 3 show high cytotoxicity against both tumor and non-tumor cell lines. The IC₅₀ values of the compound were lower than those of **Bt**, ranging from 1.88 µg/mL for HEK293 cells to 5.29 µg/mL for A375 cells. Even in this case, the SI was not significant (<3). Cisplatin, a conventional chemotherapy drug, was used as a positive control. The IC₅₀ values of cisplatin against tumor and non-tumor cell lines are listed in Table 3. They ranged from 0.32 µg/mL for HEK293 cells to 3.45 µg/mL for MDA-MB-231. In a molar comparison of the IC₅₀ values of cisplatin with those of *trans*-2-dodecenal, the latter was less cytotoxic. Notably, cisplatin as **Bt** and *trans*-2-dodecenal had an SI that was not significant (<3) for the three tumor cell lines.

To characterize the mechanism of action of **Bt** and *trans*-2-dodecenal on tumor cell lines, the cells were incubated in the presence of **Bt** and *trans*-2-dodecenal at the $2 \times \text{IC}_{50}$ concentration for 2 and 24 h and then subjected to acridine orange-ethidium bromide (AO/EB) fluorescent staining. Viable cells exclude ethidium bromide and are permeable to acridine orange, which reacts with DNA to yield green nuclear fluorescence. Non-viable cells showed red/orange fluorescence because of the entry of ethidium bromide, which reacts with DNA [27]. After 2 h of incubation of the cells in the presence of **Bt** and *trans*-2-dodecenal, no morphological modifications occurred, and the green fluorescence of the cells excluded the necrotic action of the samples. Figure 4 shows the morphological changes in cells after 24 h of incubation, as observed by light microscopy. The images revealed that **Bt** and *trans*-2-dodecenal caused clear morphological changes, including cell detachment and shrinkage, as well as a reduction in cellular density compared to untreated cells. Characteristic nuclear features with chromatin condensation, which reached a late stage with dense orange areas and apoptotic bodies, were observed in AO/EB fluorescence staining, mainly in cells treated with *trans*-2-dodecenal. The effect on **Bt** was less evident, with morphological changes in cell shape and reduced presence of dense orange areas, probably due to the different concentrations of *trans*-2-dodecenal in **Bt**. These data suggest that *trans*-2-dodecenal acts on tumor cell lines through an apoptotic mechanism.

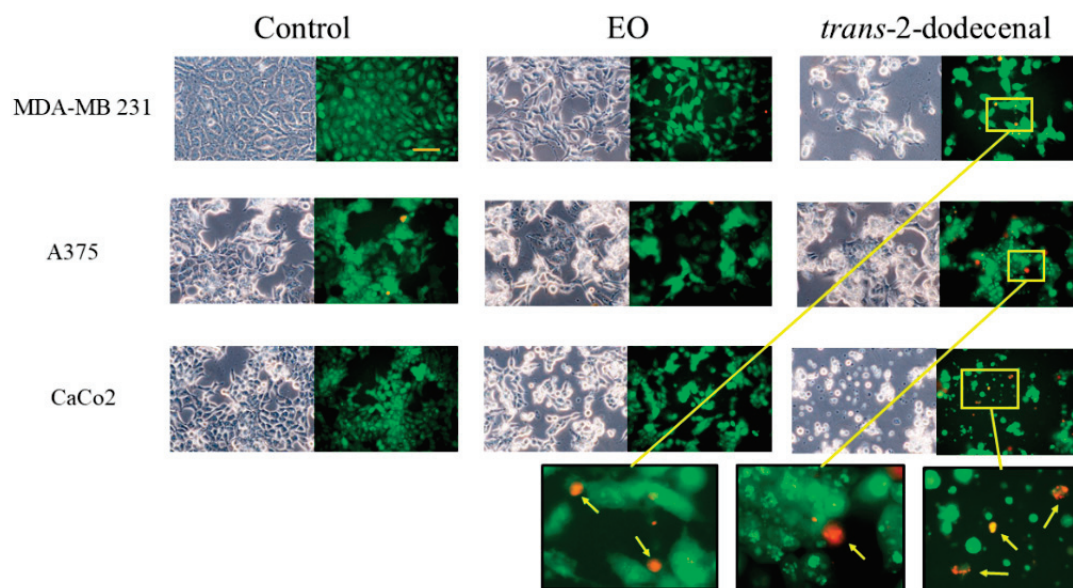


Figure 4. Morphological changes consistent with apoptosis were observed in MDA-MB 231, A375, and CaCo2 cells after 24 h of exposure to *Bt* and *trans*-2-dodecenal at $2 \times \text{IC}_{50}$ concentrations. Cells were stained with acridine orange/ethidium bromide and observed using phase-contrast and fluorescence microscopy. Arrows indicate apoptotic bodies; yellow boxes indicate magnified regions. Scale bar: 50 μm .

The treated cells were stained with fluorescent Hoechst 33258, which binds to DNA, showing the morphology and chromatin structure of the cell nuclei to further investigate the occurrence of chromatin condensation (Figure 5).

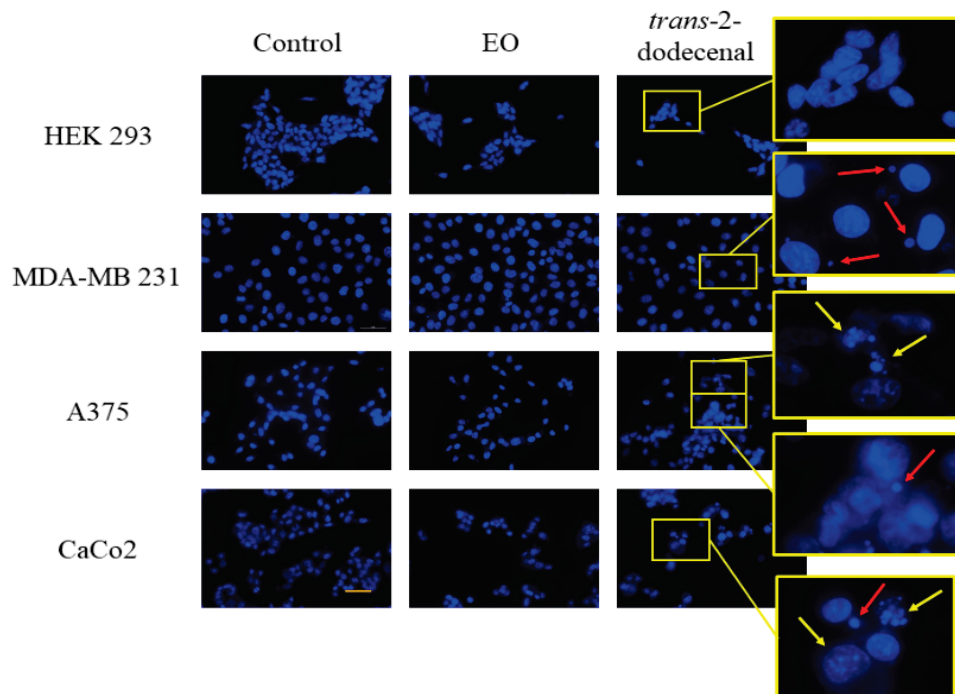


Figure 5. Nuclear changes induced by *B. testiculata* essential oil and *trans*-2-dodecenal in MDA-MB 231, A375, and CaCo2 cells following 24 h of treatment at $2 \times \text{IC}_{50}$. Chromatin condensation and fragmentation were visualized using Hoechst 33258 staining under a DAPI filter on an Olympus inverted fluorescence microscope. Yellow arrows indicate apoptotic bodies; red arrows indicate micronuclei. Scale bar: 50 μm .

As shown in Figure 5, treatments with **Bt** had less effect on nuclear modification than treatments with *trans*-2-dodecenal. Chromatin and nuclear shapes appeared substantially ineffective in comparison with the untreated cells. In contrast, nuclei of tumor cells treated with *trans*-2-dodecenal showed chromatin condensation with the presence of apoptotic bodies in A375 and CaCo2 cell lines. In MDA-MB 231 and A375 cells, Hoechst staining revealed the presence of micronuclei in the cytoplasm of the cells, indicating exposure to a toxic substance. Micronuclei formation is associated with chromosomal instability after genotoxic stress [28]. *trans*-2-Dodecenal possesses an α,β -unsaturated aldehyde structure, and thus, can potentially act as an alkylating agent on proteins and nucleic acids [29]. Aldehydes have been reported to have cytotoxic, mutagenic, genotoxic, and carcinogenic effects [30]. Aldehydes also react with proteins, disrupting their biological activity and causing inactivation. α,β -Unsaturated aldehydes target the thiol (cysteine), imidazole (histidine), and ϵ -amino (lysine) groups of proteins to form Michael addition-type or Schiff base adducts [31]. The depletion of intracellular glutathione and oxidation of thioredoxin 1 partially account for the DNA damage-independent cytotoxicity of aldehydes [32]. These effects lead to the formation of reactive oxygen species (ROS), advanced lipoxidation end-products, and advanced glycation end-products [33]. Oxidative stress induced by **Bt** and *trans*-2-dodecenal was assessed in MDA-MB 231, A375, CaCo2, and HEK293 cells by incubating the cells in the presence and absence of *N*-acetyl-L-cysteine (NAC). The antioxidant compound was used to investigate the possible involvement of ROS in the cytotoxic effects triggered by **Bt** and its main component. As shown in Figure 6, NAC was able to reverse the cytotoxic effects of **Bt** and *trans*-2-dodecenal, even at concentrations that caused a reduction in cellular viability close to 0%. These data suggest that the cytotoxic activity of **Bt** on cell lines can be attributed mainly to the α,β -unsaturated structure of *trans*-2-dodecenal, which triggers oxidative stress. However, it cannot be excluded that other aldehydes/aliphatic acid compounds present in **Bt** might contribute to the final cytotoxic activity observed.

Although the cytotoxic activity of **Bt** is significantly relevant to tumor cell lines, its specificity is not very high. In fact, its cytotoxic activity on the normal human embryonic kidney cell line HEK293 was higher than that on tumor cell lines. However, this finding requires further investigation. HEK293 cells are immortalized cells [34] that may not correspond to the characteristics of a non-tumor cell line. Thus, it would be useful to extend the study to other tissue-specific non-tumor cell lines, such as MCF-10A (Human Mammary epithelial cells), NHEM (Normal Human Epidermal Melanocytes), and FHC (Fetal Human Colon). The mechanisms underlying the cytotoxic activity need to be examined in more detail to consider **Bt** and *trans*-2-dodecenal as potential chemotherapeutic agents. Although α,β -unsaturated aldehydes, such as citral and acrolein, are toxic [29], some long-chain α,β -unsaturated aldehydes, such as 4-hydroxynonenal, may play a regulatory role in oxidative stress [35]. Thus, it would be interesting to evaluate the role of **Bt** and its main components in cellular redox regulation.

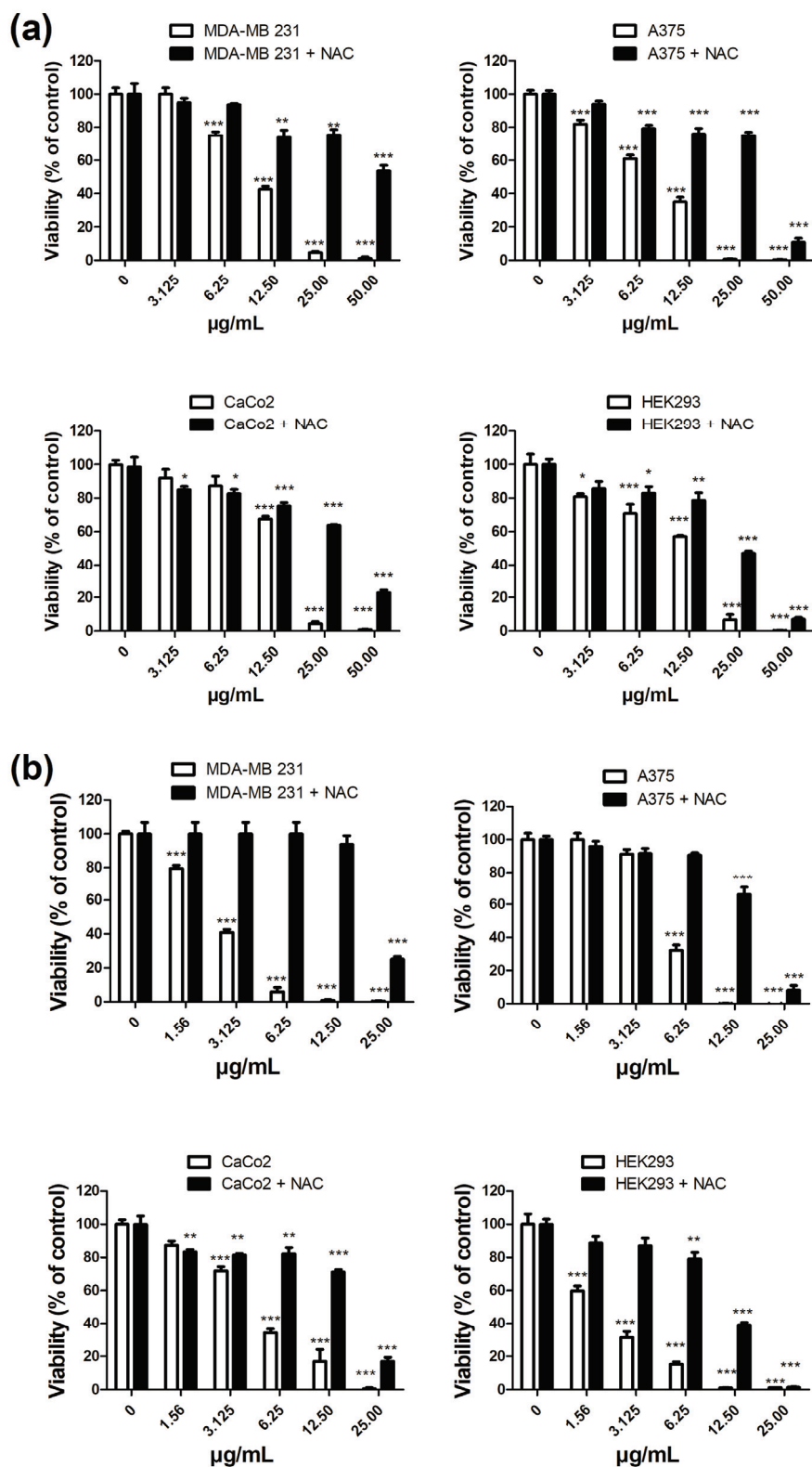


Figure 6. Evaluation of oxidative stress involvement in the cytotoxic effects of Bt (a) and *trans*-2-dodecenal (b). Cell viability was assessed in MDA-MB 231, A375, CaCo2, and HEK293 cell lines using the MTT assay after 72 h of treatment with increasing concentrations of essential oil and *trans*-2-dodecenal, in the presence (■) or absence (□) of 5 mM *N*-acetyl-L-cysteine (NAC). The results are expressed as the mean $\pm$ SE of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (vs. vehicle).

3. Materials and Methods

3.1. Plant Materials

The aerial parts (flowers, leaves, fruits, and stems) of *B. testiculata* were collected in the area called Serre di Villalba, in the province of Caltanissetta, Sicily, Italy (37°38'19" N 13°50'42" E), about 830 m a.s.l., in May 2023. One of the samples, identified by Prof. Vincenzo Iardi, was stored in the Herbarium Mediterraneum Panormitanum (PAL, voucher no. 109790) of the Botanical Garden of the University of Palermo, Italy.

3.2. Isolation of EO

Aerial parts (300 g) were subjected to hydrodistillation using distilled water for 3 h, according to the standard procedure described in the European Pharmacopoeia [36]. Sample yielded 0.12% of **Bt**.

3.3. GC-MS Analysis

The analysis of **Bt** was performed according to the procedure reported by Vaglica et al. [37]. GC-MS analysis was performed using an Agilent 7000C GC system fitted with a fused silica Agilent DB-5 MS capillary column (30 m × 0.25 mm i.d.; 0.25 µm film thickness), coupled to an Agilent quadrupole Mass Selective Detector MSD 5973 (ionization voltage 70 eV; electron multiplier energy 2000 V; transfer line temperature, 295 °C; Solvent Delay: 4 min). The oven program was as follows: temperature was increased to 40 °C for 5 min, at a rate of 2 °C/min up to 260 °C, and then isothermal for 20 min. Helium was used as the carrier gas (1 mL/min). The injector and detector temperatures were set at 250 °C and 290 °C, respectively. The EO solution (1 µL) (3% EO/hexane *v/v*) was injected in splitless mode; MS range 40–600.

The identification of peaks was carried out by comparison with their mass spectra and relative retention indices using the WILEY275, NIST 17, ADAMS, and FFNSC2 libraries. Kovats indices (KI) were determined using the retention times of *n*-alkanes (C₈–C₄₀) (49452-U, Supelco, Sigma-Aldrich, Milan, Italy).

3.4. Cell Culture

MDA-MB 231 (human breast adenocarcinoma cell line; American Type Culture Collection, ATCC, Manassas, VA, USA) and A375 (human malignant melanoma cell line; Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, IZSLER, Brescia, Italy) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin (Corning, Manassas, VA, USA), and supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS; Corning, Manassas, VA, USA). CaCo2 (human colon adenocarcinoma cell line; American Type Culture Collection, Manassas, VA, USA) was cultured in DMEM with 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, 1% Non-Essential Amino Acid (NEAA), and supplemented with 10% HI-FBS. HEK293 (human embryonic kidney cell line; kindly donated by Dr. Michela Buccioni, School of Pharmacy, University of Camerino, Camerino, Italy) was cultured in Eagle's Minimum Essential Medium (MEM) with 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and supplemented with 10% HI-FBS. The cells were maintained in an incubator at 37 °C in a humid atmosphere enriched with 5% CO₂ and 95% relative humidity.

3.5. MTT Assay

The cytotoxic activity of **Bt** and its main component (*trans*-2-dodecenal, Merck Life Science S.r.l., Milano, Italy) was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) method [38,39].

The reduction of tetrazolium salt to formazan by mitochondrial succinate dehydrogenase was used to evaluate the viability of cancer cells, as previously described [40]. MDA-MB 231, A375, CaCo2, and HEK293 cells (2×10^4 cells/mL) were added to 96-well plates, incubated for 24 h, and exposed to different concentrations of **Bt** (0.78–200 µg/mL; vehicle: ethanol) and *trans*-2-dodecenal (0.39–200 µg/mL; vehicle: ethanol) for 72 h in a humidified atmosphere of 5% CO₂ at 37 °C. At the end of the incubation period, each well received 10 µL of MTT (5 mg/mL in phosphate-buffered saline, PBS), and the plates were incubated for 4 h at 37 °C. The supernatant was then removed, and 100 µL of dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to dissolve the formazan crystals. Absorbance was measured using a microplate reader (FLUOstar Omega, BMG Labtech, Milan, Italy) at 540 nm. The percentage of viability was calculated using the following equation:

$$Viability \% = \frac{A_{sample} - A_{blank}}{A_{vehicle} - A_{blank}} \times 100\%$$

A = Absorbance at 540 nm.

In a parallel experiment, **Bt** and its main component were incubated in the presence of 5 mM NAC (N-acetyl-L-cysteine; Sigma-Aldrich). The anti-cancer drug cisplatin (0.01–50 µg/mL) was used as a positive control. The experiments were conducted in triplicate. The 50% cytotoxic concentration (IC₅₀) was defined as the compound concentration required to reduce cell viability by half. The IC₅₀ values were determined using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). IC₅₀ values were obtained from graphs plotted using a non-linear regression equation log (inhibitor) versus response—variable slope.

The selectivity index (SI) was calculated as the ratio of the IC₅₀ value for HEK293 cells divided by the IC₅₀ values for A375, MDA-MB 231, or CaCo2 cells. The SI value indicates the degree of selective toxicity of a given **Bt** or its main components to the cancerous cell lines tested, relative to that of the non-tumor cell line. Any sample of an SI value > 3 was considered to indicate high selectivity [24].

3.6. Acridine Orange and Ethidium Bromide Staining

Characteristic apoptotic or necrotic morphological changes were investigated by fluorescent microscopy using acridine orange and ethidium bromide staining. MDA-MB 231, A375, and CaCo2 cells were seeded at a density of 2.5×10^5 cells/mL. After 24 h, the cells were treated with $2 \times IC_{50}$ values of **Bt** and *trans*-2-dodecenal for 2 and 24 h. After incubation, the cells were washed with PBS and mixed with 20 µL of a fluorochrome mixture (50 µg/mL acridine orange and 50 µg/mL ethidium bromide in PBS) in 500 µL of PBS for 3 min. The samples were examined under an Olympus IX71 fluorescent inverted microscope with an appropriate combination of filters and recorded using an Olympus DP90 digital camera. Viable cells had intact, bright green nuclei. Early apoptotic cells contained a bright green nucleus with condensed chromatin, whereas late apoptotic cells contained a red/orange nucleus showing chromatin condensation [41].

3.7. Hoechst Nuclear Staining

MDA-MB 231, A375, CaCo2, and HEK293 cells (1×10^6 cells/mL) were exposed to **Bt** and its main component for 24 h at a concentration corresponding to $2 \times IC_{50}$ values and then fixed with methanol/acetic acid (3:1). Samples of fixed cells were incubated with Hoechst 33258 nuclear stain (0.05 µg/mL in PBS) (Sigma-Aldrich, St. Louis, MO, USA) for 10 min. The stain was removed, and the coverslip was mounted in a drop of buffered glycerol mountant (0.4 M Na₂HPO₄, 0.2 M citric acid, and 50% glycerol). Nuclei were

observed using an Olympus IX71 (Olympus Italy s.r.l., via San Bovio 1, Segrate, MI, Italy) inverted fluorescence microscope and recorded using an Olympus DP90 digital camera.

3.8. Statistical Analysis

Statistical analyses were performed using one-way ANOVA, followed by Tukey's post-hoc test to test for statistical differences between groups. Significance was represented by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The statistical analysis software used was GraphPad InStat v3.0 (GraphPad Software, San Diego, CA, USA).

4. Conclusions

This study provides new insights into the chemical composition of *Bifora testiculata* essential oil, confirming its aldehyde-rich nature and highlighting its potential as a source of bioactive compounds. The predominant presence of *trans*-2-dodecenal (67.49%) distinguished the Sicilian accession and aligned with previous findings from a Greek population, reinforcing the idea that aldehydes are key chemotaxonomic markers within the *Bifora* genus. However, notable differences in minor constituents suggest potential chemotype variability among different accessions. Given the known bioactive properties of *trans*-2-dodecenal and structurally related aldehydes, the essential oil and its main component exhibit remarkable cytotoxic activity against tumor and non-tumor cell lines. Preliminary data suggest that **Bt** and its main compound, *trans*-2-dodecenal, act on cancer cell lines, triggering apoptosis through ROS stimulation. However, more research is needed to demonstrate the safety of essential oils and *trans*-2-dodecenal in non-tumor cells, as well as to elucidate their role in cellular redox regulation. In light of these results, the elucidation of the molecular mode of action of **Bt** and *trans*-2-dodecenal and the pathways involved in the apoptotic mechanism will be investigated in future works. In addition, the study will be extended to other tumor and non-tumor cell lines to better clarify their anti-tumor potential.

Author Contributions: Conceptualization, M.B. (Maurizio Bruno), F.M. and L.Q.; methodology, M.B. (Maurizio Bruno), L.Q.; software, L.Q. and M.B. (Massimo Bramucci); validation, A.V., A.P., L.Q. and M.B. (Maurizio Bruno); formal analysis, A.P., L.Q., A.V. and M.B. (Massimo Bramucci); investigation, A.V., A.P., F.M., V.I. and L.Q.; resources, M.B. (Massimo Bramucci) and M.B. (Maurizio Bruno); data curation, A.V., L.Q., M.B. (Massimo Bramucci) and M.B. (Maurizio Bruno); writing—original draft preparation, A.V., M.B. (Massimo Bramucci), M.B. (Maurizio Bruno) and F.M.; writing—review and editing, A.V., A.P., M.B. (Maurizio Bruno) and M.B. (Massimo Bramucci); visualization, M.B. (Maurizio Bruno) and F.M.; supervision, N.B., M.B. (Maurizio Bruno) and F.M. All authors have read and agreed to the published version of the manuscript.

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Article

An Investigation into the Biological Activities of Four Lamiaceae Essential Oils Against *Thrips flavus*, Crops, and Weeds

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Abstract: In recent years, with the increasing awareness of environmental protection and food safety, essential oils (EOs) have gained significant attention as safer and more environmentally friendly alternatives. This study investigated the insecticidal activity of four Lamiaceae EOs (patchouli oil, catnip oil, lavender oil, and mint oil) against *Thrips flavus* and their effects on crops and weeds. The results show that patchouli oil, catnip oil, and lavender oil exhibited better insecticidal activity, with patchouli oil having the strongest toxicity, with an LC₅₀ value of 0.31 mg/mL. Additionally, catnip oil and lavender oil had significant attractive effects on *T. flavus*, where lavender oil only had a significant attractive effect on male *T. flavus*, with an attraction rate of 71.88% ($p = 0.03$), suggesting that it could be a potential alternative to insect lures. In pot experiments, these EOs demonstrated sustained insecticidal effects and varied impacts on crops. Lavender oil only significantly affected the shoot length of soybeans (*Glycine max*), while mint oil did not significantly affect the growth of *G. max*. Finally, we preliminarily analyzed the chemical composition of the EOs to provide insights into their active components. These findings indicate that EOs have potential applications as natural agrochemicals, but further research on their mechanisms and application conditions is required.

Keywords: *Thrips flavus*; Lamiaceae; essential oil; *Glycine max*; *Zea mays*; weeds

1. Introduction

Lamiaceae, also known as the mint or sage family, is a large group of plants characterized by their distinctive labiate corolla. This family includes approximately 240 genera, with over 7000 species distributed globally [1,2]. Lamiaceae plants are renowned for their aromatic properties and medicinal values, making them widely used in food flavorings, cosmetics, and traditional medicine [3,4]. In recent years, they have also gained attention due to the insecticidal activities of their extracted essential oils (EOs) [5,6]. For example, *Mentha piperita* wild-type EO exhibits contact toxicity against *Aphis gossypii* and *Psylla Chinesis* [7]; patchouli oil repels *Aedes aegypti* and has toxic effects, making it a potential insecticide and repellent [8,9]; catnip oil repels subterranean termites [10] and *Stomoxys calcitrans* [11]; and lavender oil shows fumigant toxicity against *Orgyia trigotephras* [12]. Moreover, EOs have distinct advantages over chemical insecticides due to their high volatility and degradability, as well as their sensitivity to temperature, ultraviolet light, and sunlight, which result in lower persistence in the air and

environment [13]. Given these characteristics, EOs are considered safer and more environmentally friendly than synthetic insecticides and pesticides, and they exhibit lower toxicity to mammals [14,15]. As natural insecticides, EOs have made some progress in commercialization, but their market penetration and application scope still have significant room for growth [16]. One key issue is that scholars have paid relatively little attention to the insecticidal effects of EOs.

Thrips flavus, belonging to the order Thysanoptera and the family Thripidae, is a worldwide pest widely distributed in Asian and European countries [17]. It can transmit plant viruses, posing a severe threat to agricultural yields [18,19]. In China, *T. flavus* is considered a major pest of flowering plants such as those in the Compositae and Leguminosae families in the northern regions [19–21]. Currently, the control of *T. flavus* primarily relies on chemical insecticides, but the excessive use of these chemicals leads to environmental pollution and the development of pest resistance [17]. Therefore, in recent years, EOs have become potential substitutes for chemical insecticides due to their environmental friendliness [22].

Some EOs, in addition to their insecticidal properties, can also serve as potential herbicides [23]. For example, the EO of the Lamiaceae plant *Thymus daenensis* reduces the germination rates of four types of weeds: *Amaranthus retroflexus*, *Avena fatua*, *Datura stramonium*, and *Lepidium sativum* [24]. EO extracted from *M. piperita* significantly inhibits the seed germination and early seedling development of the invasive weeds *Erigeron bonariensis* and *Araujia sericifera* [25]. Furthermore, some studies have shown that the herbicidal mechanism of EOs is involved in causing electrolyte leakage in plants, ultimately leading to plant death [26,27]. Although EOs have a certain degree of selectivity in their effects on plants [28], there is a concern about whether they may also harm crops. Currently, there is limited research on the impact of EOs on crop growth and development [1,29]. Therefore, in addition to assessing the effects of EOs on weed growth and development, we also evaluated their impact on crops. In this study, we selected soybean (*Glycine max*), a host crop of *T. flavus* [1], and corn (*Zea mays*), which is commonly intercropped with *G. max* [30], as well as two major weeds: barnyard grass (*Echinochloa oryzoides*) [31] and purslane (*Portulaca oleracea*) [32]. By evaluating the effects of EOs on both crops and weeds, this study aimed to understand their practical applications and to explore the potential development of herbicides while addressing the research gap regarding the impact of EOs on crop growth and development.

This study aimed to systematically evaluate the insecticidal effects and behavioral impacts of four Lamiaceae EOs on *T. flavus* through indoor bioassays, olfactory behavior response tests, and a chemical component analysis of the plant EOs. Additionally, we assessed the effects of these EOs on crops and weeds to provide theoretical support for the development of commercial insecticides and herbicides derived from EOs. This research helps to understand the effectiveness of EOs in practical applications. Finally, we also analyzed the compositional components of the EOs to provide insights into their active ingredients (Figure 1).

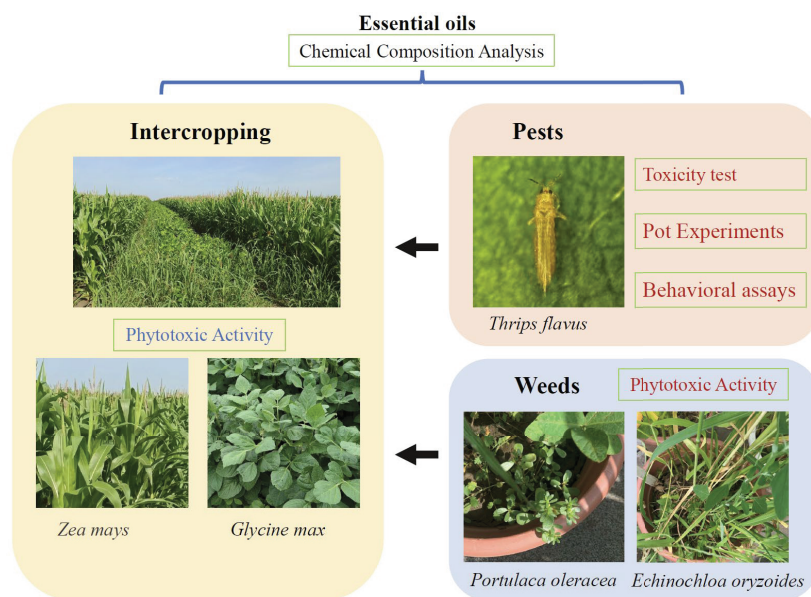


Figure 1. A comprehensive evaluation of four EOs. This study provides a systematic evaluation of the insecticidal effects and behavioral impacts of four EOs on *T. flavus*, utilizing indoor bioassays, olfactory behavior response tests, and a chemical component analysis. Moreover, it examines the effects of these EOs on crops (*G. max* and *Z. mays*) and weeds (*E. oryzoides* and *P. oleracea*). Finally, the compositional components of the four Lamiaceae EOs are analyzed.

2. Results

2.1. Toxicity of EO on *T. flavus* in Lab

Patchouli oil showed the strongest toxicity, with the lowest LC₅₀ value of 0.31 mg/mL and a 95% confidence interval that did not overlap with the others. Next were catnip oil and lavender oil, with LC₅₀ values of 0.33 mg/mL and 0.36 mg/mL, respectively. Mint oil had the highest LC₅₀ value at 0.52 mg/mL, and its 95% confidence interval did not overlap with that of the other three EOs, indicating that it had significantly lower toxicity. The positive control, 45% malathion EC, exhibited higher toxicity to *T. flavus* than to the four EOs, with an LC₅₀ of 0.0127 mg/mL (Table 1).

Table 1. Toxicity of four EOs and malathion to *T. flavus*.

Number	EOs	Regression Equation	Correlation Coefficient	LC50 (mg/mL)	95% Confidence Interval	χ^2	df
1	Patchouli oil	$y = 6.6940 + 3.3029x$	0.96	0.31	0.22–0.38	2.29	3
2	Catnip oil	$y = 6.8508 + 3.8827x$	0.99	0.33	0.26–0.40	0.58	3
3	Lavandula oil	$y = 6.3242 + 2.9614x$	0.78	0.36	0.27–0.43	7.36	3
4	Mint oil	$y = 6.1968 + 4.2378x$	0.88	0.52	0.45–0.60	4.44	3
5	45% Malathion EC	$y = 9.7959 + 2.5307x$	0.94	0.0127	0.0068–0.0168	1.97	3

2.2. Toxicity of EOs to *T. flavus* in Pots

The pot experiments of the four EOs at different concentrations showed significant differences over time after application (Figure 2). At a concentration of 180.00 g a.i.·hm^{−2}, 7 days after application, the control efficacy of lavender oil was the highest at 65.38 ± 3.85%, significantly higher than that of catnip oil and mint oil ($F = 37.171$, $p < 0.001$, Figure 2A). At a concentration of 360.00 g a.i.·hm^{−2}, 7 days after application, the control efficacy of lavender oil was the highest at 83.33 ± 3.39%, significantly higher than that of patchouli oil and mint oil ($F = 35.265$, $p < 0.001$, Figure 2B). At a concentration of 540.00 g a.i.·hm^{−2}, 3 days after application, the control efficacy of catnip oil and lavender oil was 72.16 ± 5.06% and 78.2 ± 6.79%, respectively, significantly higher than that of mint oil ($F = 31.043$, $p < 0.001$,

Figure 2C). At a concentration of $720.00 \text{ g a.i.}\cdot\text{hm}^{-2}$, 3 days after application, the control efficacy of lavender oil was the highest at $88.23 \pm 4.24\%$, significantly higher than that of mint oil ($F = 28.600$, $p < 0.001$, Figure 2D). At a concentration of $900.00 \text{ g a.i.}\cdot\text{hm}^{-2}$, 7 days after application, the control efficacy of patchouli oil, catnip oil, and lavender oil was $96.2 \pm 2.19\%$, $96.2 \pm 3.8\%$, and 100% , respectively, significantly higher than that of mint oil ($F = 51.068$, $p < 0.001$, Figure 2E).

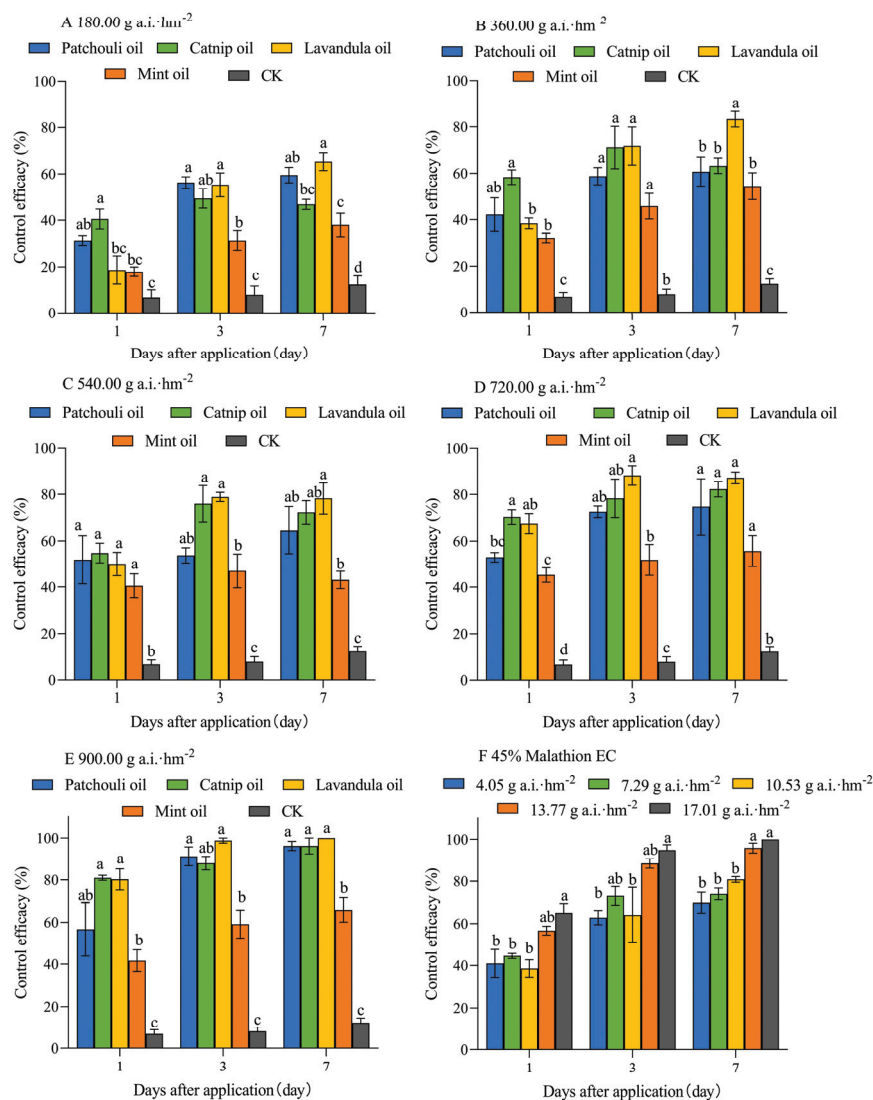


Figure 2. The pot experiments of the four plant EOs against *T. flavus* at different concentrations. (A) pot experiments of the four plant EOs at $180.00 \text{ g a.i.}\cdot\text{hm}^{-2}$; (B) pot experiments of the four plant EOs at $360.00 \text{ g a.i.}\cdot\text{hm}^{-2}$; (C) pot experiments of the four plant EOs at $540.00 \text{ g a.i.}\cdot\text{hm}^{-2}$; (D) pot experiments of the four plant EOs at $720.00 \text{ g a.i.}\cdot\text{hm}^{-2}$; (E) pot experiments of the four plant EOs at $900.00 \text{ g a.i.}\cdot\text{hm}^{-2}$; (F) pot experiments of 45% malathion EC. Different lowercase letters indicate significant differences among the efficacies of the four oils ($p < 0.05$).

2.3. Behavioral Effects of EOs on *T. flavus*

Catnip oil significantly attracted both male and female adult *T. flavus*, with attraction rates of 71.88% ($\chi^2 = 4.693$, $p = 0.030$) and 67.74% ($\chi^2 = 3.903$, $p = 0.048$), respectively. Lavandula oil significantly attracted male *T. flavus* only, with an attraction rate of 69.44% ($\chi^2 = 4.064$, $p = 0.044$), but it did not significantly attract female *T. flavus*. The remaining EOs did not have a significant effect on either male or female adult *T. flavus* (Figure 3).

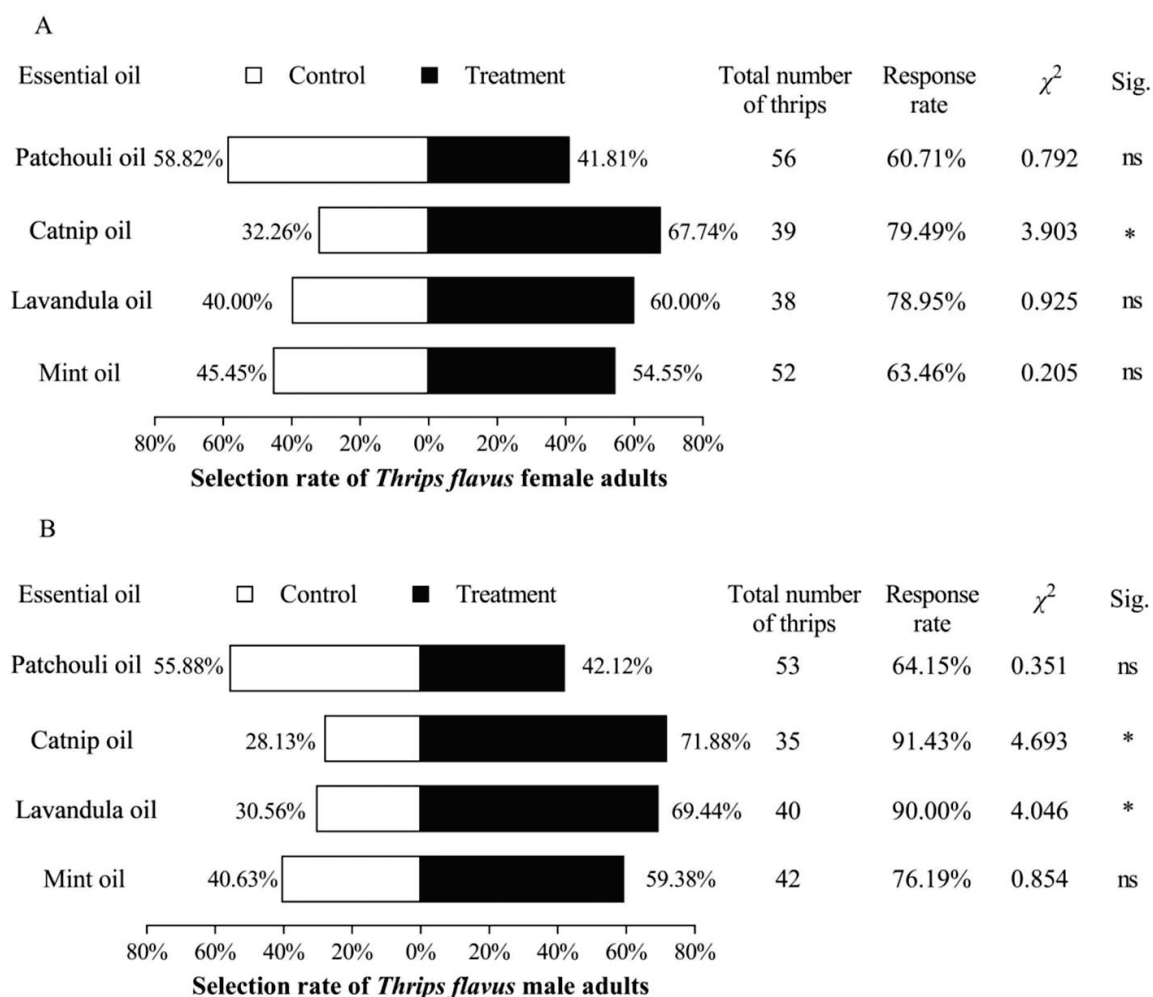


Figure 3. The olfactory behavior response of *T. flavus* to four plant EOs. (A) The olfactory behavior response of female adult *T. flavus* to the four EOs; (B) the olfactory behavior response of male adult *T. flavus* to the four EOs. “ns” indicates no significant difference between the control and treatment groups. “*” indicates a significant difference between the control and treatment groups ($p < 0.05$).

2.4. Effects of Essential Oils on Plants

After treatment with different concentrations of the four EOs, there were significant differences in the germination potential, germination rate, germination index, and shoot length of the four plants. Patchouli oil had a significant impact on the germination potential of *G. max* ($F = 3.549$, $p = 0.034$), the germination index of *Z. mays* ($F = 4.631$, $p = 0.014$), the germination potential of *P. oleracea* ($F = 4.641$, $p = 0.014$), and the germination index of *P. oleracea* ($F = 4.407$, $p = 0.016$) (Figure 4A–D). Catnip oil showed significant differences in the germination potential of *G. max* ($F = 4.483$, $p = 0.015$), the germination index of *E. oryzoides* ($F = 4.662$, $p = 0.013$), and the shoot length of *P. oleracea* ($F = 8.614$, $p = 0.001$). This EO also had a significant effect on the germination rate ($F = 5.645$, $p = 0.007$), germination potential ($F = 3.183$, $p = 0.047$), and germination index ($F = 4.089$, $p = 0.021$) of *Z. mays* (Figure 4E–H). Lavender oil had a significant effect on the shoot length of *G. max*, where the shoot length at 0.8 mg/mL was significantly higher than at 0.4 mg/mL ($F = 3.353$, $p = 0.04$), but it did not have a significant impact on the other indicators of the four plants (Figure 4I–L). Mint oil had a significant effect on the germination index of *P. oleracea* ($F = 6.198$, $p = 0.005$); it also had a significant effect on all four indicators of *Z. mays*, where the shoot length at 0.8 mg/mL was significantly lower than that of the CK and at 0.2 mg/mL ($F = 4.662$, $p = 0.013$) (Figure 4M–P).

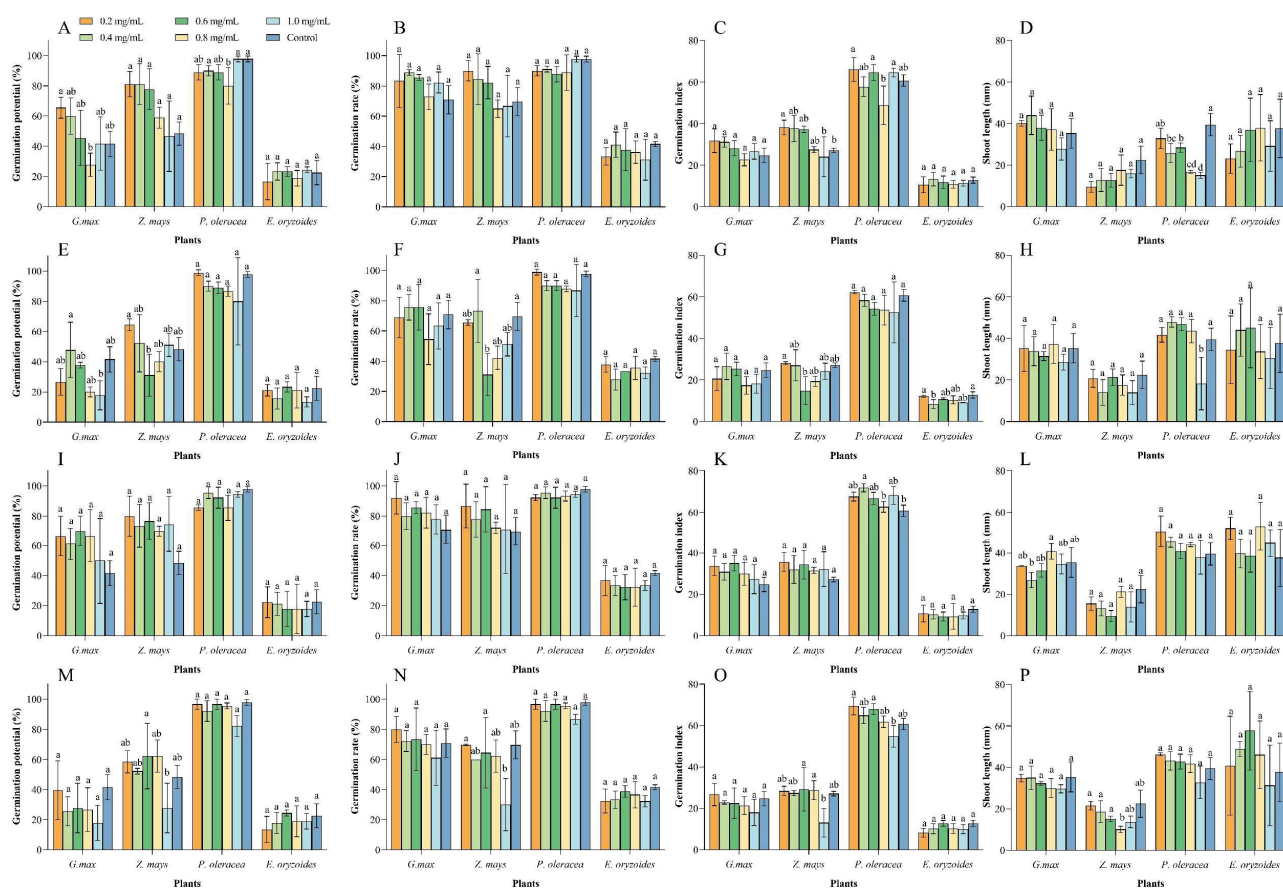


Figure 4. The phytotoxicity of the four essential oils on four plant species. (A–D) present the germination potential, germination rate, germination index, and radicle length of the four plants after treatment with patchouli essential oil. (E–H) correspond to the germination potential, germination rate, germination index, and radicle length of the four plants after treatment with nepeta essential oil. (I–L) show the germination potential, germination rate, germination index, and radicle length of the four plants after treatment with lavender essential oil. (M–P) indicate the germination potential, germination rate, germination index, and radicle length of the four plants after treatment with mint essential oil. Different lowercase letters indicate significant differences ($p < 0.05$) among the indices at different concentrations.

2.5. Chemical Composition of Four EOs

Patchouli oil contains 23 compounds, including terpenes, alcohols, and aldehydes, as well as derivatives of cyclobutane and naphthalene. The most abundant compound is patchoulol, with a relative content of 29.54%, followed by β -humulene, α -guaiane, α -bulnesene, and α -amorphene, with relative contents of 16.33%, 12.43%, 9.59%, and 8.36%, respectively (Table 2).

Table 2. Chemical composition of patchouli oil.

Number	Retention Index	Relative Percentage (%)	Name of Constituent
1	935	0.08	α -pinene
2	974	0.17	β -pinene
3	1340	0.14	δ -elemene
4	1387	3.22	β -patchoulene
5	1391	1.46	β -elemene
6	1415	1.08	thujopsen
7	1422	4.18	β -caryophyllene
8	1440	12.43	α -guaiane
9	1447	8.36	α -panasinsene

Table 2. *Cont.*

Number	Retention Index	Relative Percentage (%)	Name of Constituent
10	1456	1.21	α -bisabolene
11	1463	9.59	α -bulnesene
12	1471	0.63	caryophyllene
13	1478	1.05	alloaromadendrene
14	1489	0.83	α -guaiene
15	1500	5.55	longifolene
16	1506	16.33	β -humulene
17	1520	0.39	β -panasinsene
18	1528	0.14	cubebene
19	1551	0.97	isopatchoulane
20	1568	0.30	α -longipinene
21	1579	0.63	viridiflorol
22	1618	0.91	widdrol
23	1657	29.54	patchouli alcohol

Catnip oil contains seven compounds, including terpenes, phenols, aldehydes, and derivatives of benzene. Limonene has the highest relative content at 51.31%, followed by o-cymene and 2-propenal, with relative contents of 20.32% and 12.90%, respectively (Table 3).

Table 3. Chemical composition of catnip oil.

Number	Retention Index	Relative Percentage (%)	Name of Constituent
1	1013	20.32	o-cymene
2	1024	51.31	limonene
3	1051	2.65	γ -terpinene
4	1235	12.90	2-propenal
5	1332	10.45	eugenol
6	1418	1.85	β -caryophyllene
7	1454	0.51	α -caryophyllene

Lavender oil contains 17 compounds, including terpenes, alcohols, and esters. Linalyl acetate has the highest content at 17.90%, followed by terpinyl acetate at 16.66%, while octylmethacrylat has the lowest relative content at only 0.33% (Table 4).

Table 4. Chemical composition of lavender oil.

Number	Retention Index	Relative Percentage (%)	Name of Constituent
1	934	7.24	α -pinene
2	1021	4.40	1,8-cineole
3	1086	8.01	3-Octanol
4	1126	7.94	camphor
5	1136	0.76	β -terpineol
6	1159	0.75	borneol
7	1171	0.45	terpinen-4-ol
8	1179	6.16	α -terpineol
9	1185	2.42	γ -terpineol
10	1246	17.90	linalyl acetate
11	1270	4.48	cyclohexanol
12	1274	9.81	bornyl acetate
13	1318	1.12	β -terpinyl acetate
14	1335	16.66	terpinyl acetate
15	1347	0.33	octylmethacrylat
16	1408	9.95	lignyl acetate
17	1420	0.80	β -caryophyllene

Mint oil contains 19 compounds, including terpenes, alcohols, ketones, and esters. Menthol has the highest content at 27.54%, followed by isomenthone at 17.81% and limonene at 8.21% (Table 5).

Table 5. Chemical composition of mint oil.

Number	Retention Index	Relative Percentage (%)	Name of Constituent
1	933	4.55	α -pinene
2	968	0.66	α -phellandrene
3	973	4.64	β -pinene
4	983	1.77	3-octanol
5	1023	8.21	limonene
6	1140	1.69	isopulegol
7	1143	17.81	isomenthone
8	1153	10.41	menthone
9	1161	6.73	neoisomenthol
10	1168	27.54	menthol
11	1176	1.26	isomenthol
12	1179	0.91	α -terpineol
13	1214	1.97	pulegone
14	1222	1.22	valeric acid 3-hexen-1-yl ester
15	1228	1.31	piperitone
16	1280	7.86	menthyl acetate
17	1387	0.24	β -bourbonene
18	1420	0.93	β -caryophyllene

3. Discussion

In recent years, with the growing awareness of environmental protection and food safety, people have increasingly focused on plant-derived insecticides such as EOs, which are considered safer and more environmentally friendly than synthetic pesticides [14,15]. These EOs have been proven effective in managing agricultural pests [1]. In this study, we measured the toxicity of four EOs to *T. flavus*. Patchouli oil, catnip oil, and lavender oil all showed good insecticidal activity, with patchouli oil being the most potent, followed by catnip oil and lavender oil, with LC₅₀ values of 0.31, 0.33, and 0.36 mg/mL, respectively. These three EOs were significantly more toxic to *T. flavus* than mint oil. Compared to previous studies, these EOs showed higher toxicity towards *T. flavus* [19]. It has been reported that the fumigant toxicity of *Mentha pulegium* and *Thymus mastichina* EOs to *Frankliniella occidentalis* had LC₅₀ values of 3.1 and 3.6 mg/L, respectively [33]; oregano oil and savory oil showed high insecticidal activity against *Plodia interpunctella* and *Ephestia kuehniella*, reaching 100% mortality within 24 h at concentrations of 9 μ L/L air (for *P. interpunctella*) and 25 μ L/L air (for *E. kuehniella*) [34]. The difference in toxicity in our study compared to these may be due to different testing methods or insect species. While toxicity tests are often conducted under laboratory conditions, we simulated natural conditions in pot experiments [1]. In the pot experiment analysis, we found that patchouli oil, lavender oil, and catnip oil were consistent with the bioassay results and showed good pest control effects; thus, they can be developed as potential insecticides. Additionally, although EOs are generally considered highly volatile [13] and require microencapsulation for sustained release [35], in our study, catnip oil still exhibited persistent insecticidal effects after seven days. This suggests that we may need to reassess the impact of the volatilization of some EOs on practical applications.

EOs play a crucial role in mediating interactions between plants and their environment [36]. Therefore, we evaluated the behavioral impacts of four EOs on *T. flavus*. Our results indicate that catnip oil and lavender oil have attractive effects on *T. flavus*, potentially enhancing insecticidal effects through attraction before killing [37]. Notably, in some studies, catnip oil could repel *A. aegypti* [38], *S. calcitrans* [11], and *Blattella germanica* [39]; this difference may be due to concentration variations. A study by Bedini et al. indirectly supports this finding, showing that the effects of six EOs on *Sitophilus zeamais* changed from attractive to repellent as the concentration increased [36]. Whether higher concentrations of catnip oil would repel *T. flavus* requires further investigation. In *Drosophila melanogaster* and *A. aegypti*, catnip oil activates the widely conserved chemosensory receptor TRPA1, and mutations in TRPA1

prevent these insects from being repelled by catnip oil. We speculate that the receptor for catnip oil in *T. flavus* is also TRPA1 [40]. Most studies on lavender oil have focused on its repellent effects, such as against Tabanidae and *Tribolium confusum* [41,42]. However, in our study, lavender oil was attractive to *T. flavus*. Interestingly, lavender oil only attracted male *T. flavus*, similar to the effects of sex pheromones on male *T. flavus* [43]. Therefore, lavender oil could serve as a potential substitute for more expensive pheromones.

In recent years, EOs have received considerable attention due to their herbicidal activity and environmental friendliness [23–25]. Some EOs cause electrolyte leakage leading to cell death in weeds [27,44]. Therefore, it is necessary to assess the safety of these EOs on crops. Our results show that all four EOs had varying negative impacts on crop growth and development, validating our concerns. We observed that the EOs had different effects on two crops and two weeds. Among them, lavender oil had the least impact on crops, only significantly affecting the shoot length of *G. max* but not impacting the weeds. Thus, lavender oil is not suitable as a potential herbicide but could be a potential insecticide. Mint oil did not adversely affect *G. max* growth but had significant effects on *Z. mays* at different growth stages, suggesting that it might be more suitable as a specific herbicide for *G. max* fields. Patchouli oil and catnip oil affected certain growth stages of crops. If these EOs are to be used as potential herbicides, care should be taken to avoid specific crops or growth stages, such as using mint oil only in *G. max* fields. Interestingly, patchouli oil and mint oil only affected the growth of *P. oleracea*. Therefore, patchouli oil and mint oil can be further developed as specific herbicides targeting *P. oleracea*.

Finally, we preliminarily analyzed the chemical compositions of the EOs to provide insights into their active components. The main chemical constituents identified in this study were consistent with those in other studies, but there were differences in their concentrations [45–49]. These differences depend on genetic, environmental, and processing factors [47]. The effects of EOs on insects and plants are mainly due to the presence of terpenoid compounds. In our study, we found a high content of terpenoids in the four EOs, including patchoulol, β -caryophyllene, α -guaiane, and α -bulnesene in patchouli oil; limonene and eugenol in catnip oil; α -pinene and camphor in lavandula oil; and menthol, menthone, and limonene in mint oil. For insects, some hypotheses support the notion that monoterpenes act on cytochrome P450, and certain terpenoids inhibit acetylcholinesterase activity [50]. For plants, some terpenes can inhibit seed germination and plant growth [51]. For example, eugenol in *Syzygium aromaticum* EO completely inhibits seed germination [52].

In summary, this study evaluated the bioactivity of four Lamiaceae EOs on *T. flavus*, crops, and weeds. We found that patchouli oil, catnip oil, and lavender oil had good insecticidal activity against *T. flavus*, with patchouli oil being the most potent. These EOs showed persistent insecticidal effects in pot experiments, indicating their potential as insecticides. Additionally, catnip oil and lavender oil had attractive effects on *T. flavus*, potentially enhancing insecticidal effects through attraction before killing. Regarding the impact on crop growth and development, lavender oil had the least impact on crops, while mint oil seemed more suitable as a specific herbicide for *G. max* fields. Finally, we analyzed the chemical compositions of the EOs to provide a basis for understanding their active components. Overall, these EOs have broad prospects as natural insecticides and herbicides, but further studies on the mechanisms and application conditions of the EOs are required in subsequent research.

4. Materials and Methods

4.1. Insects

The *T. flavus* individuals used in the experiment were collected from a *G. max* field in Changchun, Jilin Province (125°24′31″ E, 43°47′51″ N). They were captured using a

sweep net and reared in insect cages on *G. max* for 2–3 days. Referring to the method by Han et al., the thrips species was identified under a stereomicroscope to ensure that it was *T. flavus* [53]. The rearing conditions were set to a temperature of 25 ± 1 °C, a relative humidity of $70\% \pm 5\%$, and a photoperiod of 16 h/8 h.

4.2. Essential Oils

The four Lamiaceae EOs tested were patchouli oil (*Pogostemon cablin*), catnip oil (*Nepeta cataria*), lavender oil (*Lavandula angustifolia*), and mint oil (*Mentha canadensis*). All EOs were provided by Ji'an Zhongxiang Natural Plants Co., Ltd., Ji'an, China, and they were extracted using the water–steam distillation method. The purity of the EOs was 98%.

4.3. Toxicity Test on *T. flavus*

To evaluate the toxicity of the EOs to the *T. flavus* adults, a feeding assay was performed. The method was based on oral toxicity with a few modifications [1]. The four EOs were pre-dissolved in acetone (Tianjin Xintong Fine Chemical Co. Ltd., Tianjin, China, purity 99.5%) and diluted with water to concentrations of 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL, and 1.0 mg/mL (acetone content was 0.5% for all solutions); 45% malathion (purchased from Hebei Jindelun Biochemical Technology Co. Ltd., Shijiazhuang, China; emulsifiable concentrate) served as the positive control. Fresh leaves of *G. max* (variety “Jinong 28”, provided by the Jilin Agricultural University), free from disease and insect damage and of uniform size, were selected, washed with distilled water, and air-dried. The leaves were then immersed in the test solution for 10 s, removed, and allowed to air dry. They were subsequently placed in 50 mL plastic centrifuge tubes containing moist filter paper. Water containing acetone (0.5% acetone content) served as the control. Thirty 3-day-old *T. flavus* adults were introduced into the test tubes for the experiment. Each treatment was replicated three times. After 24 h at room temperature, the mortality of *T. flavus* was checked, and the total numbers of insects and dead insects were recorded.

$$M_1 = \frac{ND}{NA} \times 100 \quad (1)$$

$$M_2 = \frac{MR_1 - MR_2}{1 - MR_2} \times 100 \quad (2)$$

where M_1 is the mortality (%); ND is the number of dead thrips; NA is the total number of thrips treated; M_2 is the corrected mortality (%); MR_1 is the mortality rate of the treatment (%); and MR_2 is the mortality rate of the blank control (%).

4.4. Pot Experiments

When the second trifoliate leaf emerged on the *G. max* plants, pots with uniform growth were selected, and one healthy *G. max* per pot was retained. No pesticides were sprayed on *G. max* prior to the experiment. Before conducting the experiments, each soybean pot was watered to ensure that the plants had sufficient moisture. Subsequently, the top of the pots was sealed with a cardboard sheet to prevent the *T. flavus* from falling into the soil after death and to minimize soil moisture loss. No further watering was conducted during the experiment. Based on the indoor bioassay results, the pesticide was applied at a rate equivalent to 900 L of solution per hectare (1 hm^2). Each dose was replicated three times, with untreated controls (ddH₂O) included. Using a spray bottle, the solution was applied uniformly, with 5 mL applied to each pot, and each plant was infested with 30 *T. flavus* adults. The pots were arranged with a 1 m spacing between treatments in a randomized block design. The number of dead thrips was observed and recorded 1 day,

3 days, and 7 days after spraying, and the efficacy in the pot trials was calculated according to the following formula:

$$CE = \left(1 - \frac{AT \times BC}{BT \times AC}\right) \times 100 \quad (3)$$

where *CE* is the control efficacy (%); *AT* is the number of live thrips in the treated pots after treatment; *BC* is the number of live thrips in the control pots before treatment; *BT* is the number of live thrips in the treated pots before treatment; and *AC* is the number of live thrips in the control pots after treatment.

4.5. Behavioral Assays

The olfactory behavior responses of *T. flavus* to the plant EOs were tested using a Y-tube olfactometer [1]. The EOs were diluted to a concentration of 1.0 mg/mL in acetone. A 1 µL aliquot of the oil solution was applied to a 1 cm × 1 cm filter paper strip and placed in the odor-source bottle. Acetone alone was used as the control. The stem of the Y-tube was connected to a vacuum pump, and air was blown into the main arm of the Y-tube glass apparatus at a flow rate of 0.5 L/min. To maintain consistent lighting, the Y-tube was placed inside a light box during the experiment, with an average light intensity of 7800 to 8000 lx. Each adult thrips was observed for 5 min. When the thrips crossed the midpoint of either arm of the Y-tube, it was considered to have chosen the reagent on that side, and the attraction and repellency rates were calculated. If no choice was made within 5 min, it was recorded as no selection. Thirty male and thirty female adult thrips were tested for each EO. The sex of the thrips was determined by examining the shape of their genitalia under a stereomicroscope. The *T. flavus* individuals used in the behavioral assays were all 3 days old. The ovipositor of the female *T. flavus* consists of two pairs of sclerotized, toothed valves, with grooves between them that serve as the passage for eggs. The aedeagus of the male *T. flavus* is a slender, backward-curving structure that extends slightly upward, featuring either a pointed or blunt tip [21]. The numbers of thrips choosing the treatment and control groups were recorded, and the attraction and repellency rates were calculated using Formulas (1) and (2), respectively:

$$S = \frac{CE}{Rs} \times 100 \quad (4)$$

$$R = \frac{CC}{Rs} \times 100 \quad (5)$$

where *S* is the attraction rate (%); *CE* is the number of insects choosing the EO; *Rs* is the number of insects with a response; *R* is the repellency rate (%); and *CC* is the number of insects choosing the control.

4.6. Phytotoxic Activity of EOs

A plant toxicity test was conducted following the method described by Kasrati et al. [54]. The four EOs were mixed with Tween-80 (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) at a ratio of 1:1 (*v/v*). The mixtures were then diluted with distilled water to five concentrations: 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL. Distilled water containing Tween-80 was used as the control. Plant toxicity was tested on four plants: *Glycine max* (variety “Jinong 28”, provided by the Jilin Agricultural University), *Z. mays* (corn variety JS416, purchased from Jilin Huawei Agricultural Science and Technology Development Co., Ltd., Jilin, China), *E. oryzoides*, and *P. oleracea*. The seeds of these plants were disinfected with 1% sodium hypochlorite (Guangdong Wenlong Biotechnology Co., Ltd., Yangjiang, China); then, they were rinsed three times with distilled water and filtered. The disinfected seeds were placed in glass Petri dishes. Thirty seeds were added to each dish, and the EO solutions

were applied, with three replicates for each concentration. One layer of filter paper was placed above and below each layer of seeds. The filter papers were sprayed daily with the corresponding concentrations of EO solutions to keep them moist. Seed germination was considered to occur when the radicle broke through the seed coat and reached a length of 1 mm. All treatments were incubated in complete darkness at a temperature of 25 ± 1 °C and a relative humidity of $70 \pm 5\%$ in a controlled growth chamber. On the 7th day, the shoot lengths (SLs) were measured using a vernier caliper. The germination rate, germination potential, and germination index were calculated.

The calculation formulas for the germination rate, germination potential, and germination index were as follows:

$$GR = \frac{G_1}{G_0} \times 100 \quad (6)$$

$$GP = \frac{G_2}{G_0} \times 100 \quad (7)$$

$$GI = \sum \frac{Gt}{Dt} \quad (8)$$

where *GR* is the germination rate, G_1 is the number of germinated seeds, G_0 is the total number of seeds, *GP* is the germination potential, G_2 is the number of seeds germinated by day 3, *GI* is the germination index, *Gt* is the number of seeds germinated on the day, and *Dt* is the number of days of germination.

4.7. Chemical Composition Analysis

The chemical components of the EOs were analyzed using gas chromatography–mass spectrometry (GC-MS) equipped with a DB-5 capillary column (30 m × 0.25 mm i.d., 0.25 µm). The injection mode and temperature program followed the methods described by Pei et al. [1]. Compound identification was performed using GC-MS software applications by making a comparison with the mass spectral libraries NIST 147 and NIST 27.

4.8. Statistics

The toxicity regression equation was fitted using DPS 13.50 [53] (Hangzhou Ruifeng Information Technology Co., Ltd., Hangzhou, China, <http://www.dpsw.cn>, accessed on 14 September 2024) to obtain the correlation coefficient, calculate the LC_{50} value, and determine the 95% confidence interval. The pot experiment results were subjected to arcsine square root transformation using IBM SPSS Statistics (version 23.0, International Business Machines Corporation, Armonk, NY, USA), and significant differences among treatments were compared using Tukey's test in a one-way ANOVA. The results of the olfactory behavior response tests were analyzed using a chi-square test to compare the significant differences between the treatment and control groups. Graphs were created using GraphPad Prism 9.50 (GraphPad Software, Boston, MA, USA).

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Article

Antifungal Activity of Citrus Essential Oil in Controlling Sour Rot in Tahiti Acid Lime Fruits

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Abstract: Sour rot, caused by *Geotrichum citri-aurantii*, is a significant post-harvest disease in citrus, resulting in economic losses due to the lack of effective fungicides. This study investigates the antifungal activity of citrus essential oils in controlling sour rot in Tahiti acid lime fruits. Essential oils were extracted via hydrodistillation with chemical composition analyzed by CG-MS and tested in vitro and in vivo. In vitro assays evaluated mycelial growth inhibition at 2 to 32 $\mu\text{L mL}^{-1}$ concentrations. In vivo trials involved preventive and curative treatments on artificially inoculated fruits stored at $25\text{ }^{\circ}\text{C} \pm 2$, and the results showed that Pera IAC sweet orange oil, at 32 $\mu\text{L mL}^{-1}$, reduced disease severity by 96% in curative treatments. In contrast, Late IAC 585 willowleaf mandarin oil demonstrated moderate inhibition (44%) at the highest concentration in vitro. The oils did not affect key fruit quality parameters such as juice yield and total soluble solids. These findings suggest that citrus essential oils could be natural alternatives to synthetic fungicides for post-harvest sour rot management, combining effectiveness with maintaining fruit quality.

Keywords: natural fungicides; post-harvest; biocontrol; agricultural sustainability

1. Introduction

Citrus farming plays a crucial role in global agriculture, with Brazil standing out as one of the leading citrus producers. In 2022, Brazil ranked as the fourth-largest global producer of limes and lemons, with an estimated production of 1.06 million tons [1]. Brazilian production is significant for the domestic fresh fruit market and a major exporter of frozen concentrated orange juice in volume and value [2]. Brazil exported, in 2023, about 167 thousand tons of Tahiti acid lime, mainly to Europe, representing an increase of 113% in the last 10 years [3]. However, India leads the global market, producing 3.77 million tons of limes and lemons in the same year [1].

In citrus, post-harvest diseases are a significant factor that reduces the quantity of fruit available to consumers, resulting in a decline in fruit quality and quantity from harvest to the point of sale [4]. Sour rot and green and blue molds, caused by *Geotrichum citri-aurantii*, *Penicillium digitatum*, and *Penicillium italicum*, are the main infections affecting citrus fruit post-harvest, leading to losses ranging from 25% to 50% of total production [5,6].

Sour rot is one of the most prevalent citrus diseases globally, affecting all cultivated varieties, including Tahiti acid lime, and primarily infecting fruit through wounds. Therefore, careful harvesting, handling, and storage are crucial to reducing the incidence of this

disease. Many chemical fungicides used in post-harvest treatments, such as Thiabendazole and Imazalil, commonly used to control other post-harvest diseases (green or blue mold), are ineffective against *G. citri-aurantii* [7]. Moreover, some of these products face restrictions in certain countries, contribute to water and soil pollution, and promote increased pathogen resistance [8]. As a result, it is essential to find new, natural, safe, and environmentally friendly compounds to control sour rot during the post-harvest phase.

Studies indicate that essential oils extracted from plants act as natural fungicides, inhibiting fungal growth without leaving harmful toxic residues [9,10]. These oils work directly by inhibiting mycelial growth and spore germination and stimulating the production of phytoalexins in plants, acting as elicitors [11,12].

Essential oils can be extracted from plants through steam distillation, the most common method, and by cold pressing the peel of citrus fruits. These oils have perfumery, cosmetics, and food applications and are also used as herbicides, insecticides, and fungicides [13]. The compounds in essential oils can act directly on pathogens, altering the transmembrane potential, reducing ATP synthesis, and causing significant damage to DNA and mitochondria [14,15].

Due to the lack of effective commercial fungicides for controlling *G. citri-aurantii* [7], citrus essential oils are expected to replace traditional fungicides in managing post-harvest diseases due to their excellent multifaceted properties. In this study, we investigated the inhibitory efficacy of citrus essential oils against *G. citri-aurantii* in vitro, their effectiveness in controlling sour rot in vivo in Tahiti acid lime fruits, and their impact on fruit quality.

2. Results

2.1. Yield of Essential Oils (Eos)

The essential oil extraction yield from each variety investigated Late IAC 585, BRS Rainha, and Rio IAC 194 willowleaf mandarins (*Citrus × deliciosa*), Murcott IAC 221 tanger (*C. × sinensis × C. reticulata*), and Pera IAC sweet orange (*Citrus × sinensis*) and was quantified and expressed as a percentage according to Equation (1). The sample with the highest extraction yield was Late IAC 585 (0.16 mL 100 g^{−1}), followed by BRS Rainha (0.13 mL 100 g^{−1}) and Pera IAC (0.11 mL 100 g^{−1}). Murcott IAC 221 tanger and Rio IAC 194 willowleaf had lower extraction yields, with 0.08 and 0.06 mL 100 g^{−1}, respectively.

2.2. Chemical Composition of EOs by GC-MS

The chemical compositions of the EOs were determined using GC-FID and GC-MS chromatographic analyses, in which 38 volatile compounds were identified and 1 unidentified (Table 1), highlighting monoterpenes, sesquiterpenes, aldehydes, and alcohols, among others. The highest number of compounds were found in the Pera IAC oil (25 compounds) and BRS Rainha (28 compounds); on the other hand, a smaller number was observed in the Murcott IAC 221 essential oil (20 compounds), while intermediate values of components are present in the Late IAC 585 and Rio IAC 194 oils (23 compounds).

Table 1. Peel essential oil chemical composition of different citrus evaluated (IAC, Cordeirópolis-SP, Brazil).

Volatile Compounds	RI _{calc} ¹	Pera IAC	Late IAC 585	Rio IAC 194	BRS Rainha	Murcott IAC 221
		Relative Percentage (%)				
2-Thujene	926	0.36	0.48	0.49	0.52	0.00
α-Pinene	933	1.09	1.91	1.41	1.41	0.48
Sabinene	973	0.27	0.40	0.27	0.27	0.41
β-Pinene	977	1.28	0.91	1.14	1.56	0.06
β-Mircene	991	2.01	2.59	2.12	1.81	2.00
Octanal	1003	0.15	0.00	0.16	0.15	2.30

Table 1. Cont.

Volatile Compounds	RI _{calc} ¹	Pera IAC	Late IAC 585	Rio IAC 194	BRS Rainha	Murcott IAC 221
		Relative Percentage (%)				
α-Phellandrene	1006	0.09	0.84	0.07	0.07	0.00
α-Terpinene	1017	0.49	0.30	0.32	0.46	0.00
o-Cymene	1025	0.65	0.92	2.51	3.10	0.00
Limonene	1032	66.81	76.53	71.24	62.40	81.92
trans-β-Ocimene	1047	0.00	0.16	0.00	0.00	0.00
γ-Terpinene	1059	17.59	10.64	15.86	17.67	0.12
1-Octanol	1070	0.00	0.00	0.00	0.00	1.92
Terpinolene	1088	1.06	0.61	0.86	1.02	0.00
Linalool	1101	0.74	0.96	0.45	1.08	5.80
Nonanal	1105	0.00	0.00	0.00	0.00	0.39
Citronellal	1152	0.08	0.00	0.13	0.12	0.65
1-Nonanol	1171	0.00	0.00	0.00	0.00	0.10
Terpinen-4-ol	1177	0.83	0.12	0.17	1.05	0.20
α-Terpineol	1191	1.68	0.16	0.31	1.77	0.55
Decanal	1205	0.14	0.00	0.16	0.13	1.11
cis-Carveol	1219	0.00	0.00	0.00	0.08	0.00
Citronellol	1229	0.37	0.00	0.24	0.56	1.19
Carvone	1243	0.00	0.00	0.00	0.09	0.10
Geranial	1270	0.00	0.00	0.00	0.12	0.00
Perillaldehyde	1273	0.42	0.00	0.17	0.46	0.46
Limone-10-ol	1290	0.12	0.00	0.00	0.18	0.15
Thymol	1296	0.19	0.11	0.10	0.34	0.00
Neryl acetate	1365	0.00	0.00	0.00	0.00	0.10
Methyl N-methylantranilate	1406	2.86	0.00	1.24	2.86	0.00
Caryophyllene	1418	0.13	0.22	0.00	0.10	0.00
β-Farnesene	1457	0.00	0.50	0.00	0.00	0.00
Germacrene D	1479	0.00	0.17	0.00	0.00	0.00
Bicyclogermacrene	1495	0.00	0.90	0.00	0.00	0.00
α-Farnesene	1508	0.19	0.00	0.31	0.25	0.00
δ-Cadinene	1522	0.00	0.17	0.00	0.00	0.00
NI ²	1555	0.00	0.19	0.00	0.00	0.00
Spathulenol	1576	0.00	0.21	0.00	0.00	0.00
α-Sinesal	1753	0.41	0.00	0.30	0.37	0.00

¹ RI_{calc}—Retention Index calculated. ² NI—Not identified.

2.3. Bioactivity of Essential Oils on the Mycelial Growth of *G. citri-aurantii*

The Late IAC 585 and Pera IAC oils at a concentration of 32 µL mL^{−1} provided average inhibition rates of mycelial growth of *G. citri-aurantii* by direct contact of approximately 44% and 25%, respectively (Table 2).

Additionally, the oil from willowleaf mandarin (32 µL mL^{−1}) resulted in a reduction of approximately 57% (MGRI) in the speed of mycelial growth of the pathogen compared to the control (Table 2). These results suggest that essential oils of both varieties inhibit *G. citri-aurantii* growth in a dose-dependent manner in vitro, making both samples viable for in vivo trials for disease control in fruit. Although the other oil samples, Rainha, Murcott, and Rio, reduced the growth rate index compared to the control and showed slight inhibition on the plates, they were less effective at inhibiting the pathogen's mycelial growth.

Using the Biplot, it was possible to identify which compounds discriminated the samples the most and highlight the relationships between them (Figure 1). The first two components, CP1 and CP2, explained 86.1% of the variability between the descriptors, with the first component capturing 48.4% of the total data variability and the second 37.7% of this variation.

Table 2. Effect of essential oil doses on antifungal activity by contact: mycelial growth rate index (MGRI), the area under the mycelial growth curve (AUMGC), and percentage mycelial growth inhibition (PIMG) of *G. citri-aurantii* compared to the control (cm) in vitro.

Essential Oil Samples	Doses ($\mu\text{L mL}^{-1}$)	MGRI	AUMGC	PIMG
BRS Rainha willowleaf	2	1.41 bc *	25.13 bc	2.4 ab
	4	1.67 ab	27.50 ab	1.2 ab
	8	1.57 abc	25.38 bc	6.2 ab
	16	1.63 ab	25.94 bc	2.5 ab
	32	1.76 a	22.77 bc	12.0 a
	Tween	1.37 c	24.49 bc	10.6 a
	Control	1.64 ab	30.75 a	
	CV (%) **	7.2	7.1	
Murcott IAC 221 tangor	2	1.81 a	27.69 ab	0.70 a
	4	1.52 b	21.83 c	1.64 a
	8	1.19 c	17.92 d	1.87 a
	16	1.25 c	18.64 d	2.25 a
	32	-	-	-
	Tween	1.64 ab	27.08 b	0.70 a
	Control	1.64 ab	30.75 a	
	CV (%)	5.88	5.91	
Rio IAC 194 Willowleaf	2	1.82 c	23.61 bc	12.5 a
	4	1.92 bc	24.83 bc	8.48 ab
	8	1.83 bc	23.30 bc	8.95 ab
	16	1.81 c	22.99 c	10.63 ab
	32	1.92 bc	24.80 bc	5.13 ab
	Tween	2.06 ab	25.54 b	3.53 b
	Control	2.29 a	29.39 a	
	CV (%)	6.1	4.64	
Late IAC 585 willowleaf	2	1.71 ab	23.18 a	0.63 c
	4	2.04 a	26.30 a	4.5 abc
	8	1.95 a	24.89 a	7.75 bc
	16	2.12 a	28.33 a	11.0 b
	32	0.99 b	14.00 b	44.38 a
	Tween	2.06 a	25.55 ab	2.12 bc
	Control	2.29 a	29.38 a	
	CV (%)	21.46	20.89	
Pera IAC	2	1.72 a	22.09 b	0 b
	4	1.56 ab	30.38 b	0.73 b
	8	1.71 a	19.28 b	0 b
	16	1.23 b	13.43 c	23.70 a
	32	1.27 b	11.51 c	24.83 a
	Tween	1.64 ab	21.53 b	0 b
	Control	1.83 a	25.66 a	
	CV (%)	8.70	5.79	

* The means in columns for each essential oil followed by the same letter do not differ according to the Tukey test at a significance level of 5%; ** Coefficient of variation.

In general, it is possible to identify the presence of three segregated groups. The first group, formed by the Late sample, is represented almost entirely by the substances β -Mircine, Bicyclogermacrene, α -Phellandrene, and β -Farnesene. The second group, formed by the Rio, Pera, and Rainha samples, corresponds to the compounds γ -Terpinene, Methyl N-methylanthranilate, α -Terpineol, β -Pinene, Terpinolene, Terpinen-4-ol, o-Cymene, and α -Terpinene. Finally, the third group is formed by Murcott tangor, with its compounds grouped as Linalool, Octanal, 1-Octanol, Citronellol, Decanal, Citronellal, and Nonanal.

Using the Biplot, it was also possible to identify that the PIMG values at a dose of $32 \mu\text{L mL}^{-1}$ were grouped together in the Late mandarin oil sample, while the AUMGC and MGRI values at the same dose were grouped together in the essential oil samples from the Rio, Pera, and Rainha varieties.

For the mycelial growth rate index, in the evaluation of antifungal activity, none of the oils evaluated showed a statistically significant difference from the control (Table 3).

Table 3. Effect of essential oil doses on the antifungal activity of volatile compounds: mycelial growth rate index (MGRI), the area under the mycelial growth curve (AUMGC), and percentage mycelial growth inhibition (PIMG) of *G. citri-aurantii* compared to the control (cm) in vitro.

Essential Oil Samples	Doses ($\mu\text{L mL}^{-1}$)	MGRI	AUMGC	PIMG
BRS Rainha willowleaf	2	1.69 b *	8.02 c	28.22 a
	4	1.76 b	8.46 bc	24.48 a
	8	1.73 b	8.39 bc	25.31 a
	16	1.78 b	8.52 bc	23.23 a
	32	1.94 ab	7.69 c	27.38 a
	Tween	1.62 b	9.00 b	24.06 a
	Control	2.2 a	11.27 a	-
	CV (%) **	9.97	4.54	17.69
Murcott IAC 221 tangor	2	1.69 a	8.20 c	16.75 a
	4	1.76 a	8.46 bc	12.92 ab
	8	1.73 a	8.39 c	13.87 ab
	16	1.78 a	8.52 bc	11.48 ab
	32	-	-	-
	Tween	1.25 a	9.54 b	5.26 b
	Control	1.73 a	11.17 a	-
	CV (%)	15.08	5.60	34.80
Rio IAC 194 Willowleaf	2	2.03 a	8.37 a	4.08 a
	4	1.95 a	7.79 a	6.24 a
	8	1.95 a	8.19 a	6.02 a
	16	1.90 a	7.69 a	7.09 a
	32	1.92 a	7.86 a	11.61 a
	Tween	1.87 a	7.95 a	9.46 a
	Control	1.80 a	8.42 a	-
	CV (%)	7.86	8.33	32.62
Late IAC 585 willowleaf	2	1.67 ab	9.32 b	2.72 b
	4	1.83 a	9.35 b	5.44 b
	8	1.87 a	9.68 ab	6.46 b
	16	1.50 b	6.25 c	29.93 a
	32	1.49 b	5.84 c	30.95 a
	Tween	1.60 ab	8.74 b	6.42 b
	Control	1.65 ab	10.36 a	-
	CV (%)	6.01	4.16	18.79
Pera IAC	2	1.67 ab	8.82 a	2.72 b
	4	1.85 a	8.45 a	4.76 b
	8	1.85 a	8.16 a	7.14 b
	16	1.50 b	5.96 b	29.93 a
	32	1.49 b	5.64 b	30.95 a
	Tween	1.60 ab	8.76 a	4.76 b
	Control	1.66 ab	9.04 a	-
	CV (%)	5.77	6.08	16.88

* The means in columns for each essential oil followed by the same letter do not differ according to the Tukey test at a significance level of 5%; ** Coefficient of variation.

However, regarding the AUMGC, it was noted that Late willowleaf mandarin and Pera IAC sweet orange oils, at doses of 16 and 32 $\mu\text{L mL}^{-1}$, significantly differed from the control, with a mycelial inhibition percentage of *G. citri-aurantii* of approximately 31% for both doses. Rainha mandarin oil showed an inhibition percentage of around 25.7% compared to the control treatment, with no significant differences among the evaluated dosages. The other tested oils did not inhibit the pathogen's mycelial growth.

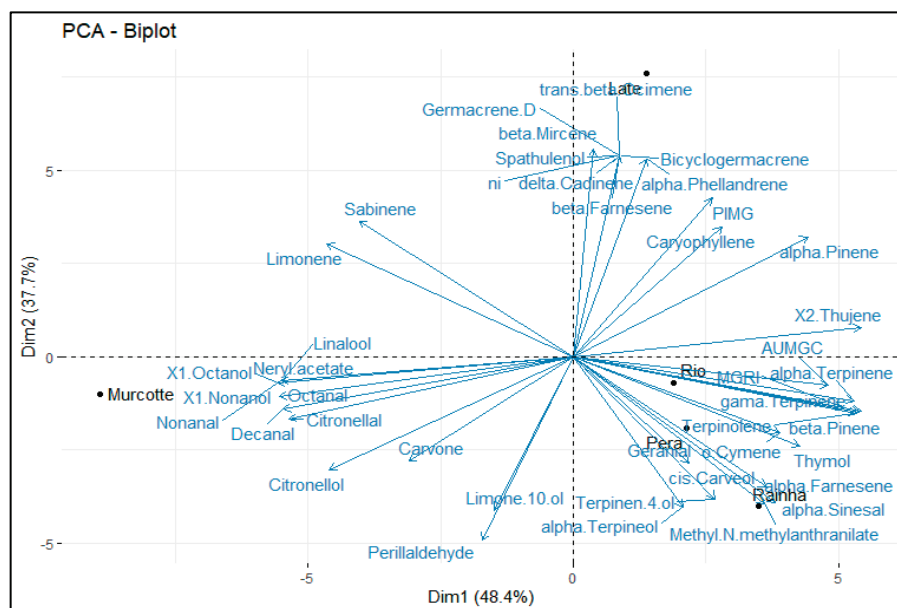


Figure 1. Biplot of 39 chemical components of the essential oils used (Late IAC 585, BRS Rainha, Rio IAC 194 willowleaf mandarins, Murcott IAC 221 tangor and Pera IAC sweet orange and MGRI, AUNG, and PIMG values at a dose 32 $\mu\text{L mL}^{-1}$).

2.4. Effectiveness of Essential Oils in Controlling Sour Rot in Post-Harvest Citrus

The results for disease severity showed that the contrast between the additional treatment (Control) and the triple factorial (Treatment $\times$ Oil $\times$ Dose) was not significant (p -value = 0.298), indicating that the means of the two groups are statistically equal. The interaction was insignificant when analyzing the triple factorial (Treatment $\times$ Oil $\times$ Dose) (p -value = 0.934). In this case, the breakdown of factors was examined: Treatment $\times$ Oils, Treatment $\times$ Doses, and Oils $\times$ Dose, where only the interactions Treatment $\times$ Doses and Oils $\times$ Dose were significant (p -value = 0.011 and p -value = 0.0068, respectively), as shown in Tables 4 and 5. The data suggest that fruits treated curatively with Pera IAC sweet orange oil at a concentration of 32 $\mu\text{L mL}^{-1}$ statistically differed from the other treatments, showing lower average lesion diameters of sour rot in Tahiti acid lime fruits stored at 25 $^{\circ}\text{C} \pm 2$.

Table 4. The area under the disease progress curve (AUDPC), evaluated by the average lesion diameter (mm) caused by *G. citri-aurantii* in Tahiti acid lime fruits treated with preventively and curatively with concentrations of 32 and 64 $\mu\text{L mL}^{-1}$.

Treatments	Doses	
	32 $\mu\text{L mL}^{-1}$	64 $\mu\text{L mL}^{-1}$
Preventive	62.31 Aa *	53.47 Aa
Curative	17.30 Bb	61.17 Aa

* Means followed by the same letter in the row or column do not differ at a 5% significance level. Tukey's test applied to columns (uppercase letters) and rows (lowercase letters).

Looking at the data in the three-fold split, it can also be inferred that in the preventive treatment with Late and Pera IAC oils, the 64 $\mu\text{L mL}^{-1}$ dose resulted in a higher AUDPC compared to the 32 $\mu\text{L mL}^{-1}$ dose, leading to a 48.84% reduction in average lesion diameter compared to the lower dose. In evaluating preventive and curative treatments at doses of 32 and 64 $\mu\text{L mL}^{-1}$, there were no statistically significant differences compared to the control group for the average lesion diameter of sour rot. In the curative treatment, it was found that Pera IAC mandarin oil at 32 $\mu\text{L mL}^{-1}$ resulted in a lower AUDPC than the higher dose, reducing disease severity by 99.3% (Supplementary Material).

Table 5. The area under the disease progress curve (AUDPC), evaluated by the average lesion diameter (mm) caused by *G. citri-aurantii* in Tahiti acid lime fruits treated with Late willowleaf mandarin and Pera IAC sweet orange essential oils at concentrations of 32 and 64 $\mu\text{L mL}^{-1}$.

Essential Oils	Doses	
	32 $\mu\text{L mL}^{-1}$	64 $\mu\text{L mL}^{-1}$
Late	56.02 Aa *	45.10 Aa
Pera IAC	23.59 Bb	69.54 Aa

* Means followed by the same letter in the row or column do not differ at a 5% significance level. Tukey's test applied to columns (uppercase letters) and rows (lowercase letters).

Regarding the incidence of sour rot, there was no significant difference between the additional treatment and the triple factorial (p -value = 0.2178). The triple interaction of factors (Treatment $\times$ Oil $\times$ Dose) was insignificant (p -value = 0.2231). However, the two-way interactions Treatment $\times$ Dose (p -value = 1×10^{-4}) and Oil $\times$ Dose (p -value = 0.0023) were significant (Tables 6 and 7). Notably, fruits treated curatively with orange oil at 32 $\mu\text{L mL}^{-1}$ showed a positive effect in controlling sour rot.

Table 6. Disease incidence was evaluated by the percentage of fruits showing sour rot symptoms caused by *G. citri-aurantii* in Tahiti acid lime fruits treated preventively and curatively with 32 and 64 $\mu\text{L mL}^{-1}$ of Late willowleaf mandarin and Pera IAC sweet orange essential oils.

Treatments	Doses	
	32 $\mu\text{L mL}^{-1}$	64 $\mu\text{L mL}^{-1}$
Preventive	72.90 Aa *	63.89 Ba
Curative	32.22 Bb	91.48 Aa

* Means followed by the same letter in the row or column do not differ at a 5% significance level. Tukey's test applied to columns (uppercase letters) and rows (lowercase letters).

Tahiti acid lime fruits were preventively treated with Late oil at a concentration of 64 $\mu\text{L mL}^{-1}$, and despite reducing the percentage of diseased fruits by 31.7%, there was no statistical difference from the control. We found that Tahiti acid lime fruits curatively treated with Pera IAC sweet orange oil at a concentration of 32 $\mu\text{L mL}^{-1}$ showed a control efficiency of approximately 96% of sour rot about the control when we observed the three-way split of the data (Figure 2).

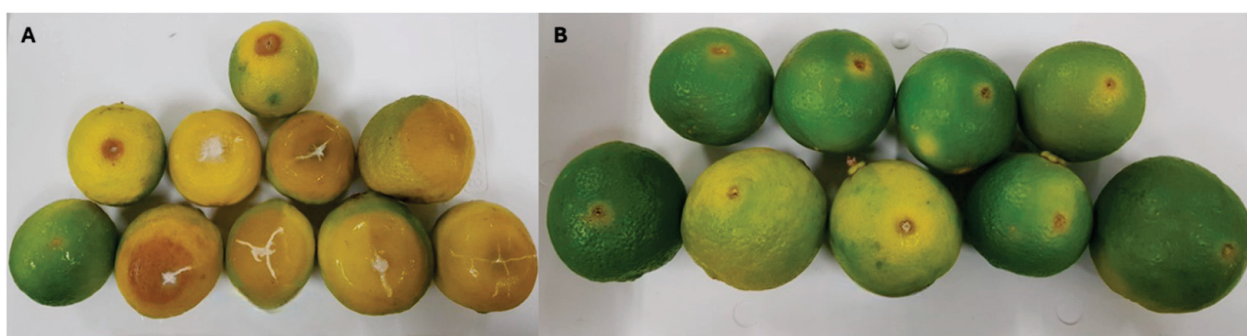


Figure 2. Tahiti acid lime fruits inoculated with *Geotrichum citri-aurantii* and curatively treated: (A) control (distilled water) and (B) Pera IAC sweet orange essential oil 32 $\mu\text{L mL}^{-1}$. They were stored for 14 days at $25\text{ }^{\circ}\text{C} \pm 2$ and 70% RH.

Table 7. Disease incidence was evaluated by the percentage of fruits showing sour rot symptoms caused by *Geotrichum citri-aurantii* in Tahiti acid lime fruits treated with Late willowleaf mandarin and Pera IAC sweet orange essential oils at concentrations of 32 and 64 $\mu\text{L mL}^{-1}$.

Essential Oils	Doses	
	32 $\mu\text{L mL}^{-1}$	64 $\mu\text{L mL}^{-1}$
Late	69.89 Aa *	70.14 Aa
Pera IAC	35.23 Bb	85.23 Aa

* Means followed by the same letter in the row or column do not differ at a 5% significance level. Tukey's test applied to columns (uppercase letters) and rows (lowercase letters).

2.5. Fruit Quality in Citrus After Oleo Essential Treatment

The application of Late willowleaf mandarin and Pera IAC sweet orange essential oils at concentrations of 32 and 64 $\mu\text{L mL}^{-1}$ did not significantly change the fruit quality parameters (average juice yield, total soluble solids (SS), total titratable acidity (TTA), and SS/TTA ratio) under commercial conditions when compared to the reference values determined by Pio et al. [16]. This indicates that the treated Tahiti acid lime fruits have commercial potential after applying the treatments (Table 8).

Table 8. Physicochemical characteristics of Tahiti acid lime fruits treated with Late willowleaf mandarin and Pera IAC sweet orange essential oils at concentrations of 32 and 64 $\mu\text{L mL}^{-1}$ and stored at $25\text{ }^{\circ}\text{C} \pm 2$, 2023 harvest (Citrus Center Silvio Moreira—Cordeirópolis, SP).

Juice Yield			
Treatments	One Day	7 Days	14 Days
Control	44.4 *	48.8	51.6
Late (32 $\mu\text{L mL}^{-1}$)	52.6	51.5	51.9
Late (64 $\mu\text{L mL}^{-1}$)	52.0	53.6	50.0
Pera IAC (32 $\mu\text{L mL}^{-1}$)	50.3	44.2	50.3
Pera IAC (64 $\mu\text{L mL}^{-1}$)	49.4	45.9	54.6
Acidity (g 100 mL^{-1})			
Treatments	1 Day	7 Days	14 Days
Control	5.98	5.96	5.28
Late (32 $\mu\text{L mL}^{-1}$)	5.83	6.00	6.27
Late (64 $\mu\text{L mL}^{-1}$)	6.78	4.14	5.19
Pera IAC (32 $\mu\text{L mL}^{-1}$)	5.92	5.98	6.36
Pera IAC (64 $\mu\text{L mL}^{-1}$)	5.81	5.28	5.67
Total Soluble Solids ($^{\circ}\text{Brix}$)			
Treatments	1 Day	7 Days	14 Days
Control	8.9	9.2	10.1
Late (32 $\mu\text{L mL}^{-1}$)	9.3	9.7	9.8
Late (64 $\mu\text{L mL}^{-1}$)	6.78	9.1	9.5
Pera IAC (32 $\mu\text{L mL}^{-1}$)	5.92	9.4	9.9
Pera IAC (64 $\mu\text{L mL}^{-1}$)	5.81	9.2	9.5
Ratio			
Treatments	1 Day	7 Days	14 Days
Control	1.5	1.5	1.9
Late (32 $\mu\text{L mL}^{-1}$)	1.6	1.6	1.6
Late (64 $\mu\text{L mL}^{-1}$)	1.4	2.2	1.8
Pera IAC (32 $\mu\text{L mL}^{-1}$)	1.6	1.6	1.6
Pera IAC (64 $\mu\text{L mL}^{-1}$)	1.5	1.7	1.7

* Mean values of samples containing ten fruits.

3. Discussion

Essential oils from Late IAC 585 willowleaf mandarin and Pera IAC sweet orange exhibited a slight inhibitory effect on mycelial growth in vitro using the agar diffusion method and volatile compound exposure assay, as well as a reduction in *G. citri-aurantii* sporulation, a pathogen that causes sour rot disease in citrus, one of the most challenging post-harvest diseases in citrus, leading to significant economic losses, exacerbated by the lack of effective fungicides [17]. The increasing resistance of fungi to synthetic fungicides, such as propiconazole and thiabendazole, necessitates alternative strategies that are both sustainable and environmentally safe [18]. In this context, essential oils have gained attention due to their antifungal properties and broad acceptance for controlling agricultural pathogens.

Gas chromatography coupled with mass spectrometry determined the composition of the EOs under study (Table 1). The major compounds identified were limonene (62.40–76.53%) and γ -terpinene (10.64–17.67%, except for Murcott tangor, 0.12%). Unfortunately, data on the chemical composition of the EOs alone are insufficient to associate the identified compounds with the observed biological activity.

Data from the literature demonstrate that some volatile compounds tested have activity against *G. citri-aurantii* and that, although they are present in some essential oils, their activity is concentration-dependent. Regnier et al. [19] tested 15 EOs and pure compounds and observed, for example, that citral (a mixture of geranial and neral) showed 75.6% of mycelial growth inhibition of *G. citri-aurantii*; however, *Cymbopogon citratus* (74.2% citral), *Litsea citrata* (72.9% citral), and *Lippia citriodora* (49.7% citral) showed 52.2, 56.4, and 64.0% of inhibition, respectively.

Zhou et al. [20] tested the antifungal activity of citral, octanal, and α -terpineol against *G. citri-aurantii*. They concluded that they can significantly inhibit the mycelial growth inhibition of *G. citri-aurantii*. The literature shows that essential oils [19], pure monoterpenes [20], and monoterpene mixtures (cinnamaldehyde and citral, 1:2, vv) [21] have the ability to inhibit the mycelial growth of *G. citri-aurantii*.

Our study and data from the literature [19] showed that limonene presented low inhibition (29.3%) of the mycelial growth of *G. citri-aurantii* and as it is present in high concentration in the studied samples with the best activity, Pera (*Citrus sinensis*) and Late (*C. deliciosa*), the results obtained here corroborate the limitation of associating the results of mycelial growth inhibition of *G. citri-aurantii* to the individual components present in the essential oils. However, it is important to present the chemical composition of the EOs so they can be used as a fingerprint and for future artificial intelligence studies.

Several studies have shown the effects of essential oils on the mycelial growth of phytopathogens. For example, mint oil inhibited *P. digitatum* and *G. citri-aurantii* for one week [22]. Essential oils from *Mentha piperita*, *Mentha spicata*, and *Mentha suaveolens* completely or nearly inhibited *Botryotinia fuckeliana* at 400 $\mu\text{g mL}^{-1}$, with 92–100% mycelial growth inhibition. The same authors noted that reducing the dose of *M. suaveolens* oil to 200 $\mu\text{g mL}^{-1}$ resulted in significantly lower inhibition levels [23]. Volatile citral applied at 60 mL L^{-1} showed potential for controlling sour rot; however, high concentrations of volatile citral may cause phytotoxicity symptoms in fruits [24]. According to Bhandari et al. [25], the mode of action of essential oils is not fully understood. Still, their effects on post-harvest phytopathogens are mainly attributed to their direct impact on spore germination and mycelial growth, disrupting cellular metabolism.

Previous studies have demonstrated the potential of essential oils as antimicrobial agents, likely due to their main volatile components or combinations [25–29]. Citrus peels can extract essential oils, with yields ranging from 0.2% to 1.0% depending on variety, agronomic conditions, and extraction methods [29,30]. Additionally, orange peel essential oil mainly consists of limonene, β -pinene, and myrcene [31].

Studies have shown that the volatile compounds in essential oils can cause cell membrane disruption, cytoplasmic disorganization, and inhibition of fungal reproduction, which are mechanisms that control *G. citri-aurantii* [5,32]. Cai et al. [18] reported that essen-

tial oils compounds such as D-limonene, citral, and eugenol disrupt fungal cell membranes and induce reactive oxygen species (ROS) formation, leading to cell death. These findings highlight the potential of essential oils for citrus citrus's post-harvest treatment.

The present research demonstrated that Tahiti acid lime fruits treated curatively were more effective in controlling sour rot, reducing lesion diameter by 99.3% and achieving 96% control efficacy. Conversely, Serna-Escolano et al. [33] found that the incidence and severity of sour rot in treated mature lemons were significantly higher in curative experiments than in preventive ones, with values of 58.67%, 40.33%, and 62.33% for 25, 50 mM HP- β -CD-thymol, and propiconazole, respectively. Also, Regnier et al. [19] demonstrated that essential oils from *C. citratus*, *C. martinii*, *Origanum vulgare*, and *Geranium graveolens roseum* Bourbon (1000 $\mu\text{L L}^{-1}$), incorporated into coatings or applied as curative dips, resulted in a 90% reduction in sour rot compared to the negative control.

Mandarin essential oils, such as those studied by Devite et al. [34], show great potential as alternatives to chemical fungicides for controlling phytopathogens. In their study, different concentrations of essential oils extracted from mandarin varieties effectively inhibited the mycelial growth of *Alternaria alternata*, a fungus that causes brown spots in citrus. The authors observed that essential oil from the IAC 2019Maria variety, applied at 16 $\mu\text{L mL}^{-1}$, demonstrated the highest inhibition of fungal growth, significantly reducing disease severity in curative and preventive treatments.

The large-scale applicability of these essential oils has shown promise, mainly due to their biodegradability and low toxicity. However, challenges remain concerning their volatility and the need for microencapsulation to ensure controlled and prolonged release during storage [33,35,36]. Additionally, studies have shown that fruits treated with different essential oils maintain their quality parameters, underscoring the importance of understanding optimal concentrations and the antagonistic effects against post-harvest pathogens to enhance fruit quality and shelf life [37]. Future research should focus on optimizing formulations and exploring combinations of these oils to maximize their efficacy in controlling citrus pathogens. However, further research is required to fully elucidate the mechanisms of action and optimize the application methods, including microencapsulation, to enhance efficacy and longevity during storage. Adopting essential oils in commercial citrus operations could reduce reliance on chemical fungicides, addressing fungicide resistance issues and environmental pollution.

4. Material and Methods

4.1. Microorganism

This study was conducted using the isolate *G. citri-aurantii*, registered in SISGEN under number A03215C and belonging to the Microorganism Collection of the Phytopathology and Biological Control Laboratory at the Sylvio Moreira Advanced Citrus Research Center/IAC, Cordeirópolis. Pure and active colonies of the isolate were maintained on Petri plates containing Potato Dextrose Agar (PDA) medium in a biochemical oxygen demand (BOD) incubator at 27 °C for 7 days, with a 12-h photoperiod.

In *in vitro* assays, 5 mm fungal mycelium disks were used as inocula. For *in vivo* assays, the fungal mycelium was removed from the surface of Petri plates containing PDA medium and transferred to tubes containing autoclaved distilled water. The suspension was filtered through two layers of gauze, and the final inoculum suspension was adjusted to 10^4 conidia mL^{-1} , using a Neubauer chamber.

4.2. Essential Oils

The EO extraction process was carried out in the Laboratory of Analysis of Fruit Quality (LMQF) of the Sylvio Moreira Citrus Center (IAC). The essential oils were extracted by hydrodistillation using a modified Clevenger apparatus, an extractor apparatus idealized to obtain essential oils with the characteristics that make them valuable oils with high density as much as lower density than the water. Small amounts of oil can also be easily readable on the scale. It is compact and sturdy equipment that enables lower heating on

vegetable material and makes convenient essential oil condensation possible [38]. For oil extraction, a 400 g sample of peel cut with approximately 1 cm² of fresh fruit from 20 fruits was used with 800 mL of distilled water and heated to 100 °C on a heating mantle until the mixture reached boiling. The vapor and volatile compounds were directed to the condenser for heat exchange, condensing the vapors with cooling water. At this stage, the liquid forms of essential oil and water were visualized in the separator tube of the extractor, maintaining a continuous four-hour extraction cycle [38]. The oil was stored in an amber vial, protected from light, and kept at 4 °C.

Five essential oils were used in the experiments, from the following varieties: Late IAC 585, BRS Rainha, and Rio IAC 194 willowleaf mandarins (*Citrus × deliciosa*), Murcott IAC 221 tangor (*C. × sinensis × C. reticulata*), and Pera IAC sweet orange (*Citrus × sinensis*). Ripe fruit peels were used for the oil extraction, except for Late IAC 585 mandarin and Pera sweet orange, where the extraction was performed using green fruit peels.

The essential oil yield, expressed as a percentage, was also calculated on a wet basis (BU) through the following equation [38], as follows:

$$\text{Yield (\%)} = \frac{\text{Volume of oil obtained (mL)}}{\text{Sample mass (g)}} \times 100 \quad (1)$$

4.3. Chemical Composition of EOs by GC-MS

Gas chromatography-mass spectrometry (GC-MS) analyses were carried out using a gas chromatograph Agilent 6890N with a mass spectrometer detector 5975MSD equipped with a fused silica capillary column HP5-MS (30 m × 0.25 mm × 0.25 µm). The oven temperature was programmed from 60° to 240 °C at 3 °C min⁻¹. The carrier gas was He (1 mL min⁻¹). The temperatures of both the injector and detector were 220 °C and 250 °C; 1 µL injection volume (sample 30 mg mL⁻¹ in ethyl acetate); and split ratio 40:1. The compounds were identified from their retention indexes (RIs) on the database of the NIST 11 library, using a homologous series of n-alkanes (C8–C24) and by comparison of the MS data presented in the literature [39].

4.4. Bioactivity of Essential Oils on Mycelial Growth of *Geotrichum citri-aurantii*

(a) Antifungal Activity of Essential Oils by Direct Contact

Essential oils were incorporated into molten Potato Dextrose Agar—PDA medium (50 °C), supplemented with Tween 80 (0.5% v/v) and poured into 90 mm Petri dishes. The tested oil concentrations were 0 (control), 2, 4, 8, 16, and 32 µL mL⁻¹. After 24 h, a 5 mm disc from the edge of a 7-day-old pathogen colony was placed in the center of each Petri dish, ensuring the fungal structures were in contact with the culture medium. The plates were incubated in a BOD chamber at 27 °C with a 12-h light/dark photoperiod for seven days.

The evaluation was performed by measuring the fungal colony diameter (two orthogonal measurements) daily until one of the treatments reached the total plate diameter. The values obtained were used to calculate the percentage inhibition of mycelial growth (PIMG) compared with the 0 µL mL⁻¹ (control), the mycelial growth rate index through the sum of the evaluation interval (MGRI), and the area under the mycelial growth curve (AUMGC) equation proposed by Shaner and Finney [40], as shown in the equations below:

$$\text{PIMG} = \frac{(\text{Control growth} - \text{treatment growth})}{\text{Control growth}} \times 100 \quad (2)$$

$$\text{MGRI} = \sum \frac{(\text{Current average diameter} - \text{Previous average diameter})}{\text{Number of days after inoculation}} \quad (3)$$

$$\text{AUMGC} = \sum \left[\left(\frac{Y_i + Y_{i+1}}{2} \right) \times (T_{i+1} - T_i) \right], \quad (4)$$

where Y_i and Y_{i+1} are the colony growth values observed in two consecutive evaluations, and $(T_{i+1} - T_i)$ is the time interval between evaluations.

(b) Antifungal Activity of Essential Oil Volatile Compounds

The concentrations of 0 (control), 2, 4, 8, 16, and 32 $\mu\text{L mL}^{-1}$ of each essential oil were tested to assess the effect of volatile compounds. The essential oil solution was prepared by adding Tween 80 (0.5% *v/v*) and distilled water. A 20 μL aliquot of each concentration was deposited onto a sterile filter paper disc (5 mm), which was then placed in one compartment of a two-compartment Petri dish (90 × 15 mm). In the opposite compartment, a 5 mm disc of pathogen mycelium was placed on the PDA with the mycelium facing upward. The plates were sealed with PVC film and incubated at 27 °C with a 12-h light/dark photoperiod for four days. The colony diameter was measured daily. The values obtained were used to calculate the percentage inhibition of mycelial growth relative to the 0 $\mu\text{L mL}^{-1}$ (control), the mycelial growth rate index, and the area under the mycelial growth curve (AUMGC), as described in the previous section.

4.5. Effectiveness of Essential Oils in Controlling Sour Rot in Post-Harvest Citrus

Tahiti acid lime fruits were collected, sanitized with neutral detergent, and surface disinfected with 0.1% sodium hypochlorite for two minutes. After drying, the fruits were wounded at two equidistant points on the equatorial region of the fruits using sterilized needles to a depth of 3 mm. The fruits were then inoculated at the wound site with 20 μL of a *G. citri-aurantii* spore suspension containing a concentration of 1×10^4 conidia mL^{-1} 24 h after (preventive treatment) and 24 h before (curative treatment) the application of essential oil from Late willowleaf or Pera IAC sweet orange, at doses of 32 and 64 $\mu\text{L mL}^{-1}$, selected through in vitro assays. The essential oil solution was prepared by adding Tween 80 (0.5% *v/v*) and distilled water. In the control treatment, the fruits were treated only with water. The fruits were stored under ambient conditions (25 °C ± 2 and 70% relative humidity).

The severity of the disease was evaluated daily by measuring the diameter of the lesions formed. The obtained measurements were used to determine the area under the disease progress curve (AUDPC) using the equation proposed by Shaner and Finney [40]:

$$\text{AUDPC} = \sum \left[\frac{(Y_i + Y_{i+1})}{2} \times (T_{i+1} - T_i) \right] \quad (5)$$

where Y_i is the lesion diameter at time T_i (in days) and Y_{i+1} is the lesion diameter at time T_{i+1} .

The percentage of healthy fruits determined the incidence. The experimental design for the severity and incidence measurements was completely randomized in a triple factorial scheme with additional treatment ($2 \times 2 \times 2 + 1$), where factor 1 refers to the fruit treatment (preventive and curative), factor 2 to the essential oils evaluated (Late IAC 585 willowleaf mandarin and Pera IAC sweet orange), and factor 3 to the doses (32 and 64 $\mu\text{L mL}^{-1}$), with the additional treatment being the control. Each treatment consisted of 3 replications, with 10 fruits per replication.

4.6. Fruit Quality in Citrus After Oleo Essential Treatment

The fruits were preprocessed as described in Section 2.3. Tahitian sour lime fruits were spray-treated with different concentrations (0 (control), 32, and 64 $\mu\text{L mL}^{-1}$) of Willowleaf Late mandarin IAC 585 and sweet orange IAC Pera essential oils. The quality parameters evaluated were average juice yield, total soluble solids (SS), total titratable acidity (TTA), and the SS/TTA ratio. The percentage juice yield was calculated using % juice yield = $(\text{MS}/\text{MF}) \times 100$, where MS is the juice mass and MF is the fruit mass. The total soluble solids (SS) content was measured using a digital refractometer, with results expressed in °Brix. Total titratable acidity (TTA) was determined using an automatic titrator, and the results were expressed as % (g citric acid per 100 mL sample). The SS to TTA ratio was calculated by dividing the SS content by the TTA. The average values were

determined from 10 fruits per treatment 24 h after treatment, followed by evaluations after 7 and 14 days of storage at ambient conditions ($25\text{ }^{\circ}\text{C} \pm 2$ and 70% relative humidity).

4.7. Statistical Analyses

The data obtained were initially subjected to analysis of variance (ANOVA), and subsequently, the means were compared using Tukey's test at a 5% significance level using the R software, version 4.4.1 for Windows [41]. Data normality was verified using the Shapiro–Wilk test at a 5% significance level. Principal Component Analysis (PCA) was also applied to the in vitro essay [41] to allow a global analysis of the results. The Bip-plot contain all 39 volatile compounds of the essential oils used (Late IAC 585, BRS Rainha, Rio IAC 194 willowleaf mandarins, Murcott IAC 221 tangor and Pera IAC sweet orange) and MGRI, AUMGC, and PIMG values at a dose $32\text{ }\mu\text{L mL}^{-1}$.

5. Conclusions

The study demonstrated that Pera IAC sweet orange and Late IAC 585 willow leaf essential oils offer promising potential as natural antifungal agents for controlling postharvest citrus sour rot, specifically Tahiti lime fruit. Pera IAC sweet orange essential oil, at a concentration of $32\text{ }\mu\text{L mL}^{-1}$, effectively reduced disease severity by approximately 96% in curative treatments, with no apparent adverse effects on fruit quality. The research also highlighted the importance of volatile compounds present in essential oils, which act by inhibiting the mycelial growth of the fungus *Geotrichum citri-aurantii* and their potential antifungal properties. These results suggest that citrus essential oils may be a viable alternative to synthetic fungicides; however, it is essential to continue studies on the large-scale application of these oils, exploring the encapsulation of volatile compounds to ensure a controlled and prolonged release during storage. These innovations could benefit the citrus sector by promoting safer production with fewer chemical residues, meeting the demands of consumers and exporters for sustainable practices with low environmental impact.

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Article

In Vitro Antioxidant and Antifungal Activities of Four Essential Oils and Their Major Compounds against Post-Harvest Fungi Associated with Chickpea in Storage

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Abstract: The antifungal and antioxidant properties of essential oils (EOs) derived from four plants were assessed in vitro: *Rosmarinus officinalis*, *Myrtus communis*, *Origanum compactum*, and *Eugenia aromatica*. These plants are renowned for their diverse biological activities. Antioxidant activities were evaluated using DPPH, ABTS, and TAC tests. Antifungal activity was tested against four postharvest pathogens associated with chickpea in storage: *Fusarium culmorum*, *Rhizopus oryzae*, *Penicillium italicum*, and *Aspergillus niger*, using the broth microdilution technique. Additionally, the efficacy of several major compounds against fungi found in the EOs 1,8-cineole, carvacrol, and eugenol was evaluated. Furthermore, this study explored the potential synergy of combining eugenol and carvacrol in various ratios. Based on the results, *E. aromatica* EO exhibited the highest antioxidant activity, as evidenced by its lowest IC₅₀ values for a DPPH of 0.006 mg/mL. This EO also demonstrated the best antifungal activity, with MIC values ranging from 0.098 to 0.13 µL/mL. The high concentration of eugenol in this oil was identified as a contributing factor to its potent antifungal effects. The individual application of eugenol displayed significant antifungal efficacy, which was further enhanced by incorporating carvacrol at a 1:3 ratio. This synergistic combination presents promising potential for the development of specific formulations aimed at optimizing grain protection during storage.

Keywords: essential oils; natural alternatives; antioxidant activity; antifungal activity

1. Introduction

The food industry is actively researching alternative preservation methods that are crucial for mitigating food waste and ensuring food security [1,2]. Seed storage is particularly vulnerable to oxidation and fungal infection during storage, resulting in significant quantity and quality losses. Free radical oxidative damage results in the degradation of lipids and proteins in seeds [3]. This oxidative process produces aldehydes or ketones, which cause irreversible alterations in the taste, flavor, color, and texture of food products, in addition to a decline in nutritional quality and seed viability [4,5]. Moreover, reactive radicals generated by oxidation can initiate a chain reaction in the human body, disrupting the structure and function of healthy cells [1,6]. Furthermore, fungal infection during post-harvest storage can result in substantial losses in seed quality and quantity. Grain storage fungi can produce a range of mycotoxins that adversely affect human health [3,7], including aflatoxins, trichothecenes, and malformin.

Exploring natural bioactive substances like essential oils (EOs) presents a promising approach for improving post-harvest quality without resorting to chemical methods, offering a sustainable and environmentally conscious alternative [4]. EOs are recognized

for their wide array of biological activities, such as antioxidant, antibacterial, antiviral, insecticidal, and antifungal properties [5,8,9]. They offer potential substitutes for synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which are associated with significant health risks and toxicity [10,11]. Additionally, EOs exhibit potent antifungal activities against various storage fungi, and their relatively harmless, biodegradable, and non-toxic nature makes them widely accepted by consumers [12–14]. EOs consist of complex mixtures of volatile organic compounds, including terpenoids and phenylpropanoids. Terpenoids, classified as monoterpene, sesquiterpene, or diterpene, consist of two, three, or four isoprene units [15]. The major elements of EOs have undergone extensive investigation in the pharmaceutical and cosmetic sectors [16,17] as well as for use as flavorings and preservatives in the food industry [18,19]. The combination of these key compounds offers a novel approach to enhancing their antimicrobial and antioxidant activities [20].

The increasing need to explore natural products with versatile functions for food preservation is evident [21]. These compounds can simultaneously serve as preservatives, antioxidants, and antimicrobial agents, fulfilling the demand for clean-label and environmentally friendly food products for consumers [22]. Extracts derived from plants containing polyphenols and EOs exemplify these adaptable natural products, providing diverse advantages for improving food safety and quality [23]. Accepting these compounds will pave the way for a safer, healthier, and more sustainable food industry, aligning with evolving consumer preferences [24,25].

This study aims to develop effective formulations to optimize the protection of grain storage by (1) evaluating in vitro the antioxidant and antifungal activities of EOs extracted from four aromatic and medicinal plant species, *Rosmarinus officinalis*, *Myrtus communis*, *Origanum compactum*, and *Eugenia aromatica*; (2) investigating the antifungal activity of some major components of the EOs cineol, carvacrol, and eugenol against storage fungi; and (3) assessing the potential synergy of combining eugenol and carvacrol in varying ratios to optimize the antifungal activity.

2. Results

2.1. Essential Oil Yields

The evaluation of EO quantities in relation to dry plant matter revealed a significant range of yield variability among distinct plant species (Table 1). The yields ranged from 0.4% to 15%, highlighting the diversity among species. Notably, *E. aromatica* demonstrated the highest yield, while the lowest yield (0.4%) was observed for *M. communis*.

Table 1. EO yields extracted from four species of aromatic and medicinal plants.

Essential Oils	Extraction Yield (%)
<i>Rosmarinus officinalis</i>	2.52
<i>Origanum compactum</i>	1.6
<i>Eugenia aromatica</i>	15
<i>Myrtus communis</i>	0.4

2.2. Chemical Composition of Essential Oils

The results of the chemical composition analysis of the EOs are concisely summarized in Table 2. The EOs displayed a complex combination of monoterpene hydrocarbons, oxygenated monoterpenes, and sesquiterpene hydrocarbons. In *R. officinalis*, a substantial percentage of 1,8-cineole (48.22%) was identified, along with a notable quantity of camphor (22.58%). Similarly, *M. communis* exhibited significant content of 1,8-cineole (42.22%), along with α -pinene (17.56%) and other compounds listed in Table 2. The EO of *E. aromatica* was characterized by the notable presence of eugenol (96.29%). Remarkably, *O. compactum* exhibited a high level of carvacrol (79.20%). These findings emphasize the distinct chemical

profiles of each botanical species, distinguished by specific compounds that likely play essential roles in their biological activities. The major constituents of these EOs, namely 1,8-cineole, carvacrol, and eugenol (Figure 1), were chosen to investigate their antifungal effectiveness against post-harvest fungi.

Table 2. Relative percentages of the main components identified in EOs.

Essential Oils	Compounds	Molecular Formula	Composition (%)
<i>Rosmarinus officinalis</i>	α -Pinene	C ₁₀ H ₁₆	2.45 ^a
	Camphene	C ₁₀ H ₁₆	3.77 ^c
	β -Myrcene	C ₁₀ H ₁₆	2.80 ^b
	1,8-Cineole	C ₁₀ H ₁₈ O	48.22 ^f
	Camphor	C ₁₀ H ₁₆ O	22.58 ^e
	α -Terpineol	C ₁₀ H ₁₈ O	4.36 ^d
<i>Myrtus communis</i>	α -Pinene	C ₁₀ H ₁₆	17.56 ^f
	1,8-Cineole	C ₁₀ H ₁₈ O	42.22 ^g
	Linalool	C ₁₀ H ₁₈ O	2.80 ^b
	α -Terpineol	C ₁₀ H ₁₈ O	3.31 ^c
	Myrtenol	C ₁₀ H ₁₆ O	3.32 ^d
	Myrtenyl acetate	C ₁₂ H ₁₈ O ₂	15.06 ^e
	Geranyl acetate	C ₁₂ H ₂₀ O ₂	1.92 ^a
<i>Eugenia aromatica</i>	Eugenol	C ₁₀ H ₁₂ O ₂	96.29 ^d
	Eugenyl acetate	C ₁₂ H ₁₄ O ₃	0.72 ^b
	α -Humulene	C ₁₅ H ₂₄	0.54 ^a
	Caryophyllene	C ₁₅ H ₂₄	2.02 ^c
<i>Origanum compactum</i>	α -Thujene	C ₁₀ H ₁₆	2.26 ^c
	Myrcene	C ₁₀ H ₁₆	4.85 ^e
	p-Cymene	C ₁₀ H ₁₄	3.91 ^d
	γ -Terpinene	C ₁₀ H ₁₆	0.33 ^a
	Carvacrol	C ₁₀ H ₁₄ O	79.20 ^f
	Caryophyllene	C ₁₅ H ₂₄	1.55 ^b

The letters represent significant differences between the compounds of EOs, determined by one-way ANOVA followed by Tukey's post hoc comparison test, with a significance level of $p < 0.05$.

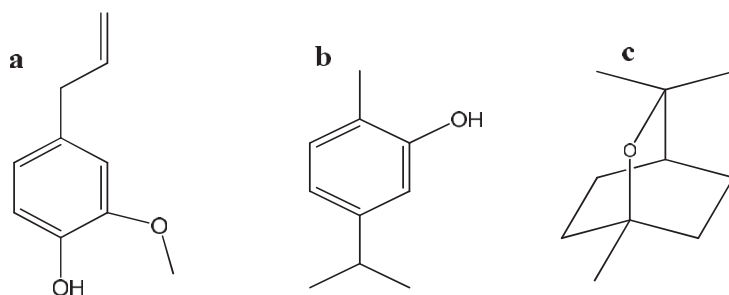


Figure 1. Molecular structures of three monoterpenes using ChemDraw (version 12.0). (a) Eugenol; (b) carvacrol; (c) 1,8-Cineole.

2.3. Antioxidant Activity of Essential Oils

Among the four species tested, the EO of *E. aromatica* demonstrated significant antioxidant effectiveness, even surpassing that of ascorbic acid, as shown in Table 3. The IC₅₀ values for the DPPH and ABTS assays were 0.006 mg/mL and 0.024 mg/mL, respectively. In contrast, *O. compactum*, *R. officinalis*, and *M. communis* exhibited relatively lower antioxidant activity (IC₅₀ > 150 µg/mL) in both DPPH and ABTS assays. These results emphasize the notable ability of all analyzed EOs to scavenge DPPH and ABTS radicals. Additionally, the evaluation of reducing capacity further confirmed the strength of *E. aromatica*, demonstrating the highest reducing capacity, equivalent to 748.36 ascorbic acid units. In comparison, *O. compactum*, *R. officinalis*, and *M. communis* displayed moderate antioxidant capacity.

Table 3. Evaluation of antioxidant activity of EOs using free radical DPPH, ABTS, and TAC assays.

Essential Oils	Reducing Capacity TAC (mgAE/gEO)	Scavenging Capacity	
		DPPH IC ₅₀ (mg/mL)	ABTS IC ₅₀ (mg/mL)
<i>R. officinalis</i>	199.952 ± 7.393 ^b	91.117 ± 3.180 ^b	48.940 ± 2.484 ^b
<i>O. compactum</i>	154.238 ± 12.957 ^a	3.937 ± 0.111 ^a	1.813 ± 0.042 ^a
<i>E. aromatica</i>	748.365 ± 3.016 ^c	0.006 ± 0.001 ^a	0.0241 ± 0.0001 ^a
<i>M. communis</i>	200.429 ± 3.898 ^b	91.312 ± 1.146 ^b	98.501 ± 1.105 ^c
Ascorbic acid	-	0.037 ± 0.003 ^a	0.132 ± 0.000 ^a

IC₅₀ = Half maximal inhibitory concentration. The presented results are expressed as mean values ± standard deviation (*n* = 3). The letters represent significant differences between the oils for each column. The test of comparison was determined by the Student–Newman–Keuls test at a significance level of *p* < 0.05.

2.4. Antifungal Activity

2.4.1. Antifungal Activity of Essential Oils

The antifungal activity of EOs extracted from four distinct plant species was assessed against four fungal strains (*F. culmorum*, *R. oryzae*, *P. italicum*, and *A. niger*) (Figure 2). Table 4 displays the values for the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC). All tested extracts demonstrated inhibitory effects on the fungal strains. *E. aromatica* EO showed the highest antifungal effectiveness, with MIC values ranging from 0.098 to 0.13 µL/mL. Interestingly, *E. aromatica* EO performed better than the commercial fungicide (Azoxystrobin) against most of the tested fungi. However, it is important to note that *E. aromatica* EO showed a fungistatic effect, limiting fungal growth without complete eradication. *O. compactum* EO also showed significant efficacy, with MIC values ranging from 0.781 to 3.125 µL/mL and with a fungicidal effect. In contrast, the extracts of *R. officinalis* and *M. communis* showed relatively modest antifungal activity, characterized by MIC values ranging from 6.25 to 25 µL/mL, indicating reduced effectiveness against the tested fungi.

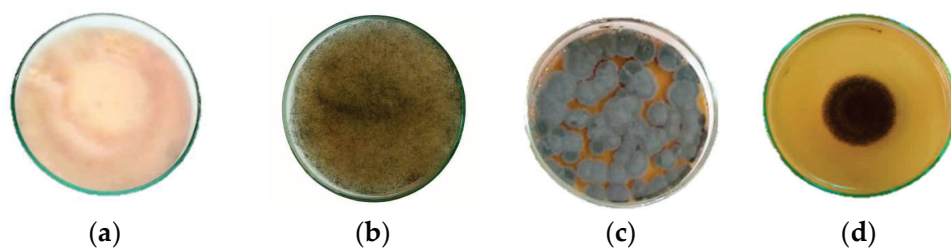


Figure 2. Cultures of selected fungal species on potato dextrose agar: (a) *Fusarium culmorum*, (b) *Rhizopus oryzae*, (c) *Penicillium italicum*, (d) *Aspergillus niger*.

Table 4. Evaluation of MIC and MFC of EOs against four fungi species.

Essential Oils		<i>F. culmorum</i>	<i>R. oryzae</i>	<i>P. italicum</i>	<i>A. niger</i>
<i>R. officinalis</i>	MIC (μL/mL)	6.25 ^c	6.25 ^d	8.33 ± 2.08 ^b	12.50 ^c
	MFC (μL/mL)	6.25 ^b	25.00 ^c	25.00 ^c	50.00 ^c
	MFC/MIC	1.00	4.00	3.00	4.00
	Interpretation	Fungicide	Fungicide	Fungicide	Fungicide
<i>O. compactum</i>	MIC (μL/mL)	0.781 ^b	3.125 ^c	0.781 ^a	1.562 ^b
	MFC (μL/mL)	1.302 ± 0.260 ^a	6.25 ^b	3.125 ^b	3.125 ^b
	MFC/MIC	1.67	2.00	4.00	2.00
<i>E. aromatica</i>	Interpretation	Fungicide	Fungicide	Fungicide	Fungicide
	MIC (μL/mL)	0.13 ± 0.03 ^a	0.098 ^a	0.13 ± 0.03 ^a	0.098 ^a
	MFC (μL/mL)	0.651 ± 0.130 ^a	0.781 ^a	1.562 ^a	0.781 ^a
	MFC/MIC	4.99	7.97	11.98	7.97
<i>M. communis</i>	Interpretation	Fungistatic	Fungistatic	Fungistatic	Fungistatic
	MIC (μL/mL)	12.50 ^d	25.00 ^e	12.50 ^b	12.50 ^c
	MFC (μL/mL)	25.00 ^c	50.00 ^d	50.00 ^d	50.00 ^c
	MFC/MIC	2.00	2.00	4.00	4.00
	Interpretation	Fungicide	Fungicide	Fungicide	Fungicide
Azoxystrobin	MIC (μL/mL)	1.302 ± 0.451 ^b	0.520 ± 0.226 ^b	0.651 ± 0.226 ^a	0.098 ^a

MIC = minimum inhibitory concentration; MFC = minimum fungicidal concentration. The presented results are expressed as mean values ± standard deviation ($n = 3$). The letters indicate significant differences between the oils for each fungus, determined by a one-way ANOVA followed by a Tukey post hoc comparison test, with a significance level of $p < 0.05$.

2.4.2. Antifungal Activity of the Main Components of EOs

The antifungal activity of the main components of the tested EOs (1,8-cineole, carvacrol, and eugenol) was evaluated against the four post-harvest fungi (Table 5). Notably, carvacrol demonstrated very high antifungal activity, with MIC values ranging from 0.098 to 0.13 μL/mL. Moreover, fungicidal effects were observed at concentrations ranging from 0.195 to 0.39 μL/mL. Similarly, eugenol showed high antifungal activity, displaying MIC values ranging from 0.195 to 0.781 μL/mL, along with fungicidal attributes. In contrast, 1,8-cineole exhibited restricted antifungal potential, characterized by higher MIC values. All tested compounds exhibited fungicidal properties against all fungal strains.

Table 5. Evaluation of the MIC and MFC of the main components of EOs against four fungi.

Main Components		<i>F. culmorum</i>	<i>R. oryzae</i>	<i>P. italicum</i>	<i>A. niger</i>
1,8-Cineole	MIC (μL/mL)	12.50 ^c	10.437 ^c	17.39 ± 3.47 ^b	41.75 ^c
	MFC (μL/mL)	25.00 ^c	41.75 ^c	83.5 ^c	167.00 ^c
	MFC/MIC	2.00	4.00	4.00	4.00
	Interpretation	Fungicide	Fungicide	Fungicide	Fungicide
Carvacrol	MIC (μL/mL)	0.098 ^a	0.098 ^a	0.13 ± 0.03 ^a	0.098 ^a
	MFC (μL/mL)	0.195 ^a	0.195 ^a	0.39 ^a	0.195 ^a
	MFC/MIC	1.99	1.99	3.00	1.99
	Interpretation	Fungicide	Fungicide	Fungicide	Fungicide
Eugenol	MIC (μL/mL)	0.39 ^b	0.39 ^b	0.195 ^a	0.781 ^b
	MFC (μL/mL)	0.781 ^b	1.562 ^b	0.781 ^b	1.562 ^b
	MFC/MIC	2.00	4.01	4.01	2.00
	Interpretation	Fungicide	Fungicide	Fungicide	Fungicide

MIC = minimum inhibitory concentration; MFC = minimum fungicidal concentration. The presented results are expressed as mean values ± standard deviation ($n = 3$). The letters indicate significant differences between the oils for each fungus, determined by a one-way ANOVA followed by a Tukey post hoc comparison test with a significance level of $p < 0.05$.

2.4.3. Antifungal Activity of the Main Component Mixture

The antifungal activity of binary mixtures of eugenol and carvacrol against four post-harvest fungi was investigated (Table 6). The results indicate that mixture 2, composed of

25% eugenol and 75% carvacrol (in a ratio of 1:3), demonstrates greater efficacy than the other mixtures. The MIC value for this mixture was determined to be 0.098 $\mu\text{L/mL}$ against all four fungi. Mixture 1, containing an equal proportion of the two components, showed a slightly reduced level of effectiveness.

Table 6. Evaluation of MIC and MFC of the three mixtures of carvacrol and eugenol against four fungi.

Mixtures		<i>F. culmorum</i>	<i>R. oryzae</i>	<i>P. italicum</i>	<i>A. niger</i>
Mix. 1	MIC ($\mu\text{L/mL}$)	0.260 $\pm$ 0.113 ^{a,b}	0.195 ^b	0.163 $\pm$ 0.056 ^{a,b}	0.260 $\pm$ 0.113 ^{a,b}
	MFC ($\mu\text{L/mL}$)	0.39 ^a	0.781 ^b	0.651 $\pm$ 0.226 ^a	0.39 ^a
Mix. 2	MIC ($\mu\text{L/mL}$)	0.098 ^a	0.098 ^a	0.098 ^a	0.098 ^a
	MFC ($\mu\text{L/mL}$)	0.39 ^a	0.39 ^a	0.651 $\pm$ 0.226 ^a	0.325 $\pm$ 0.113 ^a
Mix. 3	MIC ($\mu\text{L/mL}$)	0.39 ^b	0.39 ^c	0.195 ^b	0.39 ^b
	MFC ($\mu\text{L/mL}$)	0.39 ^a	0.781 ^b	1.562 ^b	0.781 ^b

MIC = minimum inhibitory concentration; MFC = minimum fungicidal concentration; Mix. 1 = 1:1 ratio; Mix. 2 = 1:3 ratio; Mix. 3 = 3:1 ratio. The presented results are expressed as mean values $\pm$ standard deviation ($n = 3$). The letters indicate significant differences between the oils for each fungus, determined by a one-way ANOVA followed by a Tukey post hoc comparison test with a significance level of $p < 0.05$.

Binary combination 2 demonstrated an additive effect, displaying fractional inhibitory concentration index (FICI) values ranging from 0.69 to 0.81 ($0.5 \leq \text{FICI} \leq 1$), while the other mixtures showed indifferent effects ($1 \leq \text{FICI} \leq 4$) (Table 7). The combination of eugenol and carvacrol resulted in enhanced antifungal effectiveness against the tested fungi.

Table 7. Evaluation of the synergistic antifungal effect of eugenol–carvacrol mixtures.

Mixtures		<i>F. culmorum</i>	<i>R. oryzae</i>	<i>P. italicum</i>	<i>A. niger</i>
Carvacrol ($\mu\text{L/mL}$)	MIC	0.098	0.098	0.13 $\pm$ 0.03	0.098
Eugenol ($\mu\text{L/mL}$)	MIC	0.39	0.39	0.195	0.781
Mix. 1	MIC of Mix 1	0.260 $\pm$ 0.113	0.195	0.163 $\pm$ 0.056	0.260 $\pm$ 0.113
	FIC of CAR	1.33	0.99	0.62	1.33
	FIC of EUG	0.33	0.25	0.42	0.17
	FICI	1.66	1.24	1.04	1.49
	Interpretation	Indifferent	Indifferent	Indifferent	Indifferent
Mix. 2	MIC of Mix 2	0.098	0.098	0.098	0.098
	FIC of CAR	0.75	0.75	0.57	0.75
	FIC of EUG	0.06	0.06	0.13	0.03
	FICI	0.81	0.81	0.69	0.78
	Interpretation	Additive	Additive	Additive	Additive
Mix. 3	MIC of Mix 3	0.39	0.39	0.195	0.39
	FIC of CAR	0.99	0.99	0.38	0.99
	FIC of EUG	0.75	0.75	0.75	0.37
	FICI	1.74	1.74	1.13	1.37
	Interpretation	Indifferent	Indifferent	Indifferent	Indifferent

FICI = fractional inhibitory concentration index; FIC = fractional inhibitory concentration; CAR = carvacrol; EUG = eugenol; Mix. 1 = 1:1 ratio; Mix. 2 = 1:3 ratio; Mix. 3 = 3:1 ratio. The results are presented as mean values $\pm$ standard deviation ($n = 3$).

2.5. Correlation Analysis between Antifungal and Antioxidant Activities

Pearson's correlation analysis revealed a positive correlation between the antioxidant activity, quantified through the DPPH and ABTS assays, and the antifungal activity against the fungal strains (*F. culmorum*, *R. oryzae*, *P. italicum*, and *A. niger*) (Figure 3). The correlation coefficients was ranged from $R^2 = 0.73$ to $R^2 = 0.99$. The ABTS assay, in particular, showed a strong correlation with all fungal MICs, with coefficient values ranging from 0.9 to 1. Furthermore, a negative correlation was observed between TAC and fungal MICs of fungi, with correlation coefficients ranging from -0.59 to -0.46 .

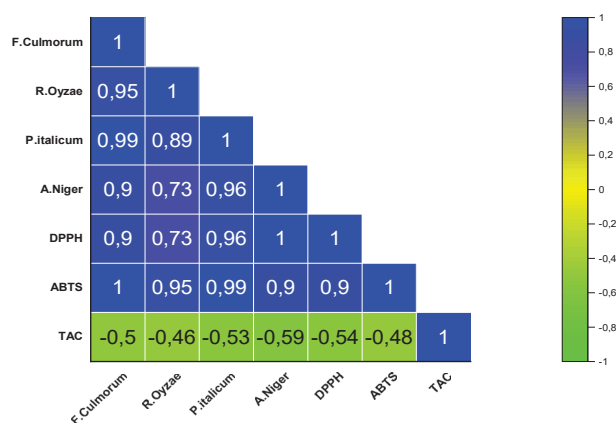


Figure 3. Pearson's correlation coefficient of the antifungal and antioxidant activities of EOs. *Fusarium culmorum*, *Rhizopus oryzae*, *Penicillium italicum*, *Aspergillus niger*; DPPH = 2,2–diphenyl–1–picrylhydrazyl; ABTS = 2,2'–azino–bis(3–ethylbenzothiazoline–6–sulfonic acid); TAC = total antioxidant capacity.

3. Discussion

The composition of essential oils (EOs) varies significantly due to factors such as plant species, harvest location, climate, and extraction techniques used [26]. EOs, rich in diverse chemical components, exhibit a range of biological activities and modes of action [27].

Among the EOs investigated in this study, *E. aromatica* EO demonstrated the highest antioxidant activity. This antioxidant potency, characterized by a low IC_{50} value of 6 $\mu\text{g/mL}$ ($<50 \mu\text{g/mL}$), aligns with the findings of Saptarini et al., 2020 [28]. *E. aromatica* EO not only displayed a high scavenging capacity but also exhibited a notable reduction ability. Conversely, EOs of *O. compactum*, *R. officinalis*, and *M. communis* exhibited comparatively lower antioxidant effects, with *R. officinalis* and *M. communis* showing similar activities. Previous studies have highlighted clove oil's excellent antioxidant properties, which corroborate our findings [29–32]. The antioxidant activity of clove oil might stem from its major component, eugenol, along with the complex interplay among its constituents, potentially involving synergistic and antagonistic effects [33]. The prominent presence of eugenol, constituting about 96% of *E. aromatica* EO's composition, contributes to its antioxidant activity. Eugenol's neuroprotective properties and its ability to inhibit lipid peroxidation are well documented [34,35]. Its structural capacity to stabilize phenoxy radicals through hydrogen atoms and neutralize damaged molecules underscores its role in preventing oxidative damage and carcinogenic mutations [36,37]. Notably, Jirovetz et al. (2006) revealed that clove EO exhibits DPPH radical scavenging activity at lower doses than eugenol alone, implying potential synergies within the EO's components [38]. Additionally, Dahham et al. (2015) showed that caryophyllene, which composes 2% of *E. aromatica*, possesses significant antioxidant activity [39]. Eugenyl acetate, accounting for 0.7% of the EO, exhibited even greater antioxidant activity than clove EO, with IC_{50} values of 367.5 $\mu\text{g/mL}$ and 283.9 $\mu\text{g/mL}$, respectively [40]. Therefore, *E. aromatica* EO's antioxidant potential can be partially attributed to eugenol and the synergistic interactions among its phenolic compounds and secondary metabolites. Interestingly, carvacrol, comprising around 79% of *O. compactum* EO, displayed important antioxidant activity when tested individually [41]. Extensive studies corroborate carvacrol's potent antioxidant properties [42,43]. Notably, oregano EO, which contains carvacrol, showcased higher antioxidant activity than carvacrol itself [44]. This suggests a synergistic action among *O. compactum* EO's constituents, with carvacrol being the key contributor. *M. communis* and *R. officinalis* EOs, both featuring 1,8-cineole as a major compound (approximately 48% and 42% of composition, respectively), exhibited similar antioxidant effects. The synergistic effects of 1,8 cineole have been documented [45]. Phenolic and flavonoid compounds often account for EOs' antioxidant attributes, acting as electron donors in free radical reactions [46,47], as also indicated by Noshad et al. (2021) [48].

Our study demonstrated significant inhibition of fungal strains by the tested EOs. Notably, *E. aromatica* EO exhibited the highest antifungal activity, even surpassing the efficacy of the fungicide azoxystrobin. *O. compactum* EO also showed pronounced fungicidal activity. In contrast, *M. communis* and *R. officinalis* EOs displayed lower antifungal effects. Consistent with previous research, clove and oregano oils are well-established for their antifungal activities [49–53]. *E. aromatica* EO's strong inhibitory effect can be attributed to its major component, eugenol, or its synergetic interactions. Eugenol alone demonstrated substantial antifungal activity against the post-harvest fungal strains, although less effectively than *E. aromatica* EO. Eugenol's minimum inhibitory concentration ranged from 0.195 to 0.781 $\mu\text{L}/\text{mL}$, while *E. aromatica* EO's MIC values ranged from 0.098 to 0.13 $\mu\text{L}/\text{mL}$. Interestingly, eugenol displayed superior fungicidal properties compared to clove oil, aligning with Schmidt et al.'s findings (2007) [54]. This suggests that other components present in *E. aromatica* EO could enhance its antifungal activity synergistically. Carvacrol, the dominant compound in *O. compactum* EO (about 79%), exhibited the highest efficacy against fungal growth, with MIC values ranging from 0.098 to 0.13 $\mu\text{L}/\text{mL}$. Carvacrol outperformed *O. compactum* EO itself, which displayed MIC values ranging from 0.781 to 3.125 $\mu\text{L}/\text{mL}$. Our findings align with Schlösser et al.'s (2018) research [55], indicating carvacrol's stronger antifungal effect compared to oregano oil. Multiple studies have highlighted carvacrol's efficacy against various fungi, with low MIC values ranging from 0.1 to 0.2 $\mu\text{L}/\text{mL}$ [56,57]. Hence, carvacrol appears to be the primary contributor to *O. compactum* EO's antifungal activity. *R. officinalis* and *M. communis* EOs demonstrated similar antioxidant activities due to the presence of 1,8-cineole, their major compound. However, 1,8-cineole exhibited lower antifungal activity compared to the oils themselves. Our findings are in accordance with Dammak et al. (2019) [58], suggesting that 1,8-cineole's antifungal potential is relatively modest compared to *L. nobilis* and *L. dentate* EOs. This indicates that other components in these EOs synergistically enhance their antifungal properties [59].

Our investigation extended to binary mixtures of eugenol and carvacrol to enhance their antifungal efficacy. A 25% eugenol to 75% carvacrol ratio exhibited the most promising results among the tested mixtures, with a MIC value of 0.098 $\mu\text{L}/\text{mL}$ against the evaluated post-harvest fungi. The synergistic combination of eugenol and carvacrol demonstrated an additive effect, enhancing the overall antifungal efficacy. Previous research has highlighted the significant inhibitory effects of binary mixtures, such as carvacrol and thymol, major components of thyme oil, against various fungi [60,61]. Eugenol and citral, major constituents of citronella and lemongrass oils, have also shown potent fungal growth inhibition that surpasses their individual effects [62]. Our results are consistent with those of Schlosser et al. (2018) [55], which highlighted the additive antifungal effect of carvacrol and eugenol on foodborne mold fungi. Properly balanced mixtures can be valuable for food applications as long as sensory qualities are maintained [55]. Synergy among natural substances enhances stability and bioactivity [63]. Combining different major compounds can optimize absorption or penetration into fungal cells, producing an impact greater than the sum of its parts. Phenolic compounds and aromatic aldehydes enhance antifungal activity due to steric hindrance [64]. According to Nieto et al. (2013) [9], Alkyl groups added to the benzene ring can also enhance antifungal activity. Notably, EOs' antimicrobial activity depends on their most common compounds' composition and concentration [24]. The synergistic action of EO constituents can disrupt fungal growth by affecting different targets or stages of the fungal life cycle, often damaging cell membranes and altering permeability, leading to leakage of intracellular contents [59,65,66]. Therefore, the combined effect of eugenol and carvacrol optimizes antifungal properties, targeting multiple aspects of fungal growth.

In this study, a strong correlation emerged between antioxidant and antifungal activities, suggesting a potential synergistic relationship. This suggests the possibility of a dual-function natural food preservative. Monoterpenes in EOs can act as pro-oxidants in fungal cells, disrupting cycles and accumulating reactive oxygen species (ROS) [67]. This

can prevent microbial adherence and biofilm formation [68]. Hence, exploring antioxidants as fungal infection treatments is promising. *E. aromatica* EO, with its dual antifungal and antioxidant actions or its major constituent, eugenol, in combination with carvacrol, holds potential applications.

While this initial study has revealed significant antioxidant and antifungal activities present in used essential oils (EOs) and their primary components, as demonstrated by in vitro analyses, further exploration through more comprehensive in vivo investigations is necessary. These investigations will assist in uncovering the most optimal approaches for utilizing their potential to effectively protect chickpea seeds.

4. Materials and Methods

4.1. Plant Materials and Extraction of Essential Oils

Four species of aromatic and medicinal plants, representing two different plant families (*Lamiaceae* and *Myrtaceae*), were harvested during the flowering season from their natural habitats at various locations in Morocco (Table 8).

Table 8. Aromatic and medicinal plant species investigated in this study.

Species Name	Family	Parts Used	Harvesting Location	Harvesting Season
<i>Rosmarinus officinalis</i>	<i>Lamiaceae</i>	Leaves + small stems	Midelt region	June–September
<i>Origanum compactum</i>	<i>Lamiaceae</i>	Leaves + small stems	Ouazzane region	June–August
<i>Myrtus communis</i>	<i>Myrtaceae</i>	Leaves	Marrakech region	May–June
<i>Eugenia aromatica</i>	<i>Myrtaceae</i>	Flowers	Herbalist	-

The essential oils (EOs) were extracted from the dried plant materials through hydrodistillation using a Clevenger apparatus following Guenther's method [69]. In this process, 250 g of plant organs were placed in a 5000 mL flask filled with distilled water. The extraction proceeded for 3 h. This procedure was repeated iteratively to accumulate the required amount of EO for subsequent chemical and natural analyses. The extracted EOs were stored at 4 °C until use. The average EO yields were calculated based on dry plant material using Equation (1):

$$\text{EO yield (\%)} = \frac{\text{Weight of EO (g)}}{\text{Weight of dried plant material (g)}} \times 100 \quad (1)$$

4.2. Gas Chromatography–Mass Spectrometry Analysis of Essential Oil

The chemical composition of the tested EOs was analyzed using gas chromatography coupled with mass spectrometry (GC–MS) in Rabat, Morocco. The identification of the different constituents of the essential oil was carried out using PerkinElmer Clarus 580 GC/Mass Spectrometer. A capillary column (30 m × 0.25 mm; 0.25 µm film thickness) was utilized for the separation of different compounds of the essential oils. The carrier gas used was helium, with a flow rate of 1 mL/min. The temperature program was fixed from 50 to 230 at 4 °C/min, with a final hold time of 5 min. The injector and detector were maintained at 235 to 240 °C. The injection volume was 0.02 µL with splitless mode. The quantification of individual components of essential oils was performed using a PerkinElmer autosystem XL gas chromatograph equipped with a flame ionization detector (GC-FID). The column used was a capillary DB5 (0.25 µm film thickness and 30 m × 0.25 mm i.d.).

4.3. Major Components of the EOs

Essential oil major components were of the highest purity available. Eugenol (≥99%), carvacrol (≥97%), and cineole (≥98%) were purchased from Fluka Chemica. These compounds were utilized to assess the antifungal activity against fungi strains. As a posi-

tive control, the commercial fungicide Azoxystrobin (250 g/L), obtained from Syngenta, was used.

4.4. Antioxidant Activity

The evaluation of the antioxidant activity of the EOs was performed using two scavenging methods: DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)). The determination of reducing capacity was carried out using the total antioxidant capacity (TAC) test. The ABTS and DPPH assays specifically target radicals, whereas the total antioxidant capacity assay offers a more comprehensive assessment of the overall antioxidant activity.

4.4.1. DPPH Radical Scavenging Activity

The DPPH assay was employed to determine the IC_{50} values, which represent the concentration of each EO required for 50% inhibition of the free radical. The methanolic DPPH solution was prepared with a concentration of 0.004% *w/v* and allowed to stabilize for one hour. Subsequently, 100 μ L of each methanolic EO solution at varying concentrations (*R. officinalis*: 10–200 mg/mL; *M. communis*: 10–200 mg/mL; *E. aromatica*: 0.01–0.05 mg/mL; *O. compactum*: 1–10 mg/mL) was mixed with 750 μ L of freshly prepared DPPH. The samples were then incubated in the dark at room temperature for 30 min. The change in color from purple to yellow was observed to assess the radical scavenging capacity of the volatiles relative to the negative control (DPPH solution only). As a positive control, a methanolic solution of ascorbic acid was prepared with concentrations ranging from 0.001 to 0.05 mg/mL. The absorbance of the samples was measured at 517 nm using a UV spectrophotometer [70]. The test was carried out in triplicate, with each experiment performing three repetitions. The antioxidant activity was calculated using Equation (2):

$$\text{Scavenging (\%)} = \left(\frac{AC - AS}{AC} \right) \times 100 \quad (2)$$

where AC and AS represent the absorbencies of the negative control and samples.

The antioxidant activity of each sample was quantified using IC_{50} values, which indicate the concentration (in mg/mL) needed to inhibit DPPH radical formation by 50%. The IC_{50} values, along with their corresponding 95% confidence intervals, were derived through linear regression analysis of the dose–response curve. In this analysis, the percentage of inhibition was plotted against the concentration.

4.4.2. ABTS Anion Radical Scavenging Activity

The ABTS solution was prepared by mixing 7 mM ABTS with 2.45 mM potassium persulfate in distilled water. The mixture was then incubated in the dark at room temperature for 16 h. The resultant ABTS+ solution was subsequently diluted with ethanol (1 mL per 1 mL) to attain an absorbance of 0.70 ± 0.02 at 734 nm [71]. For the experiment, 100 μ L of each ethanolic solution of EO at varying concentrations was combined with 2 mL of fresh ABTS solution. The EO concentrations were as follows: *R. officinalis* (10–200 mg/mL), *M. communis* (10–200 mg/mL), *E. aromatica* (0.01–0.05 mg/mL), and *O. compactum* (1–8 mg/mL). After initial mixing, the absorbance was measured 6 min later at 734 nm. As a positive control, an ethanolic solution of ascorbic acid was prepared with concentrations ranging from 0.025 to 0.2 mg/mL. The test was conducted in three repetitions, and the entire experiment was repeated in triplicate. The percentage of inhibition was calculated using Equation (2), the same equation used for the DPPH-scavenging assay.

The antioxidant activity categories based on the IC_{50} values obtained from the scavenging activity of EOs are as follows: very strong when $IC_{50} < 50 \mu\text{g/mL}$, strong when $50 \mu\text{g/mL} < IC_{50} < 100 \mu\text{g/mL}$, moderate effect when $100 \mu\text{g/mL} < IC_{50} < 150 \mu\text{g/mL}$, and low when $IC_{50} > 150 \mu\text{g/mL}$ [28].

4.4.3. Total Antioxidant Capacity

The determination of total antioxidant capacity was accomplished via the phosphomolybdenum assay [72]. The reagent solution was prepared by dissolving 4 mM ammonium molybdate, 28 mM sodium phosphate, and 0.6 M sulfuric acid in distilled water. For the assay, 25 μ L of each methanolic EO solution at a concentration of 1 mg/mL was mixed with 1 mL of the reagent solution. The samples were then incubated in a water bath at 95 °C for 90 min. The reduction of the Mo complex was indicated by a color change in the samples from transparent to green. The optical density of the samples was measured at 695 nm using a UV spectrophotometer against a blank. The test was conducted in three repetitions, and the entire experiment was performed in triplicate. The antioxidant activity was expressed in terms of ascorbic acid equivalents (mg AAE/g of extract).

4.5. Antifungal Activity

4.5.1. Fungal Isolates and Inoculum Preparation

Four fungal species from distinct genera were employed in this assay: *Fusarium* (*F. culmorum*), *Rhizopus* (*R. oryzae*), *Penicillium* (*P. italicum*), and *Aspergillus* (*A. niger*). These post-harvest fungal species were isolated from chickpea seeds after storage. Identification of the isolates was conducted using standard identification keys based on their macroscopic and microscopic features [73–75]. These fungal species were chosen due to their prevalence within the seeds and their tendency to produce mycotoxins, such as zearalenone, B trichothecene, patulin, and ochratoxin A. The strains were maintained in a solution composed of 80% sabouraud dextrose broth (SDB) and 20% glycerol at -80 °C. Fungal strains were cultured on potato dextrose agar (PDA) for 7 to 10 days at a temperature of 22 ± 2 °C.

The inoculum suspension was prepared by rinsing the surface of the agar plates with 1 mL of sterile 0.9% saline water containing 0.1% Tween 20. Conidial suspensions were quantified using a hemocytometer and diluted to achieve a working suspension of 1.10^6 spores/mL.

4.5.2. Determination of the Minimum Inhibitory Concentration (MIC)

The antifungal activity of EOs and their main components was evaluated using the broth microdilution technique in sterile 96-well microplates [57]. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of oil or main component that completely inhibited visible fungal growth. Initially, each well of the microplates received 150 μ L of SDB. Subsequently, 150 μ L of a double-concentrated emulsion of each test product was introduced to the wells of the first row of the plate. Doubling dilution series were executed to establish a concentration range from 0.004% to 5%, with preparations in dimethyl sulfoxide (DMSO) attaining a final concentration of 5% (*v/v*). Following this, 15 μ L of fungal suspension inoculum was introduced to all wells of the microplate, leading to a final concentration of 1.10^5 spores/mL. Wells containing inoculum but lacking treatment served as a negative control. A chemical treatment (azoxystrobin, 250 g/L) was utilized as the positive control. The plates were covered and incubated for 72 h at 22 ± 2 °C. Absorbance was measured at 492 nm using a microplate reader, with visual confirmation of outcomes under a microscope. Each test was replicated in triplicate, and the entire experiment was performed twice. The percentage of mycelium growth inhibition was computed using the subsequent Equation (3):

$$\text{Mycelium growth inhibition (\%)} = \left[\frac{(AC_{(72)} - AC_{(0)}) - (AT_{(72)} - AT_{(0)})}{(AC_{(72)} - AC_{(0)})} \right] \times 100 \quad (3)$$

where AC is the absorbance of the inoculum suspension in the control at $t = 0$ h and $t = 72$ h, and AT is the absorbance of the treatment at $t = 0$ h and $t = 72$ h.

4.5.3. Determination of the Minimum Fungicidal Concentration (MFC)

The minimum fungicidal concentration (MFC) was determined to assess the fungicidal or fungistatic attributes of the EOs and their main components. MFC was defined as the lowest concentration leading to complete inhibition of mycelium growth on SDB plates, as indicated by subculturing wells showing the MIC. For MFC determination, 100 μ L of contents from the MIC well and wells with concentrations surpassing the MIC were subcultured into a new microplate freshly supplemented with 150 μ L of SDB [76]. These microplates were incubated under the same conditions as the MIC microplate, and growth was assessed visually through microscopy and absorbance measurement at 492 nm. The percentage of mycelium growth was calculated using Equation (3). Each test was executed in triplicate, with the experiment repeated twice to ensure accuracy and reproducibility. To determine the fungicidal or fungistatic impact of EOs, MFC/MIC ratios were computed. A ratio of $MFC/MIC \leq 4$ denoted a fungicidal effect, while a ratio of $MFC/MIC > 4$ indicated a fungistatic effect [49].

4.5.4. Determination of the Fractional Inhibitory Concentration Index (FICI)

The fractional inhibitory concentration (FIC) was determined to reveal the synergistic antifungal effect of two main components, carvacrol and eugenol. FIC determination was accomplished via the checkerboard method within a 96-well microtiter plate, adhering to the same procedure for determining the MIC. The FIC index was ascertained using the two-fold dilution method based on the results of the MIC determination for each main component separately. Concentrations of $4 \times MIC$, $2 \times MIC$, $1 \times MIC$, $1/2 \times MIC$, $1/4 \times MIC$, $1/8 \times MIC$, and $1/16 \times MIC$ were chosen for the test [62]. Three binary combinations of eugenol and carvacrol were examined using distinct volume proportions (Table 9). The test was performed in three repetitions, and the experiment was conducted in duplicate.

Table 9. The three binary mixture ratios of eugenol and carvacrol used.

Mixture	Eugenol	Carvacrol
Mix. 1	1	1
Mix. 2	1	3
Mix. 3	3	1

The synergistic effect was expressed according to the following expression:

$$FICI = \Sigma FIC = FIC (A) + FIC (B)$$

FIC (A) = MIC of the main compound (A) in combination/MIC of the main compound (A) alone.

FIC (B) = MIC of the main compound (B) in combination/MIC of the main compound (B) alone.

The values obtained for this index determined the combined effect of both compounds: FICI–FIC index values were interpreted as follows: FICI values ≤ 0.5 indicated synergy, $0.5 \leq FICI \leq 1$ indicated an additive effect, $1 \leq FICI \leq 4$ indicated an indifferent effect, and FICI values > 4 indicated an antagonistic effect [49].

4.6. Statistical Analysis

The results were presented as the mean $\pm$ standard deviation. Mean differences were assessed using analysis of variance (one-way ANOVA), followed by the Tukey post hoc test for multiple group comparisons. A significance level of $p < 0.05$ was considered to indicate statistical significance. The statistical analysis was performed using IBM SPSS v.22 software. Pearson’s correlation test was utilized to establish correlation coefficients

between antifungal activity and antioxidant activity (DPPH, ABTS, and TAC) using Origin 2023 (10.0) software.

5. Conclusions

In conclusion, our study underscores the significant antioxidant and antifungal potential of *E. aromatica* among the tested EOs. The substantial presence of eugenol in this oil can contribute to its pronounced antifungal properties. Furthermore, the application of eugenol alone exhibited a notable antifungal effect, which was further potentiated when combined with carvacrol in a 1:3 ratio. This synergistic interaction suggests the possibility of developing customized formulations or treatments to enhance grain protection during storage. The combination of *E. aromatica* EO and carvacrol also presents a promising prospect for advancing natural preservation in diverse industries. Further investigation is required to optimize this combination and identify optimal application strategies.

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Article

Essential Oils of *Artemisia frigida* Plants (Asteraceae): Conservatism and Lability of the Composition

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Abstract: Plants of arid regions have adapted to harsh environments during the long span of their evolution and have developed a set of features necessary for their survival in water-limited conditions. *Artemisia frigida* Willd. (Asteraceae) is a widely distributed species possessing significant cenotic value in steppe ecosystems due to its high frequency and abundance. This study examines different patterns of formation of essential oil composition in *A. frigida* plants under the influence of heterogeneous factors, including climate and its integral characteristics (HTC, C_{extr}, SPEI and others). The work is based on the results of our research conducted in Russia (Republic of Buryatia, Irkutsk region), Mongolia, and China, from 1998 to 2021. A total of 32 constant compounds have been identified in the essential oil of *A. frigida* throughout its habitat range in Eurasia, from Kazakhstan to Qinghai Province, China. Among them, camphor, 1,8-cineol and bornyl acetate are the dominant components, contained in 93–95% of the samples. Among the sesquiterpenoids, germacrene D is the dominant component in 67% of the samples. The largest variability within the composition of the essential oils of *A. frigida* is associated with significant differences in the climatic parameters when plants grow in high-altitude and extrazonal conditions.

Keywords: arid territories; terpenoids; 1,8-cineol; camphor; borneol; climate; phenological phase; elevation above sea level

1. Introduction

Currently, the issue of biota response to ongoing climate warming, along with the back-reaction between “biota” and “climate”, is at the cutting edge of global biology and ecology. There is no doubt that climate change is happening and will continue to happen in the foreseeable future. Vegetation is forced to respond to biotically significant changes in climate, whether they are significant once, are episodic oscillations, or sustainable long-term trends. Therefore, a key scientific problem is the study of mechanisms of plant adaptation to the conditions of short-term or long-term exposure to adverse environmental factors.

During the current climate warming, arid territories are occupying increasingly extensive areas. Plants in arid regions have adapted to harsh environmental conditions during the long span of their evolution, and have developed a set of specific morphological, physiological and phenological features necessary for their survival in water-limited conditions. The study of these plants growing in natural populations will allow to demonstrate the stability of the plant locus with respect to drought [1].

Artemisia frigida Willd. (Asteraceae) has significant cenotic value as dominant or co-dominant in the composition of the cryophyte, true and desert steppes of Siberia, the Volga region, Kazakhstan, Central Asia, Mongolia and North America. The high plasticity of the species, as well as its ability of rapid vegetative reproduction through the development

of creeping and quickly rooting aboveground shoots, determine the competitiveness of *A. frigida* plants when pasture degradation is intensifying [2]. *Artemisia frigida* plants are heat-resistant, being able to survive in environments comparable to habitats of typical desert plants with temperatures reaching 56–57 °C [3].

In conditions of high air temperatures and aridity of the territory, volatile terpenoid compounds act as the first line of defense for the photosynthetic apparatus. Thus, the fumigation of plants with monoterpenes increases their thermal resistance. Monoterpenes, as well as many volatile sesquiterpenes found in plants, quickly react with reactive oxygen species (ROS) [4], thereby protecting the membranes of plant cells from oxidative damage. Sesquiterpenes can mitigate damage even more effectively than isoprene and monoterpenes. For example, the sesquiterpene (E)- β -caryophyllene is 43 times more reactive with ozone than the monoterpene limonene. Two other sesquiterpenes, (E)- β -farnesene and (E)- α -bergamotene, have a high potential for protecting against abiotic stress [5]. The protective mechanism is associated both with direct reactions of terpenoids with oxidants, either intracellularly or at the boundary between the leaf and the atmosphere/ boundary layer, stabilizing the membrane, and with indirect changes in ROS signal transduction [6]. Plants emit an enormous number of terpenoids annually, reaching huge quantities (500 teragrams) [7]. Climate changes affect terpenoid emissions and have significant consequences for the structure and functioning of the biosphere [8]. In addition to high temperatures, terpenoid emissions can also be induced by herbivore feeding, pathogen attack, radiation, salt stress and other abiotic stresses [9,10].

Terpenoid compounds are obtained from plants in the form of complex mixtures known as essential oils (EOs). EOs possess antibacterial, antifungal, herbicidal and other properties, and therefore have found wide applications in the pharmaceutical, food, agricultural, cosmetic and healthcare industries [11,12]. EOs are complex mixtures of both native components and artifact compounds that inevitably form because of such processes as storage, drying of plant material, extraction and analysis. Their composition is related to many variables, such as cultivar genotype [13], the geographical area of production [14], harvest year [15], and extraction system [16]. When obtaining EOs under the same conditions of storage and drying of raw materials, and using the same method of extraction and analysis of EOs under strictly defined parameters, we can observe the influence of genetic and epigenetic factors on the composition.

The aim of this study is to determine the patterns of the formation of the composition of the essential oil of *Artemisia frigida* Willd. plants, and to give possible reasons for its conservatism and lability.

2. Results and Discussion

2.1. Composition of Essential Oils of *Artemisia frigida* (Based on Plants Growing within the Territory of Buryatia (Russia) and Qinghai (China) from 2016–2021

The isolated EOs were light, mobile liquids of a pale yellow color with a yield ranging from 0.3 to 1.0% (*v/w*). A total of 179 compounds were identified in the samples of the essential oil, with the majority being monoterpenoids and sesquiterpenoids (Supplementary Materials, Table S1).

For data analysis convenience, the entire variety of identified compounds were divided into four groups, based on their frequency of occurrence in the 27 tested samples: (1)—components found in 75% or more of the samples; (2)—in 50–74% of the samples; (3)—in 26–49% of the samples; and (4)—in 25% or less of the samples.

The first group was represented by 21 compounds. Only 1 compound among these, bornyl acetate, was found in all 27 samples; 9 compounds (γ -terpinene, para-cymol, terpinen-4-ol, α -terpineol, camphor, α -pinene, germacrene D, caryophyllene oxide, spathulenol) were found in 26 samples. Another 11 compounds were found in 21–25 samples. The mass fractions of components forming the first group ranged from 26.25% to 91.17%, meaning that they were the dominant components of *A. frigida* EOs. Among them, three compounds—camphor, borneol and 1,8-cineol—were part of the top five dominant compo-

nents in 81% of the studied samples. Less frequently among the dominant compounds (in at least one of the samples), bornyl acetate, α -terpineol and terpinen-4-ol from this group were observed. There were also such compounds from this group as linalool, (E)-nerolidol, α -copaene, β -pinene, salvia-4(14)-en-1-one, and β -myrcene, which were not found as dominant in any of the studied samples (Supplementary Materials, Table S2).

The second group consisted of 19 compounds. Such components as *para-trans*-menth-2-en-1-ol, myrtenol, bicyclogermacrene, and silphiperfol-6-en-5-one were identified in 20 out of 27 samples. The remaining 15 compounds were found in 14–19 samples. The total mass fraction of compounds of the second group was 0.96–29.34%. The content of each of the components, with the exception of myrtenol, selinene, beta- and isocaryophyllene in individual samples, did not exceed 2–3%.

The third group was formed of 38 compounds. They were identified in 7–13 samples, where their total content ranged from 1.52% to 22.40%. The proportion of individual compounds in this group did not exceed 1–2%, except for (E)- β -farnesene, (Z,E)- α -farnesene, neointermedeol, and *cis*- β -p-mentha-6,8-dien-2-ol in certain samples.

The fourth group was the most numerous (101 components). However, fifty compounds were found only in one of the samples, and only five (nonanal, nerol, trans-chrysanthanol, trans-sabinene hydrate, 7-epi-silphiperfol-5-ene) were identified in six samples. The total proportion of components in this group varied from 0.72% to 59.93%. The mass fraction of most compounds in this group did not exceed 1–2%, except for two cases: the proportion of α -terpineol acetate in the sample “BURi 30.08.2017” was 6.30%, and that of α -thujone in the sample “OLNs 05.08.2017” was 10.52%.

Among the studied samples, the sample from China «CHN, 2016 4333 m» and four samples from Buryatia “BUR 1343 m 17.08.2016”, “BURss 24.08.2016”, “BURroad 19.08.2021”, and “BURE 25.08.2016” were different from others in terms of the component composition of EOs. For example, the sample “CHN, 2016 4333 m” contained only caryophyllene (17.78%) among the top five dominant components from the first group, while the rest of the dominants were from the fourth group: methyl eugenol (16.16%), caryophylla-3(15),7(14)-dien-6-ol (14.36%), β -sesquiphellandrene (8.91%) and α -fenchene (4.42%). The dominant components of the sample “BUR 1343 m 17.08.2016” were spathulenol (14.05%), germacrene D (5.58%), caryophyllene oxide (3.54%) from the first group, (E)- β -farnesene (12.88%) from the third group, and acorenone (5.25%) and (2E, 6E)-farnesol (3.43%) from the fourth group.

The “BURss 24.08.2016” sample was also different from the others, since it contained compounds from the first group—*para*-cymol (12.6%), as well as spathulenol in a noticeable amount (7.97%). In addition, the dominant components of this sample included a component from the third group, (E)- β -farnesene (7.97%). The component composition of the first six dominant components of the essential oil of the sample “BURroad 19.08.2021” included compounds from the first group (germacrene D 13.01%, spathulenol 9.84%, and caryophyllene oxide 6.94%), the second group (isocaryophyllene 10.12%, and β -selinene 5.33%) and one compound from the third group (β -elemene 5.33%). At the same time, “BURE 25.08.2016” contained isocaryophyllene (9.96%) and approximately equal amounts (5–6%) of common compounds found in most samples, such as 1,8-cineol, borneol, germacrene D, spathulenol and caryophyllene oxide.

The differences in the composition of the dominant components in the samples “CHN, 2016 4333 m” and “BUR 1343 m 17.08.2016” can be explained by the growth of plants in areas of high altitudes. The composition of the sample “BURroad 19.08.2021” components may be related to the fact that plants grew close to the federal road R258 “Baikal”, with intensive traffic. The reasons for the differences in the composition of EOs in other samples are currently unclear. In any case, it is obvious that the composition of components and the content of their EOs are changeable under the influence of different factors. In addition, let us consider some of the factors that influence the composition of EOs of *Artemisia frigida* in more detail.

2.2. Composition of Essential Oils of *A. frigida* during Ontogenesis and Phenological Development of Plants

It has been established that changes occur in the biosynthesis of plants during ontogenesis in *A. frigida*. Indeed, it has been shown with potted crops that during the first year of plant life, changes occur in nitrogen metabolism. Specifically, the nitrogen content in shoots and roots decreases throughout the vegetative period [17]. However, the genetic basis of these changes in biosynthesis during ontogenesis is currently unknown. An interesting example of this is the encoding of the biosynthesis of a large number of sesquiterpenes in patchouli plants with only five genes, which may be indirect evidence of the importance of epigenetic mechanisms in the biosynthesis of terpenoid compounds in plants [18]. The first step towards differentiating the genetic and epigenetic nature of the components of EOs may be the study of their dynamics during plant development, as it is known that the quantity and composition of EOs change not only in space but also during plant ontogeny [19,20]. Unfortunately, there are no data on the dynamics of the qualitative and quantitative composition of the essential oil at all stages of *A. frigida* ontogeny. Only the composition of the essential oil in the latent and generative phases of age development has been studied.

The latent phase of plant ontogeny is represented by fruits. In *A. frigida*, the fruit seeds contain EOs that act as anti-attractants for herbivore feeding, inhibitors of seed germination, sprout growth and reproduction of other species [21,22]. The same components identified in the EOs obtained from the aboveground parts are also found in the composition of the fruit EOs. The main components of the fruit EOs are compounds with allelopathic activity: camphor (22.58%), borneol (22.46%), 1,8-cineol (25.11%), terpinene-4 (4.79%) and bornyl acetate (3.57%). The content of the remaining components does not exceed 1.5% (Table 1).

Table 1. Chemical composition of EOs obtained from the seeds of *A. frigida* (Russia, Buryatia, Ivolginsky district, steppe. 15 September 1999).

RI *	Component	Peak Area (%)	RI *	Component	Peak Area (%)
932	pinene, α -	0.77	1162	pinocarvone	0.24
947	camphene	1.44	1166	borneol	22.46
975	pinene, β -	0.38	1177	terpinen-4-ol	4.79
991	myrcene, β -	1.13	1191	terpineol, α	1.81
1017	terpinene, α -	0.73	1287	bornyl acetate	3.57
1024	cymol, <i>para</i> -	0.63	1378	copaene, α -	0.22
1028	limonene	0.05	1387	bourbonene, β -	0.13
1031	cineol, 1,8-	25.11	1392	elemene, β -	0.10
1058	terpinene, γ -	1.33	1422	caryophyllene	0.36
1066	sabinene hydrate, <i>trans</i> -	1.44	1484	germacrene D	0.48
1088	terpinolene	0.28	1488	selinene, β -	1.18
1098	sabinene hydrate, <i>cis</i> -	1.08	1527	cadinene, δ -	0.23
1100	libalool	0.88	1580	spathulenol	0.76
1121	menth-2-en-1-ol, <i>para</i> -, <i>cis</i> -	0.43	1586	caryophyllene oxide	0.56
1144	camphor	22.58		Σ monoterpenes	91.33
	Σ sesquiterpenes				4.02

* RI, retention indices: experimental, retention index as determined on HP-5MS column using the homologous series of n-hydrocarbons.

Interestingly, unlike the aboveground parts of adult *A. frigida* plants in the generative phase of ontogeny, most of the oil in the fruits consists of monoterpenoids, which are characterized by greater volatility (91.33%). In our opinion, monoterpenoids facilitate the survival of the fruits, including protection against pests such as invertebrates (insects and others) and fungi and bacteria, as more volatile compounds interact more quickly with the surrounding environment during the early stages of seed germination and require less energy for their biosynthesis.

The composition of the essential oil changes during different phases of plant phenological development. For example, in the study [23], it was shown that as the *Lavandula × intermedia* flower develops, the camphor content in the essential oil remains unchanged, but the concentration of borneol (a precursor to camphor) in the tissues increases. However, nowadays there is no direct molecular link between plant development and terpene biosynthesis that has been revealed [18].

The compositions of the EOs of *A. frigida* plants collected in the same populations but in different phenological phases were studied. The EOs were isolated from plants collected in three populations of the Ivolginsky district of the Republic of Buryatia in 2021: steppe (“BURi 27.07.2021”, “BURi 20.08.2021”, “BURi 17.09.2021”), roadside (“BURroad 19.08.2021”, “BURroad 17.09.2021”) and hilltop (“BURhill 27.07.2021”, “BURhill 19.08.2021”). The plants collected in July were in the budding phase, in August the flowering phase, and in September the fruiting phase.

During the transition from one phase to another, the yield of essential oils did not change, but the composition slightly changed. Compounds in the first group (except β -pinene, β -myrcene) were found in all stages of plant development, and only their proportion in the essential oil changed. However, there was no specific trend of decrease or increase in the transition from one phenophase to another, meaning that the direction of change was different for each population. Monoterpenoids such as β -pinene and β -myrcene were identified in the flowering (“BURroad 19.08.2021”, “BURhill 19.08.2021”) and fruiting phases (“BURroad 17.09.2021”, “BURi 17.09.2021”). Compounds of the second group were also found in all phenophases, except myrtenyl acetate. The latter was only identified in the sample “BURi 20.08.2021”. This sample also, unexpectedly, had a high content of the monoterpene alcohol myrtenol (4.27%). In the sample “BURhill 27.07.2021”, silphiperfol-6-en-5-one (4.43%) and isocaryophyllene (7.11%) differed significantly in content compared to other samples, and the latter was also found in significant amounts in the sample “BURroad 19.08.2021” (10.12%). Compounds in the third group can be found in all phenophases (β -elemene, pentadecanal, etc.) as well as in specific phenophases (for example, β -copaene in the budding phase, and cabreuva oxide B in budding and flowering). Some of them (silphiperfol-6-ene, aristolochene, 4,5-di-epi-, teointermedeol, β -elemene, etc.) may have a content higher than 1%, reaching, for example, 5.33% for β -elemene. The majority of components in the fourth group were found in only one sample. Mintoxide, *trans*-sabinene hydrate and eugenol were identified in all phenophases. In the budding and flowering phases, 1,8-, 2,3-dehydro-cineol, and chrysanthenone were detected, and in the flowering and fruiting phases salviadienol was found. Davanafuran isomer and silphiperfol-4,7(14)-diene were detected in the budding and fruiting phases (Supplementary Materials, Table S3).

The dynamics of the content of major components (with a content of 3% and more) of the essential oil from budding to fruiting was considered using a specific steppe population as an example. The main components included compounds from the first group: bornyl acetate, terpinen-4-ol, α -terpineol, camphor, spathulenol, 1,8-cineol and borneol, as well as one component each from the second (myrtenol) and third (neointermedeol) groups. The changes in the content of camphor, spathulenol and borneol were insignificant. However, the proportion of terpinen-4-ol decreased by half during the transition from budding to flowering, while α -terpineol increased 2.6 times during fruiting compared to other phenophases. The changes in the content of components from the labile part of the *A. frigida* essential oil were more significant. For example, the content of myrtenol increased by 8.2–13.3 times during the flowering phase. Neointermedeol was not detected during the flowering phase, while its proportion was approximately the same during the budding and fruiting phases (3.30–3.61%) (Figure 1).

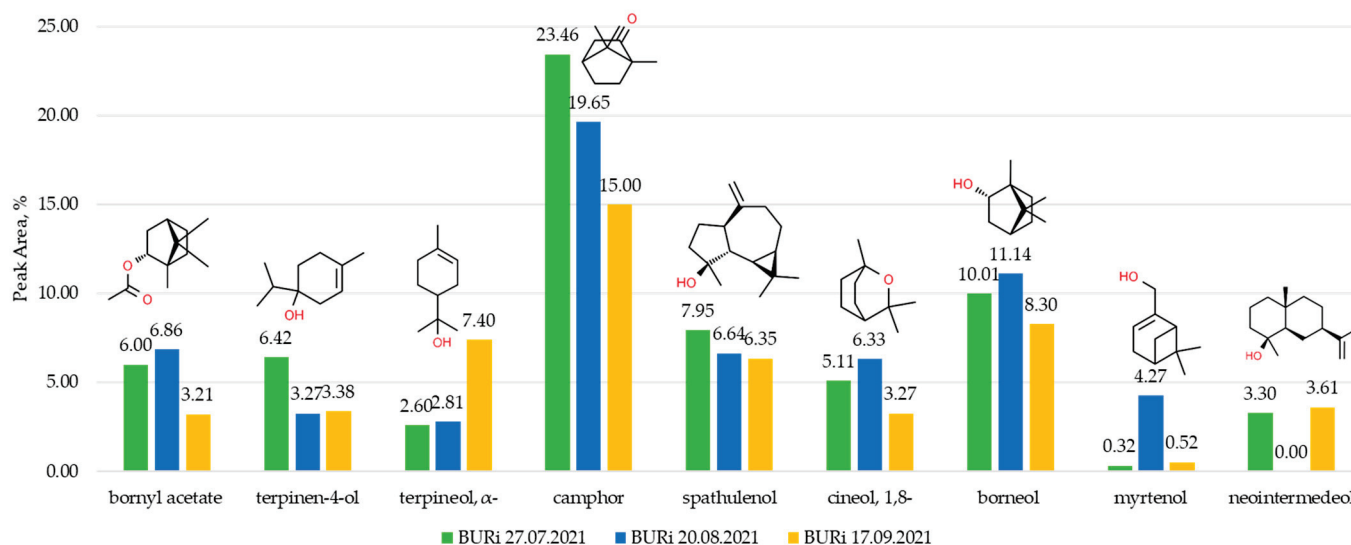


Figure 1. Content of major components of EOs of *A. frigida* plants during budding phase (green), flowering phase (blue) and fruiting phase (yellow).

As for the trends in the content of individual compounds in different phenophases, four patterns can be identified, three monomodal (Nos 1–3) and one bimodal (No 4): (1) the content of the compound is highest during the budding phase and then gradually decreases or remains at the same level (camphor, spathulenol, and terpinen-4-ol); (2) the content reaches its peak during the flowering phase (bornyl acetate, 1,8-cineol, borneol, and myrtenol); (3) the content of the compound is highest during the fruiting phase (terpineol, α-); and (4) the compound is present during the budding and fruiting phases but absent during flowering (neointermedeol). The presence of different patterns in the essential oil content during phenological development demonstrates the complexity of adaptive mechanisms.

Thus, the composition of the essential oil changed during plant development, particularly during phenological development. Compounds from the first and second groups were found in almost all phenophases, but their content in the essential oil varied. They formed a constant part of the essential oil, but no trends in the dynamics of their content were observed. It can be assumed that the formation of compounds in the constant part of the essential oil is predetermined at the genetic level, and thus this set is an immanent property of the species. On the other hand, differences in the composition of compounds from the third and fourth groups in different phases of plant development can be explained by the formation of new functional organs in which additional substances are synthesized [24]. Their formation might be associated with the species' biosynthetic reaction to certain conditions, which accordingly has an epigenetic nature, as well as with the transformations of native compounds during the processes of drying, extraction and analysis.

2.3. Composition of Essential Oils of *A. frigida* from the Same Population Depends on the Weather Conditions in Different Years

The differences in the compositions of EOs obtained from plants of the same population at different phenophases are balanced compared to the differences between the compositions of EOs of the same population in different years, i.e., under different weather conditions. Thus, on the biplot (PC1–PC2), the projections of samples of EOs were divided into two parts. One part was grouped, regardless of the different phenophases of the samples, of samples from 2021, while the other part consisted of samples from other years (Figure 2). Indeed, the year 2021 significantly differed from others in terms of temperature and moisture conditions (hydrothermal coefficient—HTC 1.6), being more humid. In contrast, the years 2014–2020 were drier (HTC 0.3–0.9). The distance between

samples collected in June and July 2015 (“15–9”, “15–18”) and July and August 2017 (“BURi 01.07.2017”, “BURi 30.08.2017”) can be explained by differences in climatic parameters from the beginning of vegetation to the date of collection. For example, in 2015, the average daily temperature from May to the date of plant collection (average $t_{veg.}$) for the “15–9” sample was 13.8 °C, while for “15–18” it was 16.1 °C. The cumulative rainfall in May until the date of plant collection ($\Sigma R_{veg.}$) for the “15–9” sample was 29.1 mm, while for “15–18” it was 62.5 mm. Correspondingly, the extremeness coefficients ($K_{extr.veg.}$) differed almost twofold (0.5 and 0.3, respectively). In contrast, in 2021, temperature fluctuations and precipitation were more evenly distributed; climatic parameters (average $t_{veg.}$, $\Sigma R_{veg.}$) did not differ significantly at different collection times, and the extremeness coefficient ($K_{extr.veg.}$) from budding to fruiting had a value of 0.1 (Supplementary Materials, Table S4).

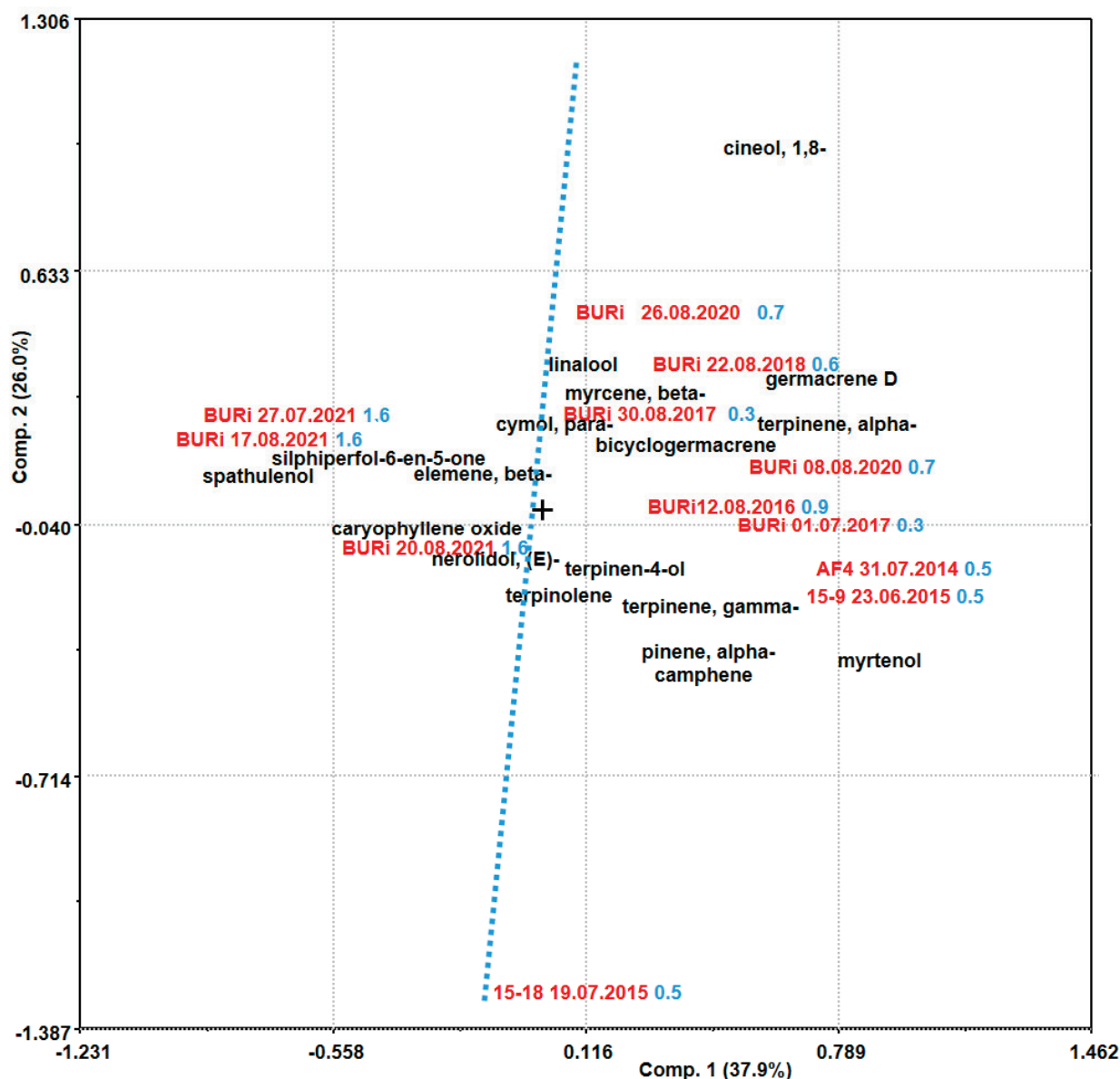


Figure 2. Biplot (PC1–PC2) of data on the content of constant compounds in the EOs of *Artemisia frigida* Willd. plants from the same population (Russia, Republic of Buryatia, and Ivolginsky district) in different years (2014–2021) (Principal Component Analysis). Samples “AF4 31.07.2014”, “15–9 23.06.2015” are previously published data [25]. Values of the hydrothermal coefficient are displayed in blue, sample codes—red.

Correlation analysis showed a high (correlation coefficient values from 0.7 to 0.9) and very high (from 0.9 to 1.0) degree of association between the values of climatic parameters and some components of the essential oil. For example, the correlation coefficient values (0.92–0.98) between HTC values and the content of spathulenol, caryophyllene oxide and silphiperfol-6-en-5-one indicated a very high positive correlation between them. The values of average t_{veg} showed a high positive correlation with the content of borneol and a negative correlation with silphiperfol-6-en-5-one. The values of $\sum R_{veg}$ positively correlate with the content of spathulenol and caryophyllene oxide, while $K_{extr.veg}$ correlated positively with α - and γ -terpinene (Supplementary Materials, Table S5). Thus, the mass content of compounds from the first and second groups in the EOs of plants from the same population depends on the weather conditions in the year of raw material collection.

2.4. Composition of Essential Oils of *A. frigida* from Different Populations in Buryatia Depends Not Only on Climatic Conditions

PCA analysis was conducted on data obtained from other populations (in Selenginsky and Eravninsky districts) and compared with the EOs of plants growing in the Ivolginsky district. The differences in the composition of EOs of plants collected in different years were less pronounced than the differences between the compositions of EOs of plants from different locations (Figure 3).

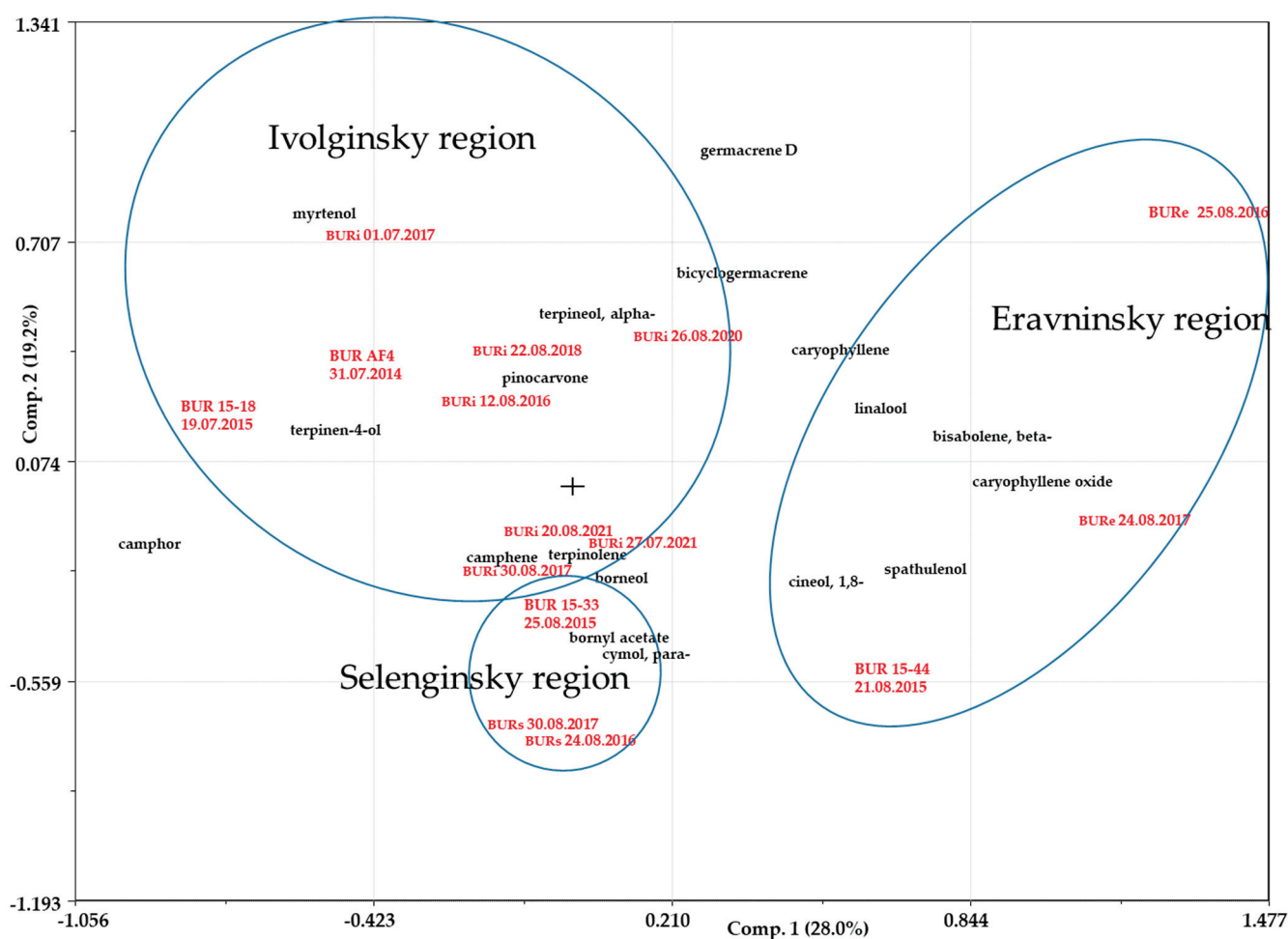


Figure 3. Biplot (PC1–PC2) of multi-year data on the content of constant compounds in the EOs of *Artemisia frigida* Willd. plants collected in the Ivolginsky, Selenginsky, and Eravninsky districts of Buryatia (Principal Component Analysis). Sample codes are displayed in red color, districts—blue circles.

The samples collected in the Eravninsky district differed the most. They were characterized by relatively low content of camphor (up to 5.5%) and noticeable amounts of certain sesquiterpenoids (β -bisabolene, caryophyllene, caryophyllene oxide, and spathulenol). In contrast, the composition of EOs from plants in the Ivolginsky and Selenginsky districts differed to a lesser extent. EOs of samples from the Selenginsky district contain higher amounts of cymol and para- and bornyl acetate compared to samples from the Ivolginsky district.

Linear regression models show that only the content of trans-carveol, para-, trans-menth-2-en-1-ol, camphor and bornyl acetate in the EOs has been changed under the influence of climatic parameters. For example, the content of trans-carveol was influenced by the climatic extremeness of the year. Among the dominant components, the content of camphor in the essential oil linked to changes in climatic parameters (Table 2).

Table 2. Content of components in the essential oil of *Artemisia frigida* Willd. plants growing in the Ivolginsky, Selenginsky and Eravninsky districts of the Republic of Buryatia, according to linear regression models.

x	a	Y	b	c	z	p-Value	R2 (adjR2)
model $z = a*x + b$							
$K_{extr.year}$	30.13		0.10		trans-carveol	0.001	0.62
model $z = a*x + b*y + c$							
$\Sigma R_{June-August}$	−0.002	$\Sigma t_{veg.}$	-3.89×10^{-4}	1.17	menth-2-en-1-ol, para-, trans-	6.70×10^{-3}	0.52
$\Sigma t_{June-August}$	0.08	$\Sigma R_{May-June}$	0.77	−155.63	camphor	7.30×10^{-4}	0.68
$\Sigma R_{June-August}$	0.03	$\Sigma t_{June-August}$	1.14×10^{-2}	−18.32	bornyl acetate	2.91×10^{-3}	0.59

The obtained results showed that the influence of climatic factors on the composition of EOs in plants was varying, which was also shown for plants of the genus *Nepeta* [26] and *Lavandula* [27]. Significant differences in the composition of EOs of plants from the Eravninsky district compared to the Selenginsky and Ivolginsky districts may be related to the fact that negative values of the average annual temperature lead to the existence of permafrost in the soils of the Eravninsky district, where permafrost has discontinuous distribution. In contrast, the Selenginsky and Ivolginsky districts belong to the zone of the only isolated distribution of permafrost. Thus, plants in the Eravninsky district experience a greater influence of low soil temperatures, which in turn is reflected in the composition of EOs. There is information in the literature about the influence of soil type [28] and substrate composition [29], but there are no data on the effect of soil temperature or permafrost on the composition of the essential oil, although the soil temperature affects the content of secondary metabolites in the plant. For example, a higher maximum soil temperature resulted in lower phenol content, while a higher minimum soil temperature and shorter harvest age increased the total flavonoids in Java tea (*Orthosiphon aristatus* B.) [30].

2.5. Composition of Essential Oils in Plants of *A. frigida* Confined to “Typical” and “Atypical” Habitats

The analysis of the composition of *A. frigida* EOs from 1998 to 2021 presented in this study and in previously published works [25,31–33], as well as literature data from different parts of the range: Siberia [34–36], Kazakhstan [35,37], Mongolia [38], Canada [39] and China [40], showed that a total of 283 compounds were identified. Camphor, 1,8-cineol and borneol were the dominant components in 93–95% of the samples, and among sesquiterpenoids, germacrene D was identified as a dominant component in 67% of the samples. The constant part of the EOs was represented by 32 components (Supplementary Materials, Table S6). It included all compounds in the first group of *A. frigida* EOs from the flora of Buryatia collected from 2016 to 2021 (Supplementary Materials, Table S2), except for salvial-4(14)-en-1-one and seven compounds from the second group: myrtenol, bicyclogermacrene, trans-carveol, humulene, β -selinene, para-cis-menth-2-en-1-ol, terpinolene, as well as some

compounds from the third group: sabinene, trans-sabinene hydrate, β -elemene, α -elemene, and pinocarvone.

Previously, it was shown for *Artemisia vulgaris* L. [41], *A. annua* L. [42], and *A. jacutica* Drob. [43] that under the influence of macroclimatic conditions, distinct “western” and “eastern” chemotypes of EOs were formed, which differed in their component composition. In the case of *A. frigida*, chemotypic differentiation was not observed, as there was no clear discrepancy between the essential oil samples obtained from “western” and “eastern” populations. In general, according to the PCA analysis in the case of *A. frigida*, most samples, regardless of their place of growth, did not show significant differences in composition. Nevertheless, there were 13 components that were most noticeably linked to climatic conditions, which was manifested in their greatest contribution to the distribution of samples on the biplot (Figure 4).

It can be noted that the majority of “western” samples were characterized by a relatively high content of monoterpenoids—camphor, 1,8-cineol and camphene. And for most “eastern” samples, in addition to monoterpenoids—bornyl acetate, terpineol-4, α -terpineol, and myrtenol—sesquiterpenoids such as spathulenol and caryophyllene oxide were presented in noticeable concentrations. However, even these features are not enough to allow for the identification of specific chemotypes. The reason may lie in the biological characteristics of *A. frigida*, which often grows on sandy and stony soils with relatively sparse vegetation cover [44]. Meanwhile, *A. frigida* plants from Western and Eastern Siberia are in similar conditions in terms of heat and moisture supply in their habitats. Thus, the values of hydrothermal coefficient (HTC) calculated from meteorological data from Western and Eastern Siberia ranged from 0.5 to 1.4, i.e., from “very arid” to “humid” conditions in both regions (Table 3).

Table 3. Hydrothermal coefficient of Selyaninov of Western and Eastern Siberia, 1999–2021 (May–August).

Year of Collection	Meteorological Station	Σt	R	HTC
Western Siberia				
1999	36038 Zmeinogorsk	2208.6	157.1	0.7
	36229 Ust-Kosa	1818.8	279.5	1.5
2000	29915 Slavgorod	2178.9	210.1	1.0
2001	36259 Kosh-Agach	1532.1	68.2	0.4
2003	360555 Kyzyl-Ozyok	1951.1	268.0	1.4
2003	36045 Soloneshnoye	2116.7	258.7	1.2
2005	29915 Slavgorod	2297.1	123.8	0.5
2007	36177 Semey	2341.9	181.3	0.8
2006	36096 Kyzyl	1884.7	193.7	1.0
Eastern Siberia				
2006	36307 Erzin	1829.8	96.7	0.5
2006	29862 Abakan	1836.5	212.3	1.2
1999	30636 Barguzin	1890.2	238.5	1.3
2014	30859 Aginskoye	1954.6	96.6	0.5
2014	30825 Ivolginsk	1881.0	97.9	0.5
2015	30745 Sosnovo-Ozerskoye	1708.3	175.9	1.0
2017	30632 Bolshoy Ushkany Island	1325.1	87.2	0.7
2017	30925 Kyakhta	2150.6	232.5	1.0
2017	30823 Ulan-Ude	2116.2	62.8	0.3
2021	30823 Ulan-Ude	1874.5	258.8	1.4

However, 10 samples of EOs of plants growing in areas where climatic parameters differed from typical ones formed a single locus (“atypical” group, Figure 4). The differences were associated with the influence of high altitude, as well as the proximity of roads, or increased insolation. This locus was formed by plant samples from China, growing at altitudes of 2735–4333 m, Mongolia, growing at 1327 m and 1854 m, Buryatia, growing

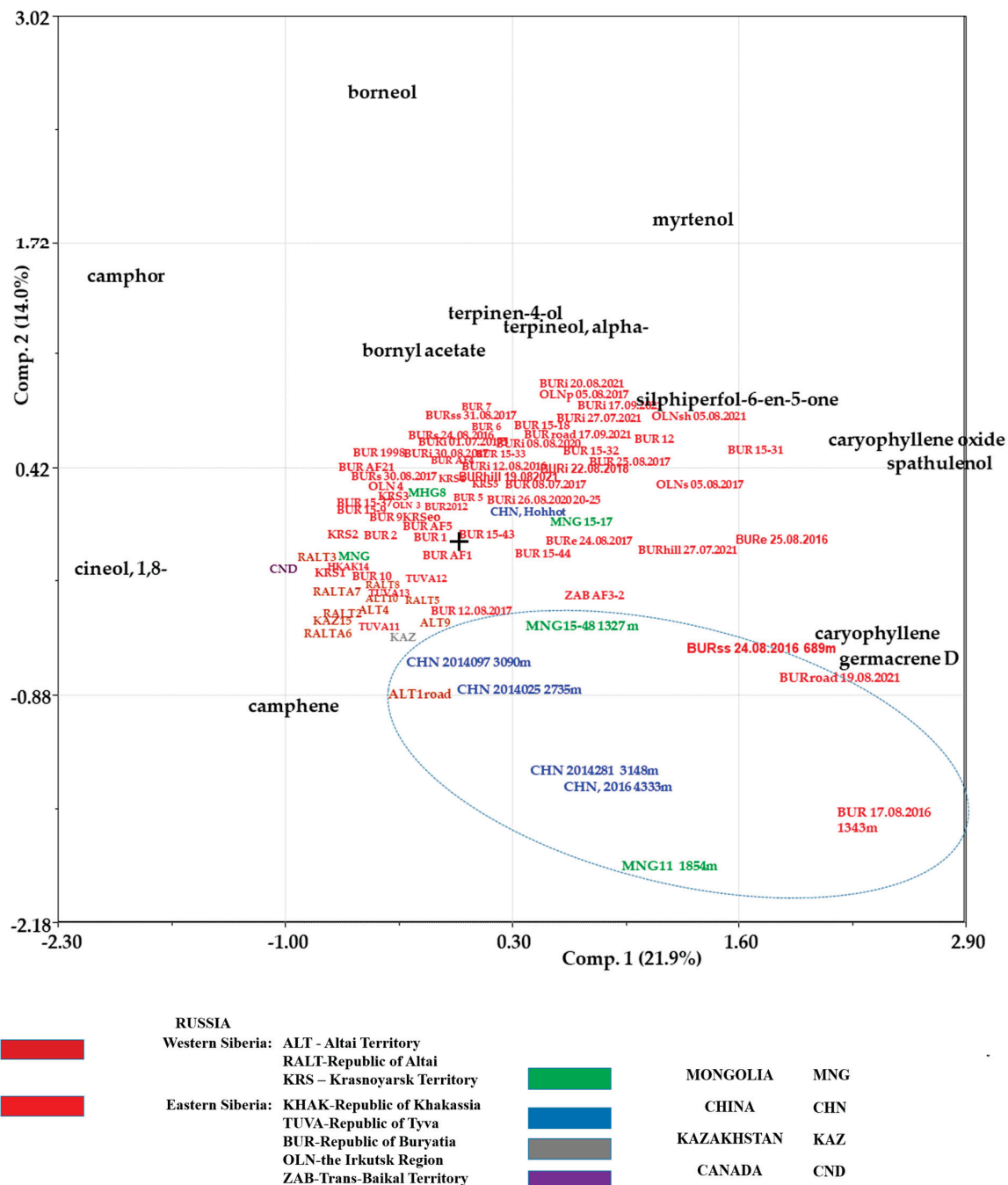


Figure 4. Biplot (PC1–PC2) of data on the content of constant compounds in the EOs of *Artemisia frigida* Willd. plants from different regions of the world (Principal Component Analysis). Designation “abbreviation and serial number”, “abbreviation and code”, “abbreviation, year”—published data, including own data from Russia (Republic of Buryatia, Irkutsk region) and Mongolia. Only designated with a code and collection date—data of 2016–2021.

If the differences in the composition of EO in plants due to the influence of altitude (which determines climatic parameters) and the proximity of roads do not require any special explanations, then the influence of the slope exposure, which is the habitat of the plants, should be considered in more detail. Essential oil samples “OLN4”, “BUR10” [25] and

“BURss 24.08.2016” were obtained from plants collected on slopes of different exposures. In the sharply continental climate of semiarid regions, including Transbaikalia and the Cis-Baikal area, the ecological conditions on different slopes differ significantly. Southern slopes receive more heat during the vegetative period. On southern insolated slopes the evaporation is much stronger, and plant communities experience a moisture deficit [45–48]. Thus, it is confirmed again that the composition of essential oil is determined by climatic parameters, in particular the ratio of heat to moisture.

All samples of the “atypical” group contained β -myrcene, terpinen-4-ol and germacrene D. If the content of terpinen-4-ol was lower than in the EOs of “typical” habitats, then the content of β -myrcene and germacrene D could reach significant levels. For example, the content of β -myrcene in the “MNG11 1854 m” sample was 7.90%, and that of germacrene D in samples “ALT1road” and “BURroad 19.08.2021” was 4.70% and 13.97%, respectively; in the sample “MNG11 1854 m” it was 20.30%. In 5–9 out of 10 samples, components were present in the EOs in noticeable concentrations. These include bicyclogermacrene (up to 2.68%), α -pinene (up to 5.90%), β -selinene (up to 6.72%), caryophyllene oxide (up to 6.94%), linalool (up to 10.31%), (E)- β -farnesene (up to 12.88%), spathulenol (up to 14.05%), 1,8-cineol (up to 22.88%), and camphor (up to 33.10%). At the same time, a number of components have been identified in half or more of the samples in this group, but the content of each of them did not exceed 1%: γ -terpinene, terpinolene, α -terpineol, (E)-nerolidol, caryophyllene, α -terpinene, camphene, bornyl acetate, β -pinene, α -copaene, and sabinene. Limonene, artemisia ketone, α -phellandrene, cis-davanone, β -elemene, and α -bisabolone oxide A were found in 3–4 samples, but their content was high. For example, in the samples “CHN2014281 3148 m”, “CHN2014097 3090 m”, and “CHN2014025 2735 m”, a significant proportion belonged to α -bisabolone oxide A (6.01%, 5.40%, and 24.25%, respectively). Acorenone was found only in two samples, and its content was significant: in “Oka 1343 m 17.08.2016” it was 5.25%, and in “CHN, 2016 4333 m” it was 8.90%. Chrysanthenone, α -thujone, α -bisabolol oxide B, and neointermedeol were also identified in only two samples, but only in one of them in large quantities. For example, the content of α -bisabolol oxide in the sample “CHN2014281 3148 m” reached 20.37%. In one of the samples of EOs of plants growing in high-altitude areas, components with a relatively high content were identified: methyl chavicol (4.30%) in the sample “MNG11 1854 m”, and α -farnesene (4.42%), (E)-methyl cinnamate (16.16%) and copaborneol (17.78%) in the sample “CHN, 2016 4333 m”. Thus, the composition of the dominant components of the EOs in this group of samples was different, especially in plants from high-altitude populations.

Samples from plants growing near the road (“ALT1road”, “BURroad 19.08.2021”) were most similar in composition and content of dominant components to the samples from “typical” habitats. Comparative analysis of the composition of EOs from *A. frigida* in “typical” and “atypical” habitats showed that 22 compounds were identified in half or more of the samples from both groups. These included camphor, 1,8-cineol, germacrene D and others. Some compounds found in the majority of EOs from “typical” habitats were found in fewer samples from “atypical” habitats. Borneol was found in all samples from “typical” habitats and in only 4 out of 10 samples from “atypical” habitats. Myrtenol was found in only one of the samples from “atypical” habitats. On the other hand, (E)- β -farnesene was found in 7 out of 10 samples from “atypical” habitats, while it occurred in only 20% of the EOs from plants in “typical” habitats. Among the other 261 components identified in less than half of the samples, 109 were found only in the EOs from “typical” habitats, 27 were found in “atypical” habitats, and 112 were common to plants from both groups. For example, α -bisabolol was specific to 18% of the samples from “typical” habitats, and yomogi alcohol was found in 3 out of 10 samples from “atypical” habitats, while trans-piperitol was identified in the EOs of both groups.

2.6. Conservatism and Lability of the Composition of *A. frigida* Essential Oils

Based on the abovementioned, it was possible to distinguish the “core” of the *A. frigida* essential oil composition, which referred to those components that were formed as part of

the genetic programs of terpenoid biosynthesis, regardless of the plant's habitat. A total of 24 such components formed the "core". However, the content of some of these components in the essential oil could vary during plant development and under the influence of climatic conditions. The total content of "core" components in the EOs of plants from "typical" habitats ranged from 48.31% to 92.60%, with an average value of 75.18%, while in the EOs of plants from "atypical" habitats it ranged from 4.82% to 77.17%, with an average value of 48.43%. The fluctuations in the content of camphor, 1,8-cineol, borneol and germacrene D were particularly noticeable.

Surrounding the "core" was a "cloud" of components whose formation was influenced by external factors. This "cloud" consisted of two concentric circles. The first concentric circle (11 compounds) included compounds that have been identified in samples from both groups but were found in the majority of essential oil samples from either "typical" or "atypical" habitats. The second concentric circle (248 compounds) comprised compounds that were found in a smaller number of samples from both groups (Figure 5). In addition to terpenoid compounds, the second concentric circle included phenylpropenes (eugenol, etc.) and acyclic compounds (capillene, etc.).

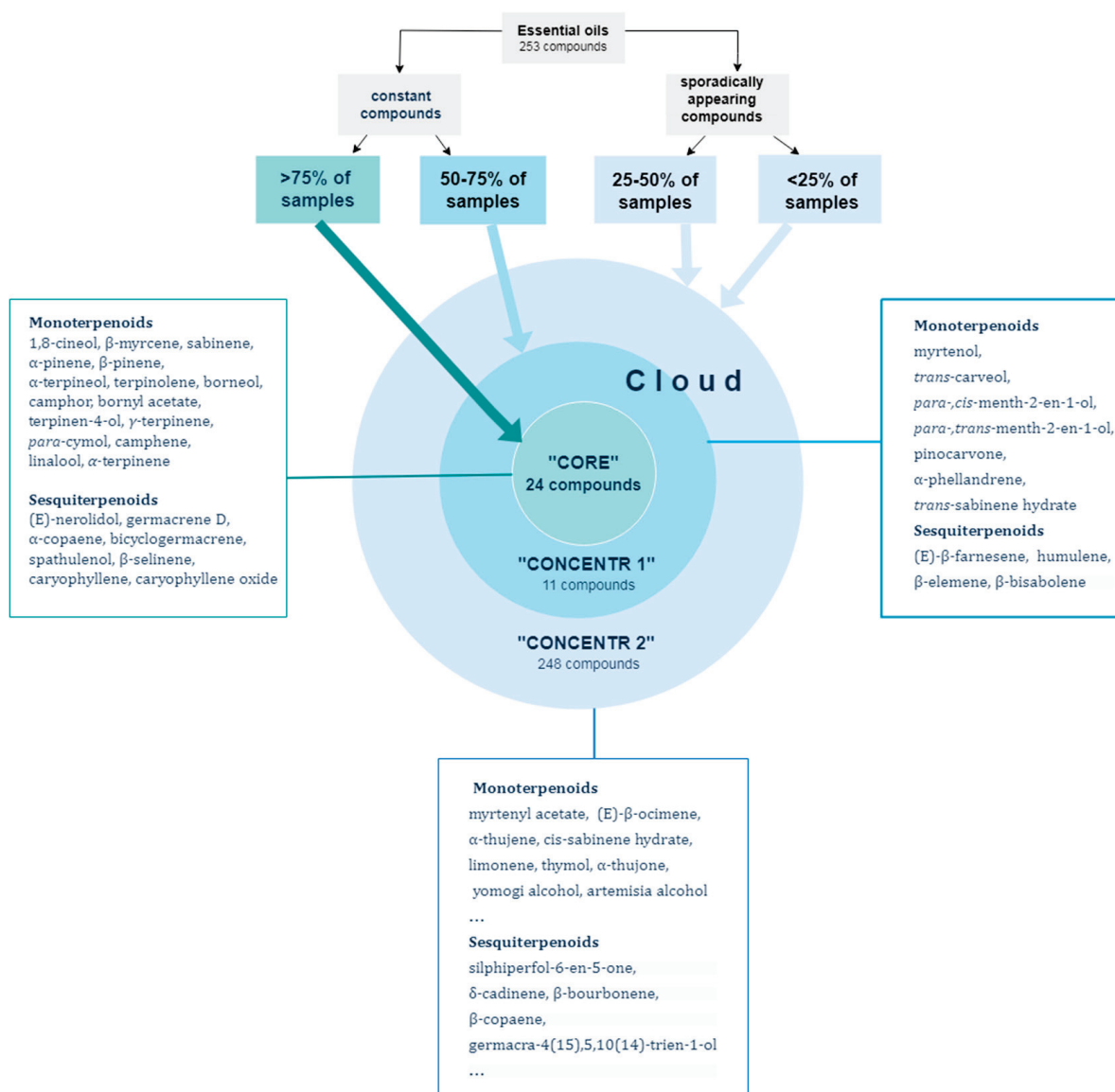


Figure 5. Conservative and labile components of essential oils of *A. frigida*.

The conservatism of the qualitative composition of the “core” was largely related to the characteristics of the compounds’ biosynthesis. For example, one of the dominant components of *A. frigida* EOs, 1,8-cineol, was always accompanied by myrcene, limonene, sabinene, α -, β -pinene, α -terpineol [49], terpinolene, (E)- β -ocimene, and α -thujene [50,51] in small quantities, because they are formed under the action of the multiproduct enzyme cineole synthase. These components are known as “cineole cassette”.

Borneol and camphene are formed from a common precursor, bornyl diphosphate, and are catalyzed by the multiproduct enzyme BOR-synthases [52]. Other compounds, such as borneol and camphor, are interconnected. The intermediate bornyl diphosphate is hydrolyzed to borneol through the catalytic activity of bornyl diphosphate synthase and borneol synthase, and then borneol is oxidized to form camphor through the catalytic activity of bornyl dehydrogenase. Previously it was considered that the enzymatic reaction catalyzed by γ -terpinene synthase lead to the formation of γ -terpinene, which further transformed into p-cymene and the phenolic terpene isomers thymol and carvacrol [53]. Now it is established that γ -terpinene is oxidized by monooxygenases of the cytochrome P450 subfamily CYP71D to form unstable intermediate cyclohexadienols, which are then dehydrogenated by short-chain dehydrogenase/reductase (SDR) to the corresponding thymol and carvacrol. The formation of p-cymene occurs due to spontaneous rearrangement of the dienolic intermediate products because of their instability under aqueous conditions [54].

According to recent studies, both 2*E*,6*E*-farnesyl pyrophosphate (*E,E*-FPP) and its isomer *Z,Z*-FPP are substrates for sesquiterpene synthases [55]. Acyclic sesquiterpenoids, such as (E)- β -farnesene and (E)-nerolidol, are formed through farnesyl and nerolidol cation, respectively. The biosynthesis of cyclic sesquiterpenoids is considered in terms of biogenetic pathways. In the essential oil of *A. frigida*, three groups (chemotypes) of sesquiterpenoids can be distinguished: germacrane, bisabolane and humulane sesquiterpenoids [56], with a predominance of germacrane compounds (Supplementary Materials, Table S7). The main compound of the germacrane chemotype in the EOs of *A. frigida* is germacrene D, which serves as a biogenetic precursor for many other sesquiterpene structures [57], including α -copaene, bicyclogermacrene, β -selinene and β -elemene. Spathulenol is formed from bicyclogermacrene during the extraction, storage and analysis of the essential oil [58]. Humulene, caryophyllene, caryophyllene oxide and triquinanes are representatives of the humulane series. All of these compounds were found in most samples and were part of the “core” and “concentric circle 1”. The exception was the group of triquinanes, which were found in specific samples. Among them, silphiperfol-6-en-5-one was the most frequently identified compound (48% of “typical” samples, and 3 out of 10 “atypical” samples). Bisabolane sesquiterpenoids were not represented in the “core”, and β -bisabolene was a part of “concentric circle 1”. The rest were included in “concentric circle 2”, but their content in the EOs of high-altitude plants was quite high. The total content of bisabolane sesquiterpenoids in samples from Qinghai province (China) ranged from 8.70% to 45.91%. “Concentric circle 2” included compounds formed as a result of further modification of terpenoids from the “core” and “concentric circle 1”. The presence of irregular monoterpenoids was observed in “concentric circle 2”, and they were formed through condensation of C5 units in a “non-head-to-tail orientation”, such as yomogi alcohol, lavandulol, artemisia alcohol and artemisia ketone.

Mono- and sesquiterpenoid compounds are multifunctional compounds. Their role in biotic interactions, such as allelochemicals, allelopathic compounds, antifeedants, attractants, and repellents, and their importance in plant defense against bacterial pathogens and fungi, has been well studied. At the same time, it is known that their emission is closely related to changes in abiotic factors in their environment. The existence of special biosynthetic pathways and the preservation of their emission, despite their “expensive” cost for plants (1–2% of photosynthetic carbon fixation) has led to the proposal of a model of a “unified biochemical mechanism for multiple physiological stressors”. It has been shown that volatile isoprenoids participate in direct reactions with oxidants and can also modify the transfer of ROS signals and/or contribute to membrane stabilization [4]. For

example, α -bisabolol demonstrates high antioxidant activity in vitro [59]. Overall, the role of isoprene in plant thermotolerance has been extensively studied, while the role of volatile terpenoids has been less explored [60]. It has been shown that terpenes can protect plants from heat damage, and there is a correlation between monoterpene emission and heat stress resistance [61]. In our opinion, the emission of terpenoids by *A. frigida* explains its thermotolerance. The remarkable fact is that the “core” predominantly consists of compounds (pinene, 1,8-cineol, camphor, spatulenol, etc.) with pronounced allelopathic activity [20]. At the same time, β -caryophyllene is involved in attracting natural enemies of herbivorous insects [62] and in the defense against microbial pathogens [63].

The components of “concentric circle 1” can act as allelochemicals (para-menth-2-en-1-ol) or can have other activities. For example, (E)- β -farnesene on one hand is secreted by aphids as a signaling pheromone, and on the other hand it is produced by infected plants to attract natural enemies of aphids [64].

On the other hand, the composition of compounds in “concentric circle 2” is determined by the conditions of specific plant habitats, including abiotic factors and interactions with other plants, microorganisms, insects and herbivores, and even humans. Pathogen attack can shift biosynthesis towards the production of sesquiterpenoid phytoalexins [65]. Triquinanes have antibiotic properties [66]. The increase in the content of copaene in EOs, an intermediate compound in the pathway of terpenoid alkaloids’ biosynthesis, may also be associated with protection against mammals [67].

Changes in the content of compounds in EOs are associated with their emission. In high-altitude conditions, for example, there seems to be a faster conversion of borneol into camphor, resulting in a tendency for a decrease in borneol content and an increase in camphor content in the essential oil. Since we extract a pool of accumulated terpenoids from the tissues in the form of essential oil, we cannot definitively say whether there is an enhancement of camphor and borneol emission or if their biosynthesis is blocked at very high altitudes (4333 m above sea level). It seems that the biosynthetic pathways of the “core” components are maintained regardless of plant habitat conditions, while the rate of their emission changes. Specific factors, often biotic, influencing plants, affect the components of “concentric circle 1 and 2”.

Thus, mono- and sesquiterpenoids serve as the first line of defense for plants against biotic and abiotic stresses. They are essential for plant survival, and their immense diversity is shaped by factors that have both a genetic and epigenetic nature. Overall, we can observe a notable change in the compositional profile of *A. frigida* EOs under the influence of climatic parameters.

3. Materials and Methods

3.1. Plant Materials

The plant material was collected in the natural steppe habitats with chestnut soils within the territory of Russia (Republic of Buryatia) from 2016 to 2021 (Table 4). The collected raw material was dried to an air-dry state (2 weeks). Herbarium specimens are stored in the Laboratory of the Chemistry of Natural Systems at Baikal Institute of Nature Management, Siberian Branch, Russian Academy of Sciences.

3.2. Essential Oil Analyses

3.2.1. Isolation of Essential Oils

A total of 30–40 g of the powdered plant material was subjected to hydrodistillation for 3 h using a Clevenger-type collector apparatus. The essential oils extracted in this research were obtained from the aerial parts of the plant. All experiments were conducted in triplicate, and the results were reported based on the air-dry weight.

Table 4. Collection information for *Artemisia frigida* used in this study for the years 2016–2021.

Sample Code	Collection Date	Location	Latitude Longitude	Altitude (m)	Yield V/M (%)
BURi 12.08.2016	12 August 2016	Russia, Buryatia, Ivolginsky district, steppe	N 51°42 E 107°12	645	0.7
BURi 01.07.2017	1 July 2017				0.6
BURi 30.08.2017	30 August 2017				0.7
BURi 22.08.2018	22 August 2018				1.0
BURi 08.08.2020	8 August 2020				1.0
BURi 26.08.2020	26 August 2020				0.7
BURi 27.07.2021	27 July 2021				0.3
BURi 20.08.2021	20 August 2021	Russia, Buryatia, Selenginsky district, steppe	N 51°22 E 106°32	618	0.3
BURi 17.09.2021	17 September 2021				0.3
BURs 24.08.2016	24 August 2016				0.3
BURs 30.08.2017	30 August 2017				0.9
BURe 24.08.2017	24 August 2017				0.3
BURe 25.08.2016	25 August 2016				0.3
BURroad 19.08.2021	19 August 2021				0.3
BURroad 17.09.2021	17 September 2021	Russia, Buryatia, Eravinsky district, steppe	N 47°47 E 107°13	926	0.3
BURhill 27.07.2021	27 July 2021				0.3
BURhill 19.08.2021	19 August 2021				0.3
BUR 1343 m 17.08.2016	17 August 2016				0.3
BURss 24.08.2016	24 August 2016				0.3
BUR 27.08.2020	27 August 2020				0.7
BUR 08.07.2017	8 July 2017				0.3
BUR 31.08.2017	31 August 2017	Russia, Buryatia, Zaigraevsky district, steppe	N 52°27 E 108°71	575	0.3
BUR 12.08.2017	2 August 2017				0.7
OLNp 05.08.2017	5 August 2017				0.9
OLNs 05.08.2017	5 August 2017				1.0
OLNsh 05.08.2021	5 August 2021				0.7
CHN, 2016 4333 m	15 July 2016				1.0
		Russia, Irkutskaya oblast, Olkhon district, steppe	N 53°02 E 106°93	469	
		Russia, Irkutskaya oblast, Olkhon district, vicinity of the Sokhor cove, steppe	N 53°02 E 106°76	491	
		Russia, Irkutskaya oblast, Olkhon district, vicinity of the Aya cove, steppe	N 52°79 E 106°60	780	
		China, Qinghai Province	N 33°08 E 96°43	4333	

3.2.2. GC-MS Analysis of Essential Oils

The composition of the EOs was determined using gas chromatography–mass spectrometry (GC-MS) analysis after obtaining EOs. An Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with an HP 5973 quadrupole mass selective detector (Hewlett-Packard, Palo Alto, CA, USA) and an HP-5MS capillary column (30 m × 0.25 mm × 0.2 µm; Hewlett-Packard) was used. The analysis was conducted with an electron impact at 70 eV and with the electron multiplier set at 2200 V. Helium was used as the carrier gas at a flow rate of 1 mL/min. The temperature of the column was programmed to increase from 50 to 240 °C at a rate of 4 °C/min, with an initial hold of 5 min, and then from 240 to 280 °C at a rate of 20 °C/min, with a final hold of 5 min. The injector and detector temperatures were set at 280 and 250 °C, respectively. The purity of the helium gas used was 99.999%. The column pressure was set to 52.8 × 10³ Pa, and the split ratio was 60:1.

3.2.3. Essential Oil Compound Identification

The identification of essential oil constituents was conducted by comparing their mass spectra with standards from the NIST and with calculated linear retention indices (RIs) found in the literature [58]. The RIs were obtained by co-injecting a mixture of linear C8-C20 hydrocarbons. Identification was considered valid when the computer matching with the mass spectral libraries had a probability of over 90%. The contents of the oil components were determined by their relative amounts (%) and expressed as a percentage peak area relative to the total peak area, based on the GC-MS analyses of the oils.

3.3. Climatic Conditions

To characterize the main climatic parameters, data provided by the All-Russia Research Institute of Hydrometeorological Information (World Data Center) were used. The downloaded data included air temperatures, daily precipitation amounts, monthly precipitation sums, monthly air temperatures, number of days with precipitation equal to or greater than 1 mm, average monthly relative air humidity, monthly sunlight hours and more. Additionally, integrated indicators such as Selyaninov's hydrothermal coefficient (HTC), temperature–humidity extremeness coefficient (C_{extr}) and the Standardized Precipitation Evapotranspiration Index (SPEI) were calculated.

The HTC was estimated to assess the levels of heat and humidity in specific regions using the formula $HTC = R \times 10 / \Sigma t$. In this equation, R represents the precipitation sum in millimeters for June–July at mean daily temperatures above +10 °C, and Σt denotes the sum of mean daily temperatures in degrees Celsius (°C) for the same period.

The C_{extr} coefficient was calculated to compare the plant habitat conditions across different years. This coefficient represents the ratio of the mean temperature value for a specific period to the total precipitation for the same period, using the formula $C_{extr} = t \text{ °C} / \text{mm}$.

Several parameters were estimated, including the sum of daily temperatures (Σt_{veg}) and the sum of precipitation (ΣR_{veg}) from the start of the vegetative phase (May) until the plant harvesting. Additionally, the sum of daily temperatures (Σt_{year}) and the sum of precipitation (ΣR_{year}) from August of the previous year to August of the year of plant harvesting were calculated. The temperature–humidity extremeness coefficients were estimated for both the period from the start of the vegetative phase until plant harvesting ($C_{extr.veg}$), and for the entire year ($C_{extr.year}$). To simplify the calculations, the resulting values were multiplied by 100 and 1000, respectively, and rounded to whole numbers.

The Standardized Precipitation Evapotranspiration Index (SPEI) was calculated using the SPEI package. This index was estimated for both the calendar year (January–December) ($SPEI_{cal}$) and the period from August of the previous year until August of the year of plant harvesting ($SPEI_{year}$).

3.4. Statistical Analysis

To determine the influence of the main climatic parameters (air temperature, atmospheric precipitation, etc.) and their integral characteristics on the composition of plant essential oil, two types of linear regression models were constructed [68]. The first type of model was represented by the equation $y = a \cdot x + b$. This type of model assumed that the content of a certain component of the essential oil, y , was linearly dependent on a certain parameter, x . The equation for the second type of model was $z = a \cdot x + b \cdot y + c$. Similar to the first case, a linear dependence was assumed between the content of a certain component of the essential oil, y , but this time on two parameters. The modeling was performed using the R programming language interpreter version 4.2.1 for the x86_64-pc-linux-gnu platform. The linear regression models were built using built-in R functions such as `lm` function and operations on data structures of the `data.frame` type. The Gauss–Markov hypothesis testing was conducted using the `gvlma` function from the “`gvlma`” package [69]. Correlation analysis was performed using the Excel2016 software package. For visualization purposes the data of the component composition of the essential oil were processed

using principal component analysis (PCA) (Sirius version 6.0 software package, Pattern Recognition Systems, a/s, Bergen, Norway).

4. Conclusions

Essential oils are an important part of a plant's protective system. The components of EOs serve as the first line of defense, primarily protecting plants against high temperatures. The component composition of EOs is most responsive to biotic interactions. Any stress triggers the activation of an antioxidant defense system in plants. The antioxidant properties of EOs are well known and undisputed [70].

The fluctuations in climatic parameters and their integral characteristics have no substantial influence on the ratio of components in the essential oil (chromatographic profile). Throughout its range, the composition of the essential oil remains similar, ensuring the best adaptation of plants to their growing conditions, and allowing *A. frigida* to exist as a dominant and co-dominant species in steppe vegetation. Noticeable changes in the component composition of *A. frigida*'s essential oil are associated with significant variations in the surrounding environment's climatic parameters due to the confinement of *A. frigida* habitats to high mountains (over 1300 m a.s.l.) and/or extrazonal habitats (insolated slopes), as well as anthropogenic impacts (nearby roads).

The EOs of *A. frigida* consist of a "core" of constant components, totaling 24 compounds, as well as first and second "concentric circles" (11 and 248 compounds, respectively), which include variable components. Both the components of the "core" and the "concentric circles" exhibit variability in their mass fraction in the essential oil composition, due to the aforementioned external factors. It can be assumed that the biosynthesis of the components in the "core" is genetically controlled, while the biosynthesis of the compounds in the "concentric circles" is determined by epigenetic mechanisms. Genetic factors obviously play a significant role in the formation of the essential oil composition. Within this framework, changes in its composition are observed during ontogenetic development (at least in the latent and active periods). Variations in the composition also occur during seasonal development, with four patterns of composition changes observed in different phenophases. Additionally, the meteorological conditions of a specific vegetative period can influence the EO composition.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/plants12193422/s1>, Table S1: Chemical composition of EOs obtained from the aerial parts of *A. frigida* from Russia and China (2016–2021). Table S2: Chemical composition of EOs obtained from the aerial parts of *A. frigida* from Russia and China (2016–2021) sorted by groups. Table S3: Chemical composition of EOs obtained from the aerial parts of *A. frigida* from Russia and China (2016–2021) sorted by groups in different phenological phases. Table S4: Values of climatic parameters in plant growing locations. Table S5: Results of Pearson's linear correlation coefficient calculations between values of climatic parameters and components of essential oils (Ivolginsky district, July–September, 2021). Table S6: Constant compounds of *A. frigida* essential oils. Table S7: Content of acyclic and cyclic sesquiterpenoids, according to biogenetic trees.

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Article

A Study of the Essential Oil Isolated from *Ageratina dendroides* (Spreng.) R.M. King & H. Rob.: Chemical Composition, Enantiomeric Distribution, and Antimicrobial, Antioxidant, and Anticholinesterase Activities

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Abstract: *Ageratina dendroides* is an aromatic species native to Ecuador. In this study, the chemical composition, enantiomeric distribution, and antifungal, antibacterial, antioxidant, and anticholinesterase activities of the essential oil isolated from aerial parts of *Ageratina dendroides* were determined. The quantitative chemical composition was determined using gas chromatography equipped with a flame ionization detector. The qualitative chemical composition was determined using gas chromatography coupled with mass spectrometry. The enantiomeric distribution was determined using an enantioselective chromatographic column. A broth microdilution method was used to determine the antibacterial activity. This antibacterial activity was tested against three Gram-negative bacilli bacteria and three Gram-positive cocci bacteria. The antifungal activity was tested against two fungi, a fungus, and a yeast. The antioxidant activity was determined using the ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) method and DPPH (2,2-diphenyl-1-picrylhydrazyl) method. The anticholinesterase activity was analyzed using the spectrophotometric method. Sixty-eight volatile compounds were identified in the essential oil. These compounds represent 99.49% of the total composition. In terms of the number of compounds and their relative abundance, the most representative group was sesquiterpene hydrocarbons, with thirty-four compounds and an abundance of 49.22%. The main constituents were found to be andro enecalinol ($14.41 \pm 1.26\%$), germacrene D ($8.86 \pm 1.06\%$), amorpho-4,9-dien-14-al ($7.68 \pm 0.70\%$), β -sesquiphellandrene ($7.01 \pm 1.49\%$), α -muurolol ($5.89 \pm 0.93\%$), and 7-*epi*- α -selinene ($5.68 \pm 0.53\%$). Five pairs of enantiomers were identified in the essential oil of *Ageratina dendroides*. The essential oil did not report antimicrobial activity at the maximum concentration tested (MIC > 4000 $\mu\text{g/mL}$) against any of the microorganisms tested. The antioxidant activity of the essential oil was strong in the ABTS method, with an SC_{50} of $33.2 \pm 1.4 \mu\text{g/mL}$. Additionally, the *Ageratina dendroides* essential oil reported moderate anticholinesterase activity, with an IC_{50} of $297.8 \pm 1.03 \mu\text{g/mL}$.

Keywords: *Eupatorium dendroides*; *Raulinoreitzia crenulata*; chiral compounds; andro enecalinol; germacrene D

1. Introduction

The Asteraceae family is one of the largest and most diverse families, including about 1778 genera and 33,994 species worldwide [1]. This family is characterized by its compound flowers, which are made up of multiple small flowers grouped together in a structure called a capitulum. The Asteraceae family is known for its wide therapeutic use and deep-rooted presence in traditional medicine. Some of its members have been cultivated for more than 3000 years, both for food and medicinal purposes [2]. Although they are more common

in the arid and semi-arid regions of subtropical areas, they are known and distributed throughout the world. Members of the Asteraceae family exhibit a wide range of beneficial activities, including anti-inflammatory, antimicrobial, antioxidant, and hepatoprotective properties [3]. In addition, in traditional medicine, this family of plants has been used as an anti-inflammatory, antipyretic, diaphoretic in fevers, hepatoprotective, astringent, muscle relaxant, nervous tonic, and laxative, and to treat bleeding, wounds, headaches, spasmodic diseases, flatulence, haemorrhoids, gangrenous ulcer, dysentery, dyspepsia, leucorrhoea, lumbago, and disorders causing cachexia [2]. Phytochemical studies carried out on the Asteraceae family have shown that many components of this family have remarkable biological activity. Some of these compounds include flavonoids, phenolic acids, coumarins, terpenoids (such as monoterpenes, sesquiterpenes, diterpenes, and triterpenes), and sterols. The diversity of the compounds in this plant family makes it an interesting source for phytochemical research and the search for new compounds with potential therapeutic applications [4]. Within the Asteraceae family, *Ageratina* is an American genus of the tribe Eupatorieae (subfamily Asteroideae), which comprises about 327 species [5].

In Ecuador, the Asteraceae family is widely represented by more than 200 genera and plays a crucial role in the plant diversity of the country. One of the most important genera of this family in Ecuador is *Ageratina*. This genus includes a variety of shrubby and herbaceous species that are distributed in different regions of the country [6]. An important species of *Ageratina* in Ecuador is *Ageratina dendroides* (Spreng.) R.M. King & H. Rob. (syn. *Eupatorium dendroides* Spreng. and *Raulinoreitzia crenulata* (Spreng.) R.M. King & H. Rob.). This endemic species, also known as “chilca” or “chilca de cerro”, grows in the mountainous areas of Ecuador between 1500 and 3500 m a.s.l., mainly in the provinces of Azuay, Cañar, Loja, and Zamora Chinchipe [7]. *Ageratina dendroides* is also a species native to the tropical regions of Central and South America, where it is found mainly in areas of humid jungle and tropical forests, both in lowlands and at higher altitudes [8].

Ageratina dendroides is a perennial shrub that reaches a height of approximately 1 to 2 m. It has a woody and branched stem, with a light brown bark. The leaves are simple, opposite, and lanceolate or oval in shape, with toothed or serrated edges. The surface of the leaves is smooth and shiny, and their color can vary between dark green and light green. The inflorescences of *Ageratina dendroides* appear in the form of terminal racemes composed of numerous small flowers. Each flower is surrounded by narrow bracts and possesses a tubular corolla composed of fused petals, which can be white, cream, or pale pink in color. The fruit is a small, dry achene containing a single seed [8]. The species *Ageratina dendroides* plays an important ecological role by providing food and shelter for a variety of pollinators, such as bees and butterflies. Their presence in ecosystems contributes to the pollination of other plant species and the conservation of biological diversity [9]. *Ageratina dendroides* has aroused the interest of the scientific community due to its possible medicinal properties. This plant species has been traditionally used in Ecuadorian folk medicine to cure blows and infections [10]. However, despite the promising characteristics of *Ageratina dendroides*, the scientific research on this species has been limited to date.

Studies carried out on alcoholic and hexanic extracts from species of the genus *Ageratina* have reported flavonoids, flavones, and flavanones as the main constituents that could have therapeutic applications in modern medicine [11,12]. Likewise, studies carried out with essential oils (Eos) obtained from the genus *Ageratina* have demonstrated the presence of several compounds, including carvacrol, spathulenol, terpinen-4-ol, thymol, nerodiol, cubebene, β -caryophyllene, β -myrcene, α -pinene, limonene, pentacosane, 8,9-epoxythymyl isobutyrate, germacrene D, thymyl isobutyrate, eupatoriocromene, and encecalol [13–15]. On the other hand, studies carried out with extracts and essential oils obtained from species of the genus *Ageratina* have revealed a variety of biological activities, including repellent, antiviral, antibacterial, and cytotoxic activity [16]. However, to date, there is no specific scientific information on the chemical composition, enantiomeric distribution, and biological activity of the essential oils of *Ageratina dendroides*. Therefore, in this scientific article, it is intended to shed light on the chemical nature of the volatile compounds that make

up the essential oil (EO) of *Ageratina dendroides* and the biological activity it possesses. This knowledge will allow for a determination of the scientific potential and possible applications of this essential oil in the field of medicine, as well as in the conservation of biodiversity through knowledge of this.

2. Results

2.1. Essential Oil Isolated

An approximate total of 15 kg (divided into three distillations) of fresh plant material (with a moisture of $63.5 \pm 1.2\%$ w/w) from aerial parts from *Ageratina dendroides* were hydrodistilled to isolate the EO. About 4.5 mL of EO was obtained from this vegetal material, which represents a yield of $0.03 \pm 0.01\%$ (v/w) or 0.3 ± 0.1 mL/kg.

2.2. Physical Properties of Essential Oil

The EO from *Ageratina dendroides* was presented as an unctuous liquid less dense than water. Table 1 shows the mean values and standard deviations (SD) of the physical properties of the EO.

Table 1. Physical properties of the essential oil of *Ageratina dendroides*.

	<i>Ageratina dendroides</i> EO	
	Mean	SD
Density, ρ (g/cm ³)	0.8816	0.0001
Refractive index, n^{20}	1.5001	0.0016
Specific rotation, $[\alpha]$ (°)	+44.6	3.6
Subjective color	light yellow	
RGB color values	R: 247, G: 240, B: 25	
CMYK color values	C: 0, M: 3, Y: 90, K: 3	
Hex Color Codes	#f7f019	

2.3. Chemical Composition of Essential Oil

The identification and quantification of the volatile compounds were carried out using gas chromatography coupled with mass spectrometry (GC-MS) and gas chromatography coupled with a flame ionization detector (GC-FID). Table 2 shows the relative abundance (%) with the standard deviation (SD), retention time (RT), chemical formula (CF), monoisotopic mass (MM), and retention indices calculated (RIC) and obtained from the literature (RIL) for each compound. Sixty-nine volatile compounds were identified in the EO of *Ageratina dendroides*. These compounds represent 99.49% of its total composition. The compounds were classified according to their chemical nature (number of carbons and presence of oxygen), and as a result, it was obtained that the compounds were found within four groups. These were a group of monoterpenes (10 carbons) that were non-oxygenated (MH), two groups of sesquiterpenes (15 carbons): oxygenated (OS) and non-oxygenated (SH), and one group of other compounds (OC, non-terpenic compounds). In terms of the number of compounds and their relative abundance, the most representative group was SH, with thirty-five compounds and an abundance of 49.22%. In fact, three of the six main compounds belonged to this group. The presence of oxygenated monoterpenes and diterpenes (oxygenated and non-oxygenated) was not determined. The main constituents (>5%) were found to be OC andro encecalinol (CN: 63, CF: C₁₄H₁₆O₂, MM: 216.11 Da) with an abundance of $14.41 \pm 1.26\%$, SH germacrene D (CN: 24, CF: C₁₅H₂₄, MM: 204.19 Da) with $8.86 \pm 1.06\%$, β -sesquiphellandrene (CN: 38) with $7.01 \pm 1.49\%$, 7-*epi*- α -selinene (CN: 37) with $5.68 \pm 0.53\%$, OS amorpho-4,9-dien-14-al (CN: 68, CF: C₁₅H₂₂O, MM: 218.17 Da) with $7.68 \pm 0.70\%$, and α -muurolol (CN: 59, CF: C₁₅H₂₆O, MM: 222.20 Da) with $5.89 \pm 0.93\%$.

Table 2. Chemical composition of essential oil from *Ageratina dendroides*.

CN	RT	Compound	RIC	RIR	%	SD	Type	CF	MM (Da)
1	11.29	<i>o</i> -Cymene	1024	1022	0.51	0.07	MH	C ₁₀ H ₁₄	134.11
2	24.36	Thymol, methyl ether	1235	1232	0.33	0.05	OC	C ₁₁ H ₁₆ O	164.12
3	27.69	<i>neoiso</i> -Verbanol acetate	1329	1328	0.18	0.01	OC	C ₁₂ H ₂₀ O ₂	196.15
4	30.25	δ -Elemene	1333	1335	2.16	0.09	SH	C ₁₅ H ₂₄	204.19
5	30.86	α -Cubebene	1344	1348	0.48	0.07	SH	C ₁₅ H ₂₄	204.19
6	31.99	Neryl acetate	1364	1359	0.06	0.00	OC	C ₁₂ H ₂₀ O ₂	196.15
7	32.36	Cyclosativene	1366	1369	0.20	0.01	SH	C ₁₅ H ₂₄	204.19
8	33.15	α -Copaene	1373	1374	0.23	0.03	SH	C ₁₅ H ₂₄	204.19
9	34.38	β -Cubebene	1385	1387	0.48	0.11	SH	C ₁₅ H ₂₄	204.19
10	34.54	β -Elemene	1388	1389	0.19	0.01	SH	C ₁₅ H ₂₄	204.19
11	34.77	α -Cedrene	1413	1410	0.15	0.01	SH	C ₁₅ H ₂₄	204.19
12	35.83	β -Funebrene	1416	1413	0.41	0.01	SH	C ₁₅ H ₂₄	204.19
13	35.96	(<i>E</i>)-Caryophyllene	1417	1417	4.45	0.70	SH	C ₁₅ H ₂₄	204.19
14	36.26	β -Cedrene	1422	1419	0.16	0.02	SH	C ₁₅ H ₂₄	204.19
15	36.38	β -Gurjunene	1428	1431	0.22	0.01	SH	C ₁₅ H ₂₄	204.19
16	37.05	6,9-Guaiadiene	1439	1442	0.35	0.08	SH	C ₁₅ H ₂₄	204.19
17	37.25	(<i>E</i>)-Cinnamyl acetate	1444	1443	0.43	0.06	OC	C ₁₁ H ₁₂ O ₂	176.08
18	37.43	Amorpha-4,11-diene	1447	1449	0.48	0.03	SH	C ₁₅ H ₂₄	204.19
19	37.47	α -Humulene	1454	1452	1.07	0.14	SH	C ₁₅ H ₂₄	204.19
20	37.59	α -Acoradiene	1458	1464	0.51	0.12	SH	C ₁₅ H ₂₄	204.19
21	37.74	Dauca-5,8-diene	1471	1471	0.64	0.08	SH	C ₁₅ H ₂₄	204.19
22	37.84	<i>trans</i> -Cadina-1(6),4-diene	1476	1475	2.42	0.62	SH	C ₁₅ H ₂₄	204.19
23	37.95	γ -Muurolene	1479	1478	0.82	0.12	SH	C ₁₅ H ₂₄	204.19
24	38.12	Germacrene D	1482	1480	8.86	1.09	SH	C ₁₅ H ₂₄	204.19
25	38.21	γ -Himachalene	1483	1481	1.83	0.13	SH	C ₁₅ H ₂₄	204.19
26	38.30	δ -Selinene	1486	1492	0.47	0.04	SH	C ₁₅ H ₂₄	204.19
27	38.45	<i>trans</i> -Muurolo-4(14),5-diene	1491	1493	0.78	0.08	SH	C ₁₅ H ₂₄	204.19
28	38.69	α -Zingiberene	1495	1493	0.51	0.08	SH	C ₁₅ H ₂₄	204.19
29	38.79	<i>epi</i> -Cubebol	1496	1493	2.39	0.76	OS	C ₁₅ H ₂₆ O	222.20
30	38.92	Bicyclogermacrene	1498	1500	0.70	0.13	SH	C ₁₅ H ₂₄	204.19
31	39.07	Epizonarene	1502	1501	0.44	0.02	SH	C ₁₅ H ₂₄	204.19
32	39.15	β -Bisabolene	1508	1505	2.87	0.80	SH	C ₁₅ H ₂₄	204.19
33	39.40	(<i>Z</i>)- γ -Bisabolene	1510	1506	0.33	0.08	SH	C ₁₅ H ₂₄	204.19
34	39.75	γ -Cadinene	1513	1513	0.32	0.04	SH	C ₁₅ H ₂₄	204.19
35	39.94	Cubebol	1517	1514	0.43	0.02	OS	C ₁₅ H ₂₆ O	222.20
36	40.08	7- <i>epi</i> - α -Selinene	1518	1520	5.68	0.53	SH	C ₁₅ H ₂₄	204.19
37	40.20	β -Sesquiphellandrene	1525	1521	7.01	1.49	SH	C ₁₅ H ₂₄	204.19
38	40.39	< γ ->Vetivenene	1533	1531	1.25	0.21	SH	C ₁₅ H ₂₂	202.17
39	40.83	<i>trans</i> -Cadina-1,4-diene	1541	1533	0.61	0.15	SH	C ₁₅ H ₂₄	204.19
40	41.00	β -Vetivenene	1548	1554	0.63	0.11	SH	C ₁₅ H ₂₂	202.17
41	41.16	Germacrene B	1559	1559	1.51	0.12	SH	C ₁₅ H ₂₄	204.19
42	41.90	<i>E</i> -Nerolidol	1563	1561	0.37	0.13	OS	C ₁₅ H ₂₆ O	222.20
43	42.06	Spathulenol	1577	1577	0.54	0.04	OS	C ₁₅ H ₂₄ O	220.18
44	42.78	Caryophyllene oxide	1585	1582	0.40	0.14	OS	C ₁₅ H ₂₄ O	220.18
45	42.89	Globulol	1589	1590	0.11	0.01	OS	C ₁₅ H ₂₆ O	222.20
46	43.00	Viridiflorol	1592	1592	1.09	0.11	OS	C ₁₅ H ₂₆ O	222.20
47	43.14	Carotol	1595	1594	0.03	0.00	OS	C ₁₅ H ₂₆ O	222.20
48	43.30	Guaiol	1599	1600	0.06	0.00	OS	C ₁₅ H ₂₆ O	222.20
49	43.44	Geranyl 2-methyl butanoate	1602	1601	0.30	0.01	OS	C ₁₅ H ₂₆ O ₂	238.19
50	43.54	1,10-di- <i>epi</i> -Cubenol	1614	1618	1.62	0.15	OS	C ₁₅ H ₂₆ O	222.20
51	43.63	10- <i>epi</i> - γ -Eudesmol	1619	1622	0.46	0.04	OS	C ₁₅ H ₂₆ O	222.20
52	43.75	1- <i>epi</i> -Cubenol	1627	1627	0.54	0.02	OS	C ₁₅ H ₂₆ O	222.20
53	43.94	γ -Eudesmol	1628	1630	0.89	0.10	OS	C ₁₅ H ₂₆ O	222.20
54	44.08	<i>cis</i> -Cadin-4-en-7-ol	1631	1632	2.00	0.45	OS	C ₁₅ H ₂₆ O	222.20
55	44.14	β -Acorenol	1634	1635	0.43	0.01	OS	C ₁₅ H ₂₆ O	222.20
56	44.26	<i>epi</i> - α -Cadinol	1637	1636	1.34	0.09	OS	C ₁₅ H ₂₆ O	222.20
57	44.35	β -Acorenol	1638	1638	0.14	0.01	OS	C ₁₅ H ₂₆ O	222.20
58	44.43	α -Muurolol (=Torreyol)	1643	1644	5.89	0.93	OS	C ₁₅ H ₂₆ O	222.20

Table 2. Cont.

CN	RT	Compound	RIC	RIR	%	SD	Type	CF	MM (Da)
59	44.63	Cubenol	1649	1645	0.63	0.04	OS	C ₁₅ H ₂₆ O	222.20
60	44.75	7- <i>epi</i> - α -Eudesmol	1663	1662	0.63	0.04	OS	C ₁₅ H ₂₆ O	222.20
61	44.85	Bulnesol	1671	1670	0.51	0.20	OS	C ₁₅ H ₂₆ O	222.20
62	44.98	Andro enecalinalol	1674	1675	14.41	1.26	OC	C ₁₄ H ₁₆ O ₂	216.11
63	45.11	Khusinol	1678	1679	0.53	0.09	OS	C ₁₅ H ₂₄ O	220.18
64	45.20	α -Bisabolol	1692	1685	3.34	0.34	OS	C ₁₅ H ₂₆ O	222.20
65	45.34	Cyperotundone	1696	1695	0.21	0.01	OS	C ₁₅ H ₂₂ O	218.17
66	45.64	Amorpha-4,9-dien-2-ol	1701	1700	1.20	0.16	OS	C ₁₅ H ₂₄ O	220.18
67	45.88	Amorpha-4,9-dien-14-al	1702	1704	7.68	0.70	OS	C ₁₅ H ₂₂ O	218.17
68	57.08	n-Tetracosane	2401	2400	0.59	0.15	OC	C ₂₄ H ₅₀	309.03
					0.51				
					49.22				
					33.76				
					16.00				
					99.49				

CN: compound number, assigned according to their elution order; RT retention time; RIC: calculated retention indices; RIR: literature retention indices; %: relative abundance; CF: chemical formula; MM: monoisotopic mass; SD: standard deviation; and Tr: traces.

2.4. Enantiomeric Analysis

Five pairs of enantiomers were separated in the *Ageratina dendroides* EO using a column with an enantioselective stationary phase. The retention time (RT), retention indices (RI), enantiomeric distribution (ED), and enantiomeric excess (e.e.) for each pair of compounds are shown in Table 3. The (+)- γ -cadinene and (−)- γ -cadinene were found to be practically like a racemic mixture (racemate) with an e.e. of 4.05%.

Table 3. Chiral compounds present in the essential oil of *Ageratina dendroides*.

RT	Enantiomers	RI	ED (%)	e.e. (%)
26.79 27.08	(+ / −)- α -Copaene	1375 1380	24.89 75.11	50.23
33.59 33.88	(+ / −)-Epizonarene	1489 1494	18.05 81.95	63.89
35.60 35.71	(+ / −)- γ -Cadinene	1524 1526	52.03 47.97	4.05
35.92 36.02	(+ / −)-Bicyclogermacrene	1629 1731	43.00 57.00	13.99
49.20 49.40	(+ / −)- α -Murolol	1769 1773	83.13 16.87	66.25

2.5. Antimicrobial Activity

The antibacterial and antifungal activities of the EO from *Ageratina dendroides* were evaluated using the microdilution broth method. A total of eight microorganisms were evaluated: three Gram-negative bacilli, three Gram-positive cocci, a fungus, and a yeast. The tested microorganisms and the values of the minimum inhibitory concentration (MIC) of the *Ageratina dendroides* EO, positive control, and negative control are shown in Table 4. It was not possible to determine the MIC value of the *Ageratina dendroides* EO against any of the microorganisms at the maximum concentration tested (400 μ g/mL). All the microorganisms showed normal growth in the negative control.

2.6. Antioxidant Activity

The ABTS and DPPH methods were used to determine the antioxidant activity of the *Ageratina dendroides* EO. The ABTS method was based on the scavenging capacity of

the EO against ion radical 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{•+}) and in the DPPH method, the EO's scavenging capacity was determined against radical 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]). The scavenging capacity (SC₅₀, µg/mL) of the EO and the positive control values with their respective standard deviations (SD) are shown in Table 5.

Table 4. Antimicrobial activity of essential oil from *Ageratina dendroides*.

Microorganism	<i>Ageratina dendroides</i>	Positive Control	Negative Control
		MIC (µg/mL)	
Gram-negative bacilli			
<i>Escherichia coli</i> O157:H7 (ATCC 43888)	>4000	1.56	+
<i>Pseudomonas aeruginosa</i> (ATCC 10145)	>4000	0.39	+
<i>Salmonella enterica</i> subs <i>enterica</i> serovar			
Thypimurium WDCM 00031, derived (ATCC 14028)	>4000	0.39	+
Gram-positive cocci			
<i>Enterococcus faecium</i> (ATCC 27270)	>4000	0.39	+
<i>Enterococcus faecalis</i> (ATCC 19433)	>4000	0.78	+
<i>Staphylococcus aureus</i> (ATCC 25923)	>4000	0.39	+
Fungi and yeasts			
<i>Aspergillus niger</i> (ATCC 6275)	>4000	0.10	+
<i>Candida albicans</i> (ATCC 10231)	>4000	0.10	+

+: normal growth.

Table 5. Antioxidant activity of essential oil from *Ageratina dendroides*.

Sample	ABTS	DPPH
	SC ₅₀ (µg/mL) ± SD	
<i>Ageratina dendroides</i> EO	33.2 ± 1.4	4586.2 ± 1.1
Trolox	29.1 ± 1.1	35.54 ± 1.1

2.7. Anticholinesterase Activity

The spectrophotometric method was used to determine the anticholinesterase (anti-AChE) activity. The IC₅₀ (half-maximal inhibitory concentration) value was measured from the corresponding rate of the reaction curve (Figure 1). The EO exhibited an IC₅₀ value of 297.8 ± 1.03 µg/mL and donepezil (positive control) reported an IC₅₀ value of 12.40 ± 1.35 µg/mL.

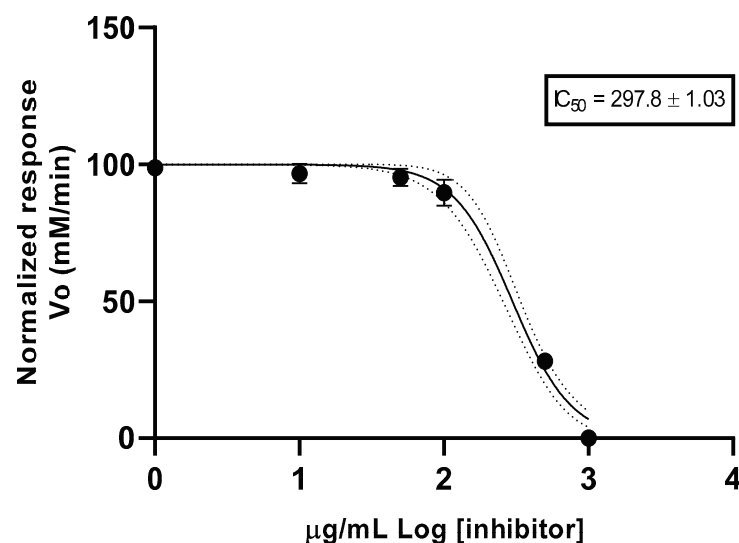


Figure 1. Anticholinesterase activity of essential oil from *Ageratina dendroides*.

3. Discussion

The yield of the *Ageratina dendroides* EO was 0.3 mL/kg, which can be considered as low according to the classification (values of <5 mL/kg are considered to be low, between 5 mL/kg and 10 mL/kg are considered to be intermediate, and values of >10 mL/kg are considered to be high) given by the categorization proposed by the Agency of Ciencia y Tecnología para el Desarrollo (CYTED) [17]. The EO yields from the leaves of *Ageratina jahnii* (B.L. Rob.) R. M. King & H. Rob. and *Ageratina pichinchensis* (Kunth) R. M. King & H. Rob. collected in Mérida, Venezuela, were 0.50% and 0.43% (*w/v*), respectively [14]. In the present study, sixty-nine volatile compounds were identified, representing more than 99% of the total composition of the essential oil from *Ageratina dendroides*. The compounds were classified into four groups according to their chemical nature. Non-oxygenated sesquiterpenes were the most representative group, with thirty-five compounds and an abundance of 49.22%. Previous studies carried out by Valarezo et al. in 2021 on the chemical composition of the EO of the leaves of *Ageratina dendroides* showed the presence of forty-five individual compounds, representing more than 99% of the total composition of the EO; in this study, nineteen compounds belonging to the SH group were identified, which represented 62.35% [15]. The main constituents in the EO of the leaves of *A. dendroides* were SH germacrene D ($29.92 \pm 0.68\%$), δ -cadinene ($9.31 \pm 0.11\%$), and *cis*-cadinene-1,4-diene ($5.48 \pm 0.04\%$) [15]. In the present study, these compounds were found, but with different percentages; these differences could be due to the different phenological state of the species at the time of collecting the plant material, since the chemical composition of an essential oil can be affected by extrinsic (shade, rain, humidity, soil, and others) and intrinsic (phenological state, age of the plant, and part of the plant used, among others) factors [18].

Within the genus *Ageratina*, the existence of EO has been reported in some of its species. In the EO from the leaves of *Ageratina jahnii*, fifteen components were identified and the major components were β -myrcene (37.6%), α -pinene (17.1%), limonene (8.8%), and pentacosane (9.2%). In the EO of *A. pichinchensis*, twenty-five components were identified, the main ones being 8,9-epoxythymyl isobutyrate (20.2%), germacrene D (19.8%), thymyl isobutyrate (10.8%), eupatoriocromene (6.5%), and encenolol (5.9%) [14]. Carvacrol was the main compound in the EO of Mexican *A. jocospecana* extracted from its leaves (30.08%) and flowers (32.5%) [13]. Seventy components were identified in the EO from *A. adenophora* and the main constituents were γ -muurolene (16.88%), o-cymene (11.17%), bornyl acetate (8.62%), and α -bisabolol (7.12%) [19]. In the EO from the leaves of *A. pentlandiana* grown in Cuzco (Peru), one hundred and fifteen compounds were identified and the main components were 2,5-dimethoxy-p-cymene (20.9%), thymol methyl ether (9.9%), and 2,5-dimethoxy-p-cymene (8.2%) [20].

This is the first report of an enantioselective GC-MS analysis of the EO from *Ageratina dendroides*, and this analysis showed the presented five pairs of enantiomeric compounds, including (+/−)- α -copaene, (+/−)-epizonarene, (+/−)- γ -cadinene, (+/−)-bicyclogermacrene, and (+/−)- α -muurolol. The ability to separate these enantiomers indicates the presence of chiral compounds in the essential oil. The enantiomeric ratio of an essential oil is important information, which could be related to the biological activity, metabolism, and organoleptic quality of the enantiomeric pairs [21]. The (+)- γ -cadinene and (−)- γ -cadinene were found almost as a racemic mixture or racemate (e.e. 4.05%), which could have important biological implications [2]. Indeed, racemic mixtures can influence the organoleptic properties and interactions with biological receptors, which may have consequences in terms of biological activity and therapeutic potential [3]. Despite the lack of complete separation in the case of (+)- γ -cadinene and (−)- γ -cadinene, the obtained results still provided valuable information on the chemical composition of the essential oil of *Ageratina dendroides*. The successful identification and separation of the other pairs of enantiomers demonstrated the complexity of the compounds present in the EO and their potential chemical diversity.

Regarding its antimicrobial activity, the EO from *Ageratina dendroides* was inactive against the microorganisms evaluated at the maximum concentration of 4000 $\mu\text{g/mL}$. To

the best of our knowledge, this is the first report on the antimicrobial activity of the EO from *A. dendroides*. In 2017, Van Vuuren and Holl established a criterium for the classification of the antimicrobial activity for extracts and essential oils, and in this sense, values of $>1001 \mu\text{g/mL}$ are considered to be inactive [22]. As a comparison, the antimicrobial results for *Ageratina* species were presented. Torres-Barajas et al., in 2013, evaluated the antibacterial activity of the Eos from *Ageratina jahnii* and *Ageratina pichinchensis* using the disc diffusion agar method and reported activity against *Staphylococcus aureus* and *Enterococcus faecalis*, with MIC values of 49.5 mg/mL for *A. jahnii* and 104 mg/mL for *A. pichinchensis* [14]. These values are higher than 4000 $\mu\text{g/mL}$, however, the methods were different, where microdilution was performed in a liquid state while the disc diffusion agar in a solid state, and this difference influenced the rate of diffusion for the EO in the microorganism medium. Solis-Quispe et al., in 2019, using the disc diffusion agar method, reported strong antibacterial activity of the EO from *Ageratina pentlandiana* against *S. aureus* (MIC 11.9 $\mu\text{L/mL}$), *Bacillus subtilis* (22.7 $\mu\text{L/mL}$), *E. coli* (64.8 $\mu\text{L/mL}$), and *Salmonella thipymurium* (50 $\mu\text{L/mL}$) [20]. However, the antibacterial activities were different due to the variation in the chemical composition.

The antioxidant activity of the EO from *A. dendroides* showed different values for the ABTS (SC₅₀ 33.2 $\mu\text{g/mL}$) and DPPH (SC₅₀ 4586.2 $\mu\text{g/mL}$) assays. The lower activity with the DPPH method may be explained by the low capacity of the non-oxygenated terpenes from donating a hydrogen atom [23]. According to Anthony et al. (2012), who studied different essential oils and their antioxidant activity, the authors proposed that an EO with SC₅₀ values less than 300 $\mu\text{g/mL}$ could be considered as exceptionally active [24]. Vasanthi and Gopalakrishnan, in 2013, reported the antioxidant activity for the EO from *Ageratina adenophora* (Spreng) as SC₅₀ for ABTS and DPPH assays with values of 71.25 $\mu\text{g/mL}$ and 60.2 $\mu\text{g/mL}$, respectively [25]. The determination of antioxidant properties allows for an evaluation of the effectivity of an EO in radical-mediated process; EOs are known to scavenge free radicals, and in vitro antioxidant assays represent the first attempt to propose their use for health benefits.

The scientific evidence about using EOs in the treatment of Alzheimer's disease has motivated the evaluation of the anti-AChE of volatile fractions of aromatic plants. Benny and Thomas, in 2018, summarized the significant effects of EOs on modulating pathologies through different mechanisms, including anticholinesterase activity [26]. This is the first report of the anti-AChE activity for the EO from *Ageratina dendroides*, with an IC₅₀ value of 297.8 $\mu\text{g/mL}$. This activity could be considered as being of moderate potency ($20 < \text{IC}_{50} < 200 \mu\text{g/mL}$), according to the scale proposed by Santos et al. in 2018 [27]. Other studies have reported the anti-AChE for the EO from *Ageratina adenophora* (Vasanthi and Gopalakrishnan, 2013), with an IC₅₀ of 92.25 $\mu\text{g/mL}$ [25]. The complexity of the chemical composition of essential oils is associated with these different values.

4. Materials and Methods

4.1. Materials

Acetylthiocholine (AcSch), acetylcholinesterase (AChE), butylated hydroxytoluene (BHT), dimethyl sulfoxide (DMSO), dichloromethane (DCM), donepezil, magnesium chloride hexahydrate, methanol (MeOH), phosphate-buffered saline (PBS), sodium sulfate anhydrous, tris hydrochloride (Tris-HCl), trolox, 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydryl (DPPH), and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich (San Luis, MO, USA). Fluid thioglycollate medium, Mueller-Hinton II broth, Mueller-Hinton broth, and Sabouraud dextrose broth were purchased from DIFCO (Quito, Ecuador). Helium was purchased from INDURA (Quito, Ecuador). The standard aliphatic hydrocarbons were purchased from ChemService (West Chester, PA, USA). All the chemicals were of analytical grade and used without further purification.

4.2. Plant Material

The aerial parts (leaves and flowers) of *Ageratina dendroides* were collected on the Villonaco hill in the canton and province of Loja. The collection was carried out in a place located at 3°59'38.3" south longitude and 79°15'49.4" west latitude at an altitude of 2700 m a.s.l. After being collected, the plant material was stored and transferred in airtight plastic containers. Botanist Vladimir Morocho made the identification of the plant material. A voucher specimen was deposited at the Herbarium of Universidad Técnica Particular de Loja (HUTPL).

4.3. Postharvest Treatments

Once the material arrived at the laboratory, one hour after being collected, the post-harvest treatment was carried out, which included the elimination of foreign or degraded plant material.

4.4. Moisture Determination

The moisture of the plant material was determined using the method of Loss on drying (Moisture) in plants (AOAC 930.04-1930), according to Equation (1). For this, an analytical balance (Mettler AC 100, Mettler Toledo, Columbus, OH, USA) was used.

$$\text{Moisture}(\%) = \frac{w_i - w_o}{w_i} * 100 \quad (1)$$

where w is the weight sample of “i” initial and “o” after drying.

4.5. Essential Oil Isolation

The extraction of the EO was carried out using hydrodistillation in Clevenger-type apparatus (80 L distiller). Initially, 16 L of water was placed in the distiller, then the plant material, and the extraction process began. The process was carried out for 3 h, counted from the fall of the first drop of distillate. The obtained steam (EO and water) was condensed and the essential oil was separated via decantation. Anhydrous sodium sulfate was used to dry the EO. Once dry, the essential oil was stored at 4 °C in amber sealed vials.

4.6. Determination of the Physical Properties of the Essential Oil

The density of the essential oil was determined using the ISO 279:1998 standard [28] (equivalent to the AFNOR NF T 75-111 standard), the refractive index (index of refraction) was determined using the ISO 280:1998 standard [29] (similar to AFNOR NF T 75-112), and the optical rotation was determined according to the ISO 592:1998 standard [30]. An analytical balance (Mettler AC 100, Mettler Toledo, Columbus, OH, USA) was used to determine the density, a refractometer (model ABBE, BOECO, Hamburg, Germany) to determine the refractive index, and an automatic polarimeter (Mrc-P810, MRC, Holon, Israel) to determine the optical rotation. The subjective color was obtained online, for which a photograph taken of the EO with a white background was uploaded to the PINETOOL website <https://pinetools.com/> (accessed on 20 February 2023). All the measurements were taken at 20 °C.

4.7. Essential Oil Compounds Identification

4.7.1. Quantitative Analysis

The quantitative analysis was performed using gas chromatography coupled with a flame ionization detector (GC-FID), for which a Thermo Scientific gas chromatography (Trace 1310, Waltham, MA, USA), a flame ionization detector (FID), a nonpolar GC column (DB-5ms, stationary phase 5%-phenyl-methylpolysiloxane, 30 m of length, 0.25 mm of diameter, and 0.25 µm of stationary layer thickness), and an automatic injector (AI 1310, Thermo Scientific, Waltham, MA, USA) were used. For the sample preparation, 1 µL of solution (1/100, v/v, EO/DCM) was injected, with a split ratio of 1:50. Helium was used as a carrier gas at 1 mL/min in constant flow mode with an average velocity of

25 cm/s. The injector and detector temperatures were 230 °C. The oven temperature program included an initial isotherm of 50 °C for 3 min, followed by a temperature ramp to 230 °C at 3 °C/min (60 min), and a final isotherm for 3 min (total run time 66 min). The relative amounts of the compounds were calculated based on the GC peak area (FID response), without using a correction factor.

4.7.2. Qualitative Analysis

The qualitative analysis was performed using gas chromatography coupled with mass spectrometry (GC-MS), for which the same equipment was used as that in the quantitative analysis, except for the detector, which was replaced by a mass spectrometer (MS) (quadrupole) detector (ISQ 7000, Thermo Scientific, Waltham, MA, USA). The sample concentrations and temperatures (ramp, injector, and detector) were the same as those in qualitative analyses. Helium was used as a carrier gas at 0.9 mL/min in constant flow mode with an average velocity of 34 cm/s. The operating conditions for the MS were as follows: electron multiplier 1600 eV, 70 eV, mass range 40–350 m/z , and scan rate 2 scan/s. Equation (2) [31] was used to determine the retention index (RI) of each compound. For the identification of the compounds, the IR and mass spectra were compared with published data [32].

$$RI = 100C + 100 \frac{(RT_x - RT_n)}{(RT_N - RT_n)} \quad (2)$$

where C is the carbon number of the aliphatic hydrocarbons (C_9 to C_{25}) that elute before the compound of interest. RT is the retention time of x compound of interest, n is the aliphatic hydrocarbons that elute before of the compound of interest, and N is the hydrocarbons that elute after of the compound of interest.

4.8. Antimicrobial Activity

4.8.1. Antibacterial Activity

The antibacterial activity of the *Ageratina dendroides* EO was tested against five strains of bacteria, two Gram-negative bacilli bacteria: *Escherichia coli* O157:H7 (ATCC 43888) and *Pseudomonas aeruginosa* (ATCC 10145), and three Gram-positive cocci bacteria: *Enterococcus faecium* (ATCC 27270), *Enterococcus faecalis* (ATCC 19433), and *Staphylococcus aureus* (ATCC 25923). The procedures were performed as previously described by Valarezo et al., 2021 [33]. Briefly, the antibacterial assay was developed into a 96 microwell plate according to the microdilution broth method. Two-fold serial dilution was used to obtain a concentration of the EO ranging from 4000 to 15.62 µg/mL. Ciprofloxacin was used as a positive control for *Escherichia coli* and *Pseudomonas aeruginosa*, and ampicillin for *Enterococcus faecium*, *Enterococcus faecalis*, and *Staphylococcus aureus*. The maximum evaluated concentration was 4000 µg/mL and DMSO at 5% was used as a negative control. The minimum inhibitory concentration (MIC), the lowest concentration of an antimicrobial that inhibits the growth of a microorganism after its incubation, was used to report the activity values.

4.8.2. Antifungal Activity

The antifungal activity of the *Ageratina dendroides* EO was tested against two strains of fungi, a fungus: *Aspergillus niger* (ATCC 6275), and a yeast: *Candida albicans* (ATCC 10231). The procedures were performed as previously described by Valarezo et al., 2021 [33]. Briefly, the MIC was determined using a final concentration of 5×10^4 spores/mL in 96 microwell plates. The EO was dissolved in Sabouraud dextrose broth with a fungal inoculum to achieve the required concentrations from 4000 to 15.62 µg/mL. Ciprofloxacin was used as a positive control and DMSO as a negative control.

4.9. Evaluation of Antioxidant Capacity

4.9.1. ABTS Radical Cation Scavenging Activity

The ABTS methods were used to determine the free radical scavenging activity of the *Ageratina dendroides* EO. For this, the reagent 2,2'-azino-bis (3-ethylbenzothiazoline-6-

sulfonic acid) was used to produce 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS^{•+}). The procedures were carried out according to those described by Valarezo et al., 2021 [34]. Briefly, the antiradical capacity of the EO was assessed against ABTS^{•+} by measuring the rate of reduction at 734 nm, using a UV spectrophotometer (Genesys 10S UV-Vis Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). Stabilized radicals in methanol, adjusted to an optical density of 1.1 ± 0.02 , were mixed with different concentrations of the EO at room temperature for one hour. The antiradical capacity was expressed as half scavenging capacity (SC₅₀), calculated from the corresponding curve fitting. Trolox was used as a positive and MeOH as a negative control. The maximum evaluated concentration was 2000 µg/mL.

4.9.2. DPPH Radical Scavenging Activity

The free radical scavenging activity of the *Ageratina dendroides* EO was also determined using the DPPH method. In the DPPH method, the reagent 2,2-diphenyl-1-picrylhydrazyl was used to produce 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[•]). The procedures were carried out according to those described by Valarezo et al., 2021 [34]. Briefly, the antiradical capacity of the EO was assessed against DPPH[•] by measuring the rate of reduction at 515 nm. The equipment, the positive and negative control, and the maximum concentration evaluated were the same as those for the ABTS radical cation scavenging activity.

4.10. Anticholinesterase Activity

An anticholinesterase assay was performed according to the principle described by Ellman et al. [35], according to the procedure described by Valarezo et al., 2021 [36], with slight modifications. The mix of the reaction, containing buffer Tris 50 mM pH 8.0, acetylthiocholine (15 mM), Ellman's reagent DTNB (3 mM), and the EO at different decreasing concentrations, was pre-incubated at 25 °C for three minutes. Later, acetylcholinesterase from electric eels (0.5 U/mL) was added to start the reaction and the progression was monitored at 412 nm in a microplate spectrophotometer (EPOCH 2, BioTek, Winooski, VT, USA). The half inhibitory concentration (IC₅₀) was extracted from the non-linear regression model (normalized response vs. log Inhibitor-variable slope). MeOH was used as a negative control and donepezil hydrochloride as a positive control.

4.11. Statistical Analysis

All the procedures were performed in triplicate, except for the identification of the essential oil compounds, enantioselective analysis, and antimicrobial activity, which were performed nine times. The data were collected in a Microsoft Excel sheet. The statistical software Minitab 17 (Version 17.1.0., Minitab LLC., State College, PA, USA) was used to calculate the measures of the central tendency and standard deviation. The data from the antiradical assays were analyzed using the GraphPad Prism, version 6.0 software (GraphPad Software Inc., San Diego, CA, USA). The data from the anticholinesterase assays were analyzed using the GraphPad Prism, version 6.0 software (GraphPad Software Inc., San Diego, CA, USA).

5. Conclusions

The enantiomeric distribution, antibacterial activity, antifungal activity, antioxidant capacity, and anticholinesterase activity of the essential oil from *Ageratina dendroides* were determined for the first time. Sixty-eight chemical compounds were identified, with androencecalinol being the main compound. The *Ageratina dendroides* essential oil exhibited exceptional antioxidant activity and moderate anticholinesterase activity. With this research, we contribute to the knowledge on the endemic aromatic plants of Ecuador. This study lays the foundation for future research on the biological properties, aroma profiles, and potential applications of the enantiomers identified in the essential oil of *Ageratina dendroides*. For future studies, it is proposed to investigate the anti-inflammatory activity of this essential oil.

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Article

Constituents and Selective BuChE Inhibitory Activity of the Essential Oil from *Hypericum aciculare* Kunth

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Abstract: A potential source of new inhibitors of cholinesterase enzymes are certain compounds of natural plant origin; therefore, in the study described herein we have determined the chemical composition and the acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory activities of the essential oil (EO) steam distilled from aerial parts of *Hypericum aciculare*, which was collected in southern Ecuador. The oil qualitative and quantitative composition was determined by GC-FID and GC-MS using a non-polar and a polar chromatographic column. A total of fifty-three constituents were identified, that accounted for about 98% of the EO content. The hydrocarbon *n*-nonane (16.4–28.7%) and the aldehyde *n*-decanal (20.7–23.1%) were the predominant oil constituents. In addition, the EO showed significant inhibition of BuChE ($IC_{50} = 28.3 \pm 2.7 \mu\text{g/mL}$) and moderate activity towards AChE ($IC_{50} = 82.1 \pm 12.1 \mu\text{g/mL}$). Thus, the EO from *H. aciculare* aerial parts is an interesting candidate to investigate the mechanism of selective ChE inhibition by the two ChE enzymes with the aim to discover potential targets to control the progression of the Alzheimer's disease (AD).

Keywords: *Hypericum aciculare*; essential oil; constituents; BuChE selective inhibitory activity

1. Introduction

The genus *Hypericum* (Hypericaceae) comprises about 500 species of herbs, shrubs, and trees, which are mainly distributed in temperate regions and on tropical mountains. They grow abundantly in Europe, Asia, Northern Africa, and America [1–3]. Several *Hypericum* taxa have a long traditional value as medicinal plants to treat inflammation, bacterial and viral infections, burns, stomach ulcers, mild depression, wounds and burns, diarrhea, pain, fevers, and poisoning by venomous animal bites [4,5]. Several biologically active secondary metabolites from *Hypericum* species exhibit a wide range of beneficial pharmacological effects, such as antimicrobial, anti-inflammatory, antioxidant, antifungal, astringent, antihyperglycemic, and hepatoprotective properties, as well as acetylcholinesterase and monoamine oxidase inhibitory activities [5,6]. Secondary metabolites isolated from *Hypericum* genus include naphthodianthrone (hypericin and pseudohypericin), ploroglucinols (hyperforin), flavonoids (quercetin, rutin, quercitrin, isoquercetin, hyperoside and amentoflavone), and essential oils [5], with antifungal [7], antibiotic [8], antiviral [9], and anticancer properties [10].

Hypericum essential oils (EOs), extracts, and isolated metabolites are reported to possess potent antidepressant, antibacterial, antifungal, antioxidant, anti-angiogenic, an-

tiviral, antimalarial, cytotoxic, neuroprotective, tyrosinase inhibition, immunomodulatory, hepatoprotective, anti-inflammatory and wound-healing effects [11–13]. Thanks to these properties, *Hypericum* plants and products are widely used in traditional medicines practiced worldwide.

Hypericum aciculare Kunth, commonly known in Perú by the indigenous names of “Hierba de las cordilleras”, “Lechuguilla”, “Hierba de iman” [14] and in Ecuador as “Bura” [15], is a 0.3–2 m tall shrub, erect, and bushy to decumbent and slender, with branches strict to ascending, lateral, and frequently pseudo-dichotomous [16]. An infusion of the plant is used by indigenous peoples who live in the Carchi province of Ecuador to treat wounds and furuncles of animals [17]. Moreover, to relieve rheumatic pains, the flowers of *H. aciculare* are crushed, mixed with animal fat, and placed on the affected part. The analgesic effect of this preparation is enhanced by adding other species of *Hypericum*, such as *H. decandrum*, that has the same therapeutic properties. *H. aciculare* is also used by different indigenous Andean communities as firewood for cooking food, to combat bad luck, and as a protector against evil spirits [15].

H. aciculare is native to the Andes. In southern Ecuador it is distributed in the provinces of Azuay and Loja, at altitudes of 1500–4000 m a.s.l, while in northeastern Ecuador, it mainly grows in more humid environments, on slopes where the bamboo also occurs [18]. *H. aciculare* has also been reported to grow on the Cordillera Oriental of Columbia as an indicator of biodegradable or polluted soils due to livestock overgrazing, surface erosion, agrochemical pollution, and frequent fires [16].

This research is part of our ongoing program on the study and valorization of essential oils from Ecuadorian aromatic plants with potential acetylcholinesterase inhibitory effects [19]. In continuation of our studies on the essential oils from species of the genus *Hypericum* [20], we examined the chemical composition and the activity of the EO steam distilled from *H. aciculare* as a potential cholinesterase inhibitor. There are two important cholinesterase enzymes in the human body: acetylcholinesterase (AChE), which is found in red blood cells as well as in the lungs, spleen, nerve endings, and the gray matter of the brain, and butyrylcholinesterase (BuChE), which occurs in the serum as well as in the liver, muscle, pancreas, heart, and white matter of the brain [21]. Acetylcholinesterase is involved in the transmission of nerve impulses by breaking down acetylcholine, a molecule that helps transmission of the signals across nerve endings. Recent evidence suggests that both enzyme (AChE and BuChE) may have roles in the etiology and progression of Alzheimer’s disease (AD) in addition to the regulation of synaptic ChE levels in healthy brain. In fact, decrease in brain acetylcholine (ACh) levels is implicated in the pathophysiology of cognitive dysfunction occurring in Alzheimer’s disease (AD). Therefore, the inhibition of the ACh catabolic enzymes, AChE and butyrylcholinesterase BuChE, can contribute to increase ACh brain levels. Moreover, it has been hypothesized that inhibition of AChE and BuChE may contribute to counter formation A β plaques and therefore to represent a disease-modifying strategy principle [22]. These features make acetylcholinesterase inhibitors (AChEi) and butyrylcholinesterase inhibitors (BuChEi) the main class of drugs currently used for the treatment of the AD dementia phase. Moreover, studies have established the therapeutic interest of the inhibition of both AChE and BuChE [22]. In fact, BuChE activity progressively increases in patients with AD, while AChE activity remains unchanged or declines in some brain areas where the BuChE:AChE ratio may shift from 0.6 to as high as 11.

The therapeutic potential of natural products against Alzheimer’s disease has been highly recognized [23,24]. However, the alkaloid galanthamine is the only naturally occurring substance that is in the market for the therapy of mild or moderate forms of Alzheimer’s disease, and other disorders of memory [22]. Recently, essential oils (EOs) isolated from medicinal and aromatic plants are receiving an increasing attention as potential neuroprotective remedies for age-related neurodegenerative diseases, due to their cholinesterase inhibitory activity against AChE and BuChE [25].

Herein, we describe the still unknown chemical composition and the inhibitory activity against AChE and BuChE of the EO obtained by steam distillation from *H. aciculare*.

2. Results

2.1. Physical Properties

Steam distillation of fresh leaves, flowers, and stems of *H. aciculare* produced a yellowish EO with a yield of 0.038 ± 0.03 % (*w/w*) on three replicates. The physical properties of the oil were: density = 0.88 ± 0.02 g/mL; refractive index = 1.46 ± 0.04 ; the specific rotation = $+0.54 \pm 0.01$ g/mL.

2.2. Chemical Composition

Fifty-three constituents were determined in the *H. aciculare* EO by GC-FID and GC-EIMS analysis, that represented 98.02% and 98.43% of the total oil analyzed on a DB-5ms and a HP-INNOWax column, respectively. The major constituents were alkanes and aldehydes, accounting for 20.97–31.62% and 21.68–23.82% of the total EO, respectively. The terpenoid fraction included sesquiterpene hydrocarbons (17.19, 21.22%), oxygenated sesquiterpenoids (3.35, 5.44%), oxygenated monoterpenoids (10.21, 10.65%), and monoterpene hydrocarbons (5.09, 7.98%).

The main identified compounds were *n*-nonane and *n*-decanal with a percent content of 28.71 and 16.36%, and 20.68 and 23.12%, respectively, in the EO analyzed on a DB-5ms capillary column and a HP-INNOWax capillary column, respectively.

The mean percent content of each identified component and the standard deviation (SD) were calculated, over three replicates, from the corresponding, electronically integrated, peak area relative to the total area of the peaks in each GC-FID chromatogram. No correction factor was applied. The chemical composition of the EO from *H. aciculare* is reported in Table 1, whereas the gas chromatograms on the two columns are shown in Figures 1 and 2.

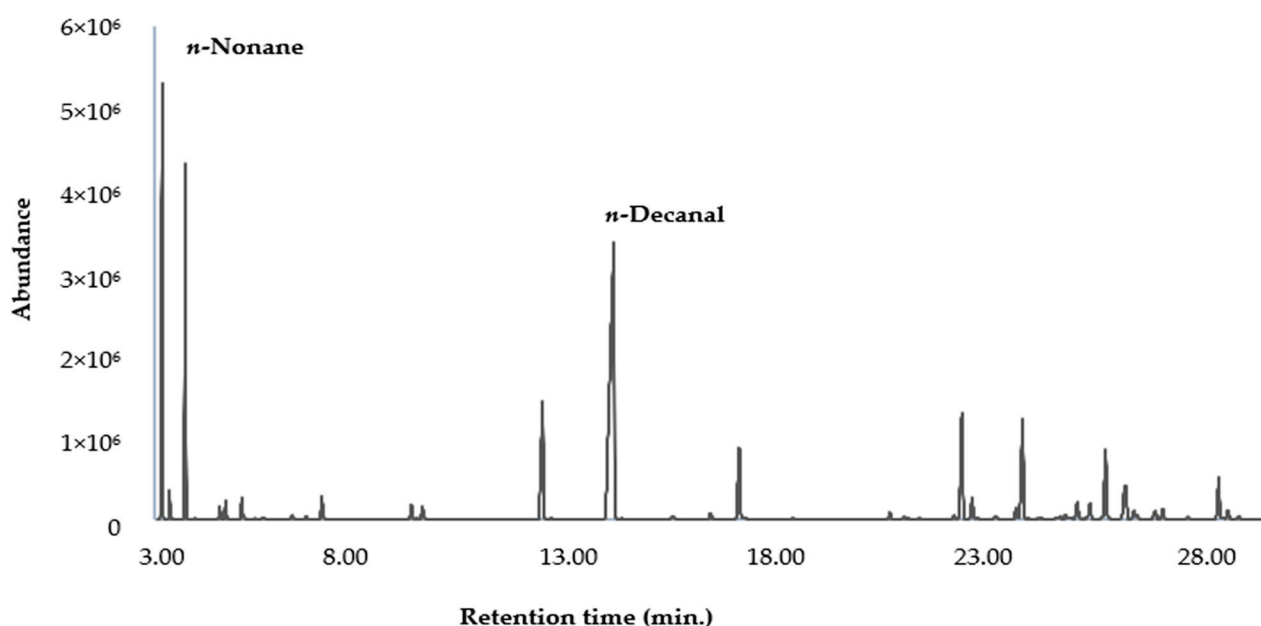


Figure 1. Gaschromatogram of the EO from *H. aciculare* aerial parts using a DB-5ms column.

Table 1. Chemical composition of *Hypericum aciculare* essential oil.

N°	Compound	LRI ^a	DB-5ms		LRI ^a	HP-INNOWax		% ± SD
			LRI ^b	% ± SD		LRI ^c	Ref.	
1	<i>n</i> -Octane	800	800	0.20 ± 0.01	-	-	-	-
2	2-Methyl-octane	855	858	1.68 ± 0.02	860	858	[26]	3.26 ± 0.18
3	<i>n</i> -Nonane	900	900	28.71 ± 0.61	904	900	[27]	16.36 ± 1.18
4	α -Pinene	930	932	2.05 ± 0.03	1046	1055	[25]	3.38 ± 0.38
5	Isoamyl propionate	968	960	0.29 ± 0.01	-	-	-	-
6	β -Pinene	975	974	0.17 ± 0.00	1109	1108	[25]	0.25 ± 0.02
7	Myrcene	988	988	0.69 ± 0.02	1161	1163	[25]	0.90 ± 0.07
8	<i>n</i> -Decane	1000	1000	0.02 ± 0.03	-	-	-	-
9	Limonene	1027	1024	0.10 ± 0.01	-	-	-	-
10	(<i>Z</i>)- β -Ocimene	1035	1032	0.15 ± 0.01	1236	1236	[25]	0.36 ± 0.02
11	(<i>E</i>)- β -Ocimene	1045	1044	1.93 ± 0.07	1254	1253	[25]	3.09 ± 0.26
12	<i>n</i> -Undecane	1100	1100	1.01 ± 0.00	1097	1100	[27]	1.35 ± 0.10
13	<i>n</i> -Nonanal	1105	1100	0.52 ± 0.03	1401	1395	[28]	0.36 ± 0.06
14	(<i>2E</i>)-Nonenol	1170	1163	4.02 ± 0.11	1457	-	-	3.85 ± 0.56
15	<i>n</i> -Decanal	1207	1201	20.68 ± 0.3	1504	1498	[28]	23.12 ± 2.73
16	Linalool	-	-	-	1566	1554	[25]	0.50 ± 0.02
17	Citronellol	1228	1223	0.53 ± 0.31	1783	1772	[25]	1.04 ± 0.06
18	1-Nonanol	-	-	-	1676	1664	[29]	0.48 ± 0.02
19	(<i>E</i>)-Ocimenone	1237	1235	3.30 ± 0.06	-	-	-	-
20	γ -Selinene	-	-	-	1698	1690	[29]	0.71 ± 0.02
21	Linalyl acetate	1251	1254	1.35 ± 0.06	-	-	-	-
22	(<i>2E</i>)-Decenal	1268	1260	0.13 ± 0.09	-	-	-	-
23	Geranial	1268	1264	4.53 ± 0.06	1740	1732	[25]	5.32 ± 0.15
24	1-Decanol	1274	1266	1.05 ± 0.04	1778	1766	[30]	2.80 ± 0.19
25	Pearlate	1278	1287	3.23 ± 0.09	-	-	-	-
26	2-Undecanone	1292	1293	0.07 ± 0.05	1603	1604	[30]	0.22 ± 0.02
27	(<i>2E</i>)-Undecenal	1364	1357	0.09 ± 0.07	-	-	-	-
28	Cyclosativene	1372	1369	0.06 ± 0.09	-	-	-	-
29	Linalyl isobutanoate	1378	1373	0.49 ± 0.02	-	-	-	-
30	β -Longipinene	1399	1400	0.07 ± 0.00	-	-	-	-
31	(<i>E</i>)- β -Caryophyllene	1414	1417	4.59 ± 0.06	1580	1593	[28]	5.76 ± 0.22
32	Dodecanal	1409	1408	0.26 ± 0.01	1713	1715	[28]	0.34 ± 0.09
33	α -Guaiene	1437	1437	0.10 ± 0.02	-	-	-	-
34	α -Humulene	1450	1452	4.92 ± 0.04	1653	1658	[25]	6.62 ± 0.27
35	9- <i>epi</i> -(<i>E</i>)-Caryophyllene	1469	1464	0.36 ± 0.36	-	-	-	-
36	γ -Himachalene	1479	1481	0.01 ± 0.07	-	-	-	-
37	β -Selinene	1483	1489	0.81 ± 0.09	1704	1708	[28]	0.99 ± 0.04
38	α -Selinene	-	-	-	1734	1744	[26]	1.75 ± 0.06
39	α -Zingiberene	1490	1493	3.79 ± 0.05	-	-	-	-
40	2-Tridecanone	1494	1495	0.23 ± 0.00	-	-	-	-
41	(<i>E,E</i>)- α -Farnesene	1503	1505	2.47 ± 0.02	1751	1751	[25]	4.47 ± 0.16
42	(<i>Z</i>)- γ -Bisabolene	1507	1514	0.01 ± 0.00	1760	1741	[25]	0.92 ± 0.02
43	Geranyl acetate	-	-	-	1766	1761	[25]	0.73 ± 0.01
44	Nerol	-	-	-	1816	1805	[25]	0.39 ± 0.02
45	Geraniol	-	-	-	1867	1854	[25]	2.67 ± 0.15
46	Caryophyllene oxide	-	-	-	1971	1967	[25]	0.40 ± 0.01
47	Methyleugenol	-	-	-	2034	2028	[27]	0.44 ± 0.03
48	(<i>E</i>)-Nerolidol	1560	1561	2.89 ± 0.03	2060	2050	[19]	5.04 ± 0.38
49	<i>trans</i> - β -Elemenone	1595	1602	0.10 ± 0.09	-	-	-	-
50	Khusimone	1604	1604	0.26 ± 0.18	-	-	-	-
51	γ -Eudesmol	-	-	-	2175	2185	[27]	0.14 ± 0.06
52	Bisaboladien-4-ol	1618	1618	0.10 ± 0.07	-	-	-	-
53	Decanoic acid	-	-	-	2280	2284	[25]	0.42 ± 0.07
Aliphatics								
Alkanes (%)				31.62				20.97
Alcohols (%)				5.07				7.13
Aldehydes (%)				21.68				23.82
Ketones (%)				0.30				0.22
Fatty acids (%)				--				0.42
Esters (%)				3.52				--
Terpenes								
Monoterpene hydrocarbons (%)				5.09				7.98
Oxygenated monoterpene hydrocarbons (%)				10.2				10.65
Sesquiterpene hydrocarbons (%)				17.19				21.22
Oxygenated sesquiterpene hydrocarbons (%)				3.35				5.44
Diterpenes (%)				-				0.14
Others (%)				-				0.44
TOTAL identified (%)				98.02				98.43

^a Calculated linear retention index; ^b linear retention index from reference [31]; ^c linear retention index from references [25–30]; %, mean percent content in the EO over three determinations; SD, mean standard deviation over three determinations.

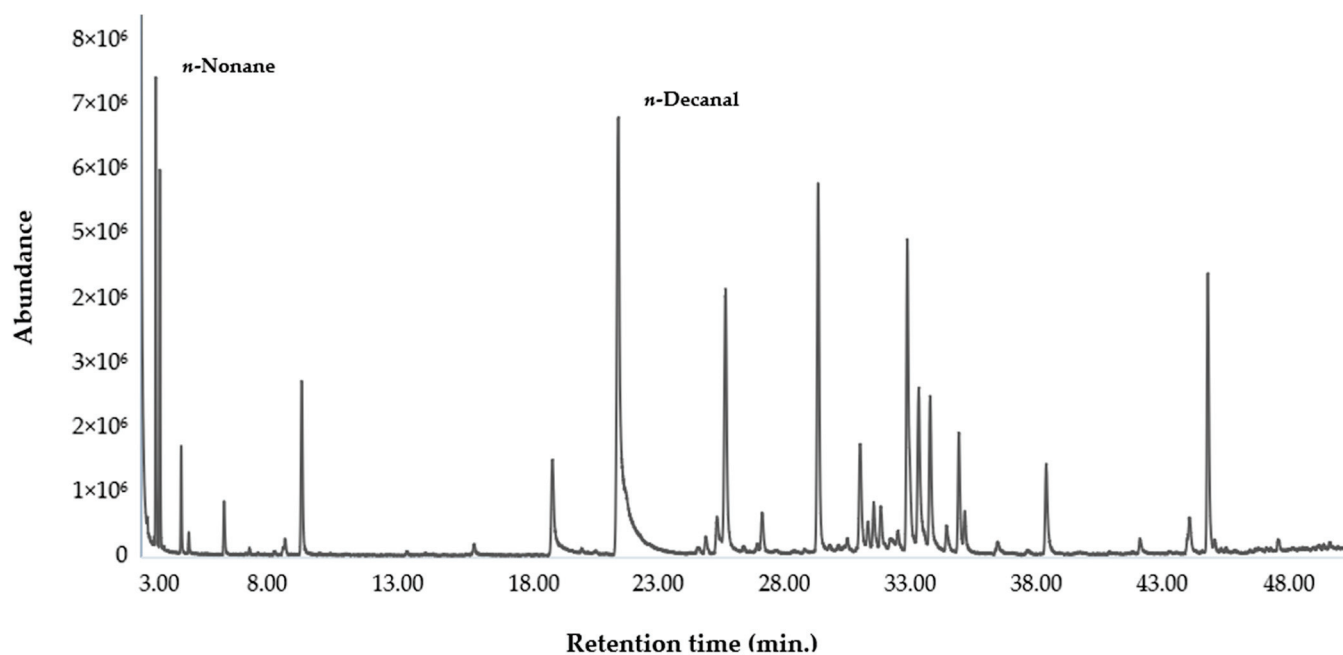


Figure 2. Gaschromatogram of the EO from *H. aciculare* aerial parts on a HP-INNOWax column.

2.3. Inhibition of Cholinesterases

The anti-cholinesterase activity of *H. aciculare* EO has been evaluated for the first time. The oil exhibited moderate inhibitory activity against AChE, with an IC_{50} value of $82.1 \pm 12.1 \mu\text{g/mL}$, and an interesting activity against BuChE, with an $IC_{50} = 28.3 \pm 2.7 \mu\text{g/mL}$ (Figure 3). The synthetic drug donepezil hydrochloride, which is employed to treat mild-moderate forms of Alzheimer's dementia, was used as the positive control in the test. It showed IC_{50} ($\mu\text{g/mL}$) values of 0.04 ± 0.02 and 3.60 ± 0.20 against AChE and BuChE, respectively [20].

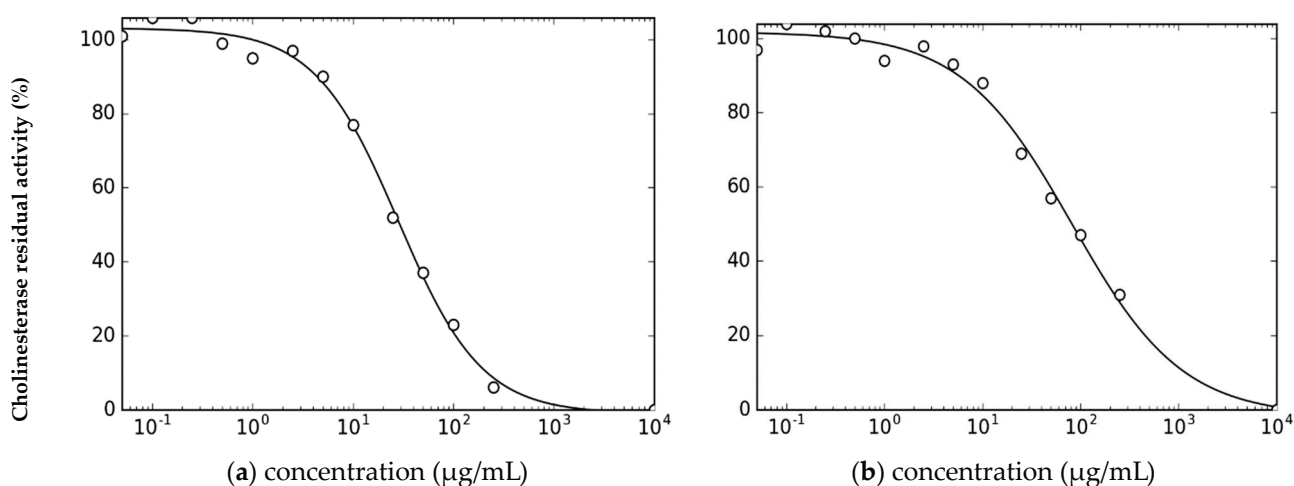


Figure 3. Curves of the cholinesterase residual activity (%) vs. the concentration ($\mu\text{g/mL}$) of the EO from *H. aciculare* aerial parts, used to calculate the IC_{50} values against (a) AChE and (b) BuChE.

To discard the possibility that the loss of enzymatic activity could be due to protein denaturation by the EO from *H. aciculare*, we determined the activity of the enzyme horseradish peroxidase (HRP) towards the substrate 2,2'-azino-di-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS), in the presence of the oil, which was compared to the enzymatic activity in the sole vehicle (DMSO). As clearly indicated by the curves reported in Figure 4,

the enzymatic activity was not reduced in the presence of the oil at the high concentration used, thus attesting that the oil do not cause protein denaturation.

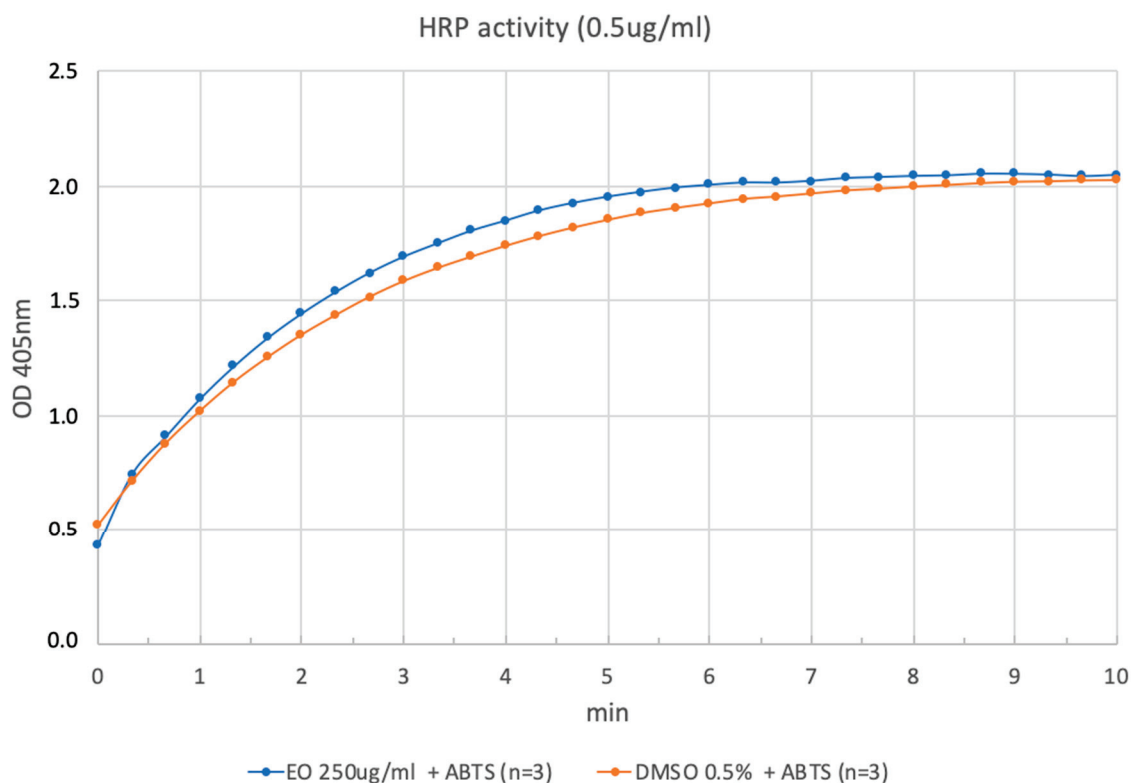


Figure 4. Curves of the horseradish peroxidase activity (HRP) vs. ABTS, in the presence of the EO from *H. aciculare* aerial parts, compared to the enzymatic activity in the sole vehicle (DMSO).

3. Discussion

The average yield of the EO steam distilled from *H. aciculare* aerial parts was 0.038% (*w/w*), confirming that *Hypericum* is generally an EO-poor genus, with yields <1% *w/w* [11,13] although in a few cases higher EO yields, up to 3%, have been reported in the literature [13]. The *H. aciculare* EO yield was even lower than those of other *Hypericum* EOs, such as *H. silenoides* (0.1–0.6%) and *H. philonotis* (0.2%), collected in central Mexico [32], or *H. laricifolium* (0.15%) collected in southern Ecuador [19]. The mean density and refractive index values were like those reported for the *H. laricifolium* EO, while the signs of the specific rotation of the two oils were opposite [19].

Yield, content, and chemical composition of *Hypericum* EOs show extensive inter- and intraspecific variation [12,13], which may be related to the influence of variables such as genetic factors, developmental stage and phenological cycle of seasonal changes, type of plant material and other conditions, such as geographical distribution [13]. An example of such variability is the significant qualitative and quantitative differences reported for the chemical profiles of the EOs from *H. perforatum* [11,13].

Regarding the chemical composition, *Hypericum* EOs can roughly be divided in three groups: (i) those in which terpenoids prevail; (ii) those in which non-terpenoid aliphatic derivatives are predominant; (iii) those containing comparable amounts of both types of compounds [11–13]. The typical most abundant terpenoids include the monoterpenes α - and β -pinenes, limonene, and β -ocimene, and the sesquiterpenes germacrene-D, cadinenes, selinenes, β -caryophyllene, and caryophyllene oxide, whereas linear C_9 – C_{12} , C_{16} , C_{25} , C_{29} , and C_{30} hydrocarbons are among the most frequently found aliphatic compounds [11–13]. The chemical composition of essential oils from the genus *Hypericum* has been used as chemotaxonomic marker to differentiate taxa of this genus. However, such a hypothesis

is no longer accepted, after that more data from a wide range of geographic distributions, taxonomic ranks, and seasonality have been examined [13].

Terpenoid constituents, particularly sesquiterpenes, were described as primary constituents in the oil of *Hypericum* collected from mountainous regions, while long-chain waxes and fats were reported as dominating in plants from lowland areas [11]. It was suggested that the increasing concentrations of monoterpenoids and sesquiterpenoids with increasing altitudes, was due to the role of these compounds in helping the plant deal with abiotic stress factors, e.g., UV radiation. Our findings, however, contradicted this hypothesis. In fact, the EO of *H. aciculare*, that was collected at an altitude of 3000 m, was significantly rich in non-terpenoid derivatives, among which *n*-nonane and *n*-decanal were predominant. However, their estimated percent content in the oil significantly depended on the type of the gas chromatographic column used for the analysis (Table 1). For example, the amount of *n*-nonane was determined as 28.71% and 16.36%, on a DB-5ms capillary column and a HP-INNOWax capillary column, respectively. Other *Hypericum* species producing *n*-nonane as the main EO component were *H. philonotis* Schltdl. & Cham (35.9%) and *H. silenoides* Juss. (31.9%) from Contepec-Mexico [32], *H. grandifolium* Choisy (42.3%) [33], *H. canariense* L. (44.3%) [33], *H. patulum* Thunb (17.1–32.6%) [34], *H. rochelii* Griseb. & Schenk (24.7%) [35], and *H. japonicum* Thunb. ex Murray (21.4%) [36]. Interestingly, EOs from *H. silenoides* [32] and *H. japonicum* [36] also contained significant amounts of *n*-decanal, 15.2 and 8.2%, respectively, thus resembling the content of *H. aciculare*.

Bioactivities of *Hypericum* EOs most frequently tested until recently are those traditionally investigated with essential oils, i.e., antimicrobial, insecticidal, antioxidant, antiviral, and cytotoxic properties [11–13]. In recent studies, other properties, such as neuroprotective, tyrosinase inhibitory, immunomodulatory, antiangiogenetic, hepatoprotective, and wound-healing effects, have been assayed. However, little is known about the cholinesterase inhibitory activity of *Hypericum* EOs, despite the increasing interest in essential oils as potential neuroprotective remedies for age-related neurodegenerative diseases [23]. In pioneering studies, the EO (0.5 mg/mL) of *H. undulatum* showed moderate inhibitory activity ($20.0 \pm 6.5\%$) against AChE, which was much lower than that of the plant extract whose inhibitory activity was $68.4 \pm 4.7\%$ at a concentration of 0.5 mg/mL [37]. At higher concentration (10 mg/mL), *H. neurocalycinum* and *H. malatyanum* inhibited 85.78 ± 4.11 and $62.24 \pm 1.81\%$, respectively, of the AChE activity [38].

In our studies of essential oils with anticholinesterase properties, we were the first to find selective inhibition of ChE enzymes by *Hypericum* EOs. In fact, the EO of *H. laricifolium* exhibited an IC_{50} of $36.80 \pm 2.4 \mu\text{g/mL}$ against BuChE which was three times lower than against AChE ($106.10 \pm 20.20 \mu\text{g/mL}$) [20]. The EO from *H. aciculare* displayed higher and more selective inhibition of ChE enzymes than the EO from *H. laricifolium*, with an $IC_{50} = 28.3 \pm 2.7 \mu\text{g/mL}$ against BuChE and an $IC_{50} = 82.1 \pm 12.1 \mu\text{g/mL}$ against AChE. This finding is highly interesting since BuChE has become an important new target in Alzheimer's disease and related dementias, due to its importance in the later stages of AD [39,40]. In both enzymes, 55% of the amino acid sequences that form them are identical; however, due to substitution by aliphatic amino acids (6 of the 14 amino acids present) at the BuChE active site, the BuChE active site has a larger volume than the AChE active site.

Consequently, the BuChE active site is larger in volume than AChE, resulting in similar, however, distinct catalytic properties, substrate specificity, kinetics, and activity in different brain regions [39]. Studies on BuChE inhibition are not as numerous as for AChE and the relationship between activity and structures of inhibitors is far from having been firmly determined, although it has been observed that the efficacy of an agent to inhibit BuChE is determined by its ability to access binding sites at the base of the large gorge of BuChE [39]. Regarding the essential oils, which are complex mixtures of compounds with different chemical characteristics, the properties of bio-inhibition can be explained due to the individual effects of some of the main components of the oil as mono- and sesquiterpenes [19]. However, monoterpenes such as α -pinene and limonene, which showed relatively good inhibitory activity against equine serum BuChE [41], are minor

components of the EO of *H. aciculare* (Table 1). Moreover, there is no information regarding specific anticholinesterase effects of the main oil constituents, *n*-nonane and *n*-decanal, that have structures and physicochemical characteristics significantly different from those of the compounds actively interacting with the binding sites of the ChE enzymes [42]. Instead, we assumed that these two compounds could have an indirect role in the modulation of the ChE activity, by facilitating the access of numerous minor components occurring in the EO (see Table 1) to the active site of the enzymes. For this reason, we didn't engage ourselves in *in silico* studies of the major components of the EO from *H. aciculare*. Moreover, *in vivo* studies have demonstrated that exposure of rats to high concentrations of *n*-nonane can cause several cerebellar damages and a significant loss of Purkinje cells [43].

Therefore, it appears more appropriate to attribute the anticholinesterase activity of the EO of *H. aciculare* to an undefined interaction of the enzymes with different oil constituents, which may include synergistic and antagonistic relationships. This hypothesis has already been proposed as a mechanism of action of other types of essential oils [44,45]. Moreover, dedicated studies are needed to evaluate the biological activity of single compounds isolated from an EO, so to determine their potential contribution to the biological effects of the entire oil. An explanatory example of such studies is the isolation of the sesquiterpene acorenone B from the EO of the Ecuadorian plant *Niphogeton dissecta* (Benth.) J.F. MacBr., that, with an IC_{50} of 10.9 $\mu\text{g/mL}$, mainly contributed to the BuChE inhibitory activity of the oil (IC_{50} of 11.5 $\mu\text{g/mL}$). [46]. As an alternative to *in vitro* studies, computational approaches may be used to assess potential biological targets for the EO components [42,47].

4. Materials and Methods

4.1. Plant Material

Aerial parts of *Hypericum aciculare* Kunth were collected on Loma Torre Mountain (Las Antenas sector), at the border between the Saraguro and San Lucas parishes, in the Loja province of Ecuador (Figure 5b), at an altitude of 3000 m above sea level (GPS coordinates: -3.681639 N, -79.228698 E). The plant (Figure 5a) was identified by Jose Miguel Andrade Morocho, botanist at the Universidad Técnica Particular de Loja (UTPL). A voucher has been deposited at the Herbarium of UTPL under the accession code HUTPL5576. The plant collection was authorized by a governmental permission (MAE-DBN-2016-048).

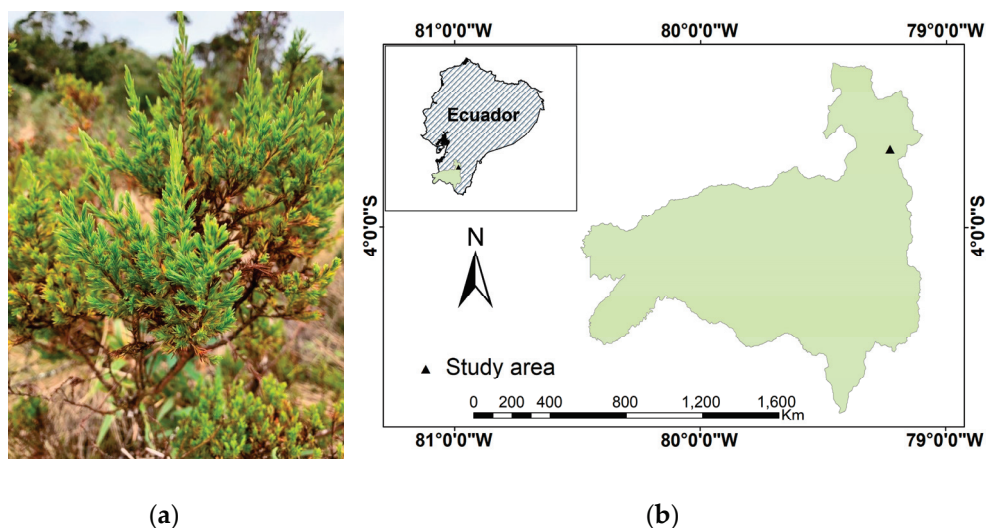


Figure 5. (a) Image of *H. aciculare* from Ecuador; (b) map of the collection site. Photographs obtained by one of the authors (C.A.).

4.2. Distillation of the Essential Oil

The fresh plant material (aerial parts) was steam distilled in a Clevenger apparatus for 3 h in three replicates of approximately 2000 g each, the three distillates were pooled, and

the pale yellow EO (approximately 0.75 g from each distillation) was dried with anhydrous Na_2SO_4 and stored in brown vials at -4°C until biological and chemical analyses [14].

4.3. Physical Properties

Three physical properties were determined for the EO: the relative density, according to standard AFNOR NF T75-111; the refractive index according to standard ANFOR NF 75-112, using an ABBE refractometer (Germany); the specific rotation $[\alpha]_D$, according to ISO 592-1998 using an automatic polarimeter Hanon P 810. For each physical property, the mean value and the SD were measured from tree replicates performed at 20°C .

4.4. Chemical Composition

According to standard procedures, the EO chromatographic analysis was performed by gas chromatography and gas chromatography-mass spectrometry (GC-MS) techniques, while a flame ionization detector (GC-FID) was used for quantification of the EO components. Each EO component was identified by comparing the mass spectrum and the calculated linear retention index (LRI) with the corresponding data reported in the literature. The LRIs were calculated according to the method of Van Den Dool and Kratz [48], using a mixture of *n*-alkanes (C9-C25) injected under the same chromatographic conditions as the EO samples.

4.4.1. GM-MS Analysis

A Thermo Scientific Gas Chromatographer (Trace 1310) with electronic pressure control was used for the gas chromatography (GC) analysis, this apparatus was coupled to a simple quadrupole mass spectrometry detector ISQ 7000 (Thermo Fisher Scientific, Waltham, MA, USA). Two columns were used: a DB-5ms column (5% phenylmethylpolysiloxane, $30\text{ m} \times 0.25\text{ mm i.d.}$, $0.25\text{ }\mu\text{m}$ film thickness) and a HP-INNOWax column (polyethylene glycol, $30\text{ m} \times 0.25\text{ mm i.d.}$, $0.25\text{ }\mu\text{m}$ film thickness). The column temperature was programmed from an initial temperature of 60°C for 5 min, followed by a gradient of $2^\circ\text{C}/\text{min}$ to 100°C , followed by a gradient of $3^\circ\text{C}/\text{min}$ to 150°C , followed by a gradient of $5^\circ\text{C}/\text{min}$ to 200°C . Finally, the temperature was increased at $15^\circ\text{C}/\text{min}$ to 250°C and held for 5 minutes. Ultrapure helium at a flow rate of $1\text{ mL}/\text{min}$ was used as a carrier gas and $1\text{ }\mu\text{L}$ of the EO solution in CH_2Cl_2 (1% *v/v*) was injected (split 40:1) [20,46,49].

4.4.2. GC-FID Analysis

For the GC-FID analysis, the gas-chromatograph and the chromatographic operating conditions were the same as those described in the previous section.

4.5. Cholinesterase Assays

The cholinesterase inhibitory activity of the *H. aciculare* EO was determined against acetylcholinesterase (AChE, from *Electrophorus electricus*, Sigma-Aldrich, C3389, St. Louis, MO, USA) and butyrylcholinesterase (BuChE, from equine serum, Sigma-Aldrich, SRE020, St. Louis, MO, USA) [49]. AChE and BuChE inhibitory effects of the oil were measured in vitro in accordance with the procedure initially designed by Ellman [50], which we subsequently adapted to essential oils, as described in [46,49,51]. Measurements were made in triplicate. Donepezil was used as the reference ChE inhibitor. False positive results, due high concentration ($>100\text{ }\mu\text{g}/\text{mL}$) of amines or aldehydes, were not excluded [20,46,49]. Used as a control, the horseradish peroxidase activity versus its substrate, 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), was determined using the increase in the absorbance at 405 nm (see Figure 4), in the equipment described for cholinesterase assays.

5. Conclusions

The physical properties, chemical composition, and anticholinesterase inhibitory properties of *Hypericum aciculare* EO were investigated and described for first time in this paper. The main component of the oil was the hydrocarbon *n*-nonane which contributed

about 30% to the oil composition. The EO showed dose-dependent inhibition of the ChE activities, with an interesting selectivity against BuChE. We believe that these findings cast the basis of future studies aimed at determining the oil component(s) responsible for the inhibitory effects and at defining the kinetic mechanisms exerted against the cholinesterase enzymes. Our ultimate goal is to explore the possibility to include the *H. aciculare* EO among the natural therapeutic remedies for the treatment of AD and other dementia diseases.

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Investigating the Biological Effects of Plant Essential Oils on Plant-Decaying Pathogens

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Abstract

Essential oils (EOs), complex volatile compounds synthesized by plants, represent a vital class of natural products that are increasingly significant in scientific research due to their diverse biological properties and broad-spectrum medicinal applications. This study provides a comprehensive overview of EOs, commencing with a historical perspective and detailing their applications. It systematically catalogs their primary botanical sources, with specific examples of the most common and important plant families, including *Lamiaceae* (e.g., sage, oregano, thyme), *Verbenaceae* (vervain), *Magnoliaceae* (magnolia), *Rutaceae* (lemon), *Myrtaceae* (eucalyptus) and *Lauraceae* (cinnamon). A key focus is their antifungal activity, including the bioactive constituents involved and their mechanisms of action, with particular emphasis on their defense against pathogenic postharvest fungi. This includes an analysis of the key bioactive constituents responsible for these bioeffects and an exploration of their possible mechanisms of action against phytopathogenic fungi, with particular emphasis on postharvest pathogens infecting several crops. The discussion further highlights the role of EOs as sustainable alternatives to synthetic fungicides for controlling plant diseases that avoid the negative ecological and public health impacts associated with conventional agrochemicals. The study addresses these objectives by describing methods for testing antimicrobial efficacy, including kill-time studies, LD₅₀ determination, growth-curve analysis, the poisoned food technique, Spore-germination assays, and metabolic CO₂ measurement. The current review also highlights some recent studies reviewing the in vitro and in vivo antifungal performance of specific EOs against postharvest diseases.

Keywords: essential oils; biological activity; biochemical characterization; plant diseases; chemical pesticides; pathogenicity; synergistic effect

1. Introduction

Essential oils (EOs) are concentrated, water-repelling liquids made up of volatile, aromatic molecules produced by plants. They are sometimes called volatile or ethereal oils. The term ‘essential’ refers to the oil’s characteristic plant aroma, not to any medical or culinary significance.

There is no doubt that EOs have important pharmacological properties, which result in their widespread use in pharmaceutical practices. Buchholtz [1] first noted the antimicrobial potential of EOs, showing that thymol inhibited bacterial growth in tobacco extract more effectively than phenol, the standard surgical antiseptic at the time [2]. Since then, extensive

studies have been undertaken to evaluate the antimicrobial potential of numerous EOs already recognized within the pharmaceutical field.

EOs' fungitoxicity is often due to interactions among their constituents, which act more effectively together than alone [3]. Their effectiveness in vapor form has also been highlighted, making EOs promising candidates for protection of stored food [3–6].

Unlike broader reviews, this review provides a focused analysis of plant EOs' effects on postharvest and plant-decaying phytopathogenic fungi. This work emphasizes economically important pathogens responsible for crop losses during both pre- and postharvest stages, with particular attention paid to fungi infecting fruits and vegetables. This review links bioactive EOs and their plant sources with current insights into antifungal mechanisms, including membrane disruption, mitochondrial impairment, oxidative stress, spore inhibition, and synergistic effects. Furthermore, the review highlights recent advances in EO-formulations and delivery strategies, such as nanoemulsions and encapsulation systems, and discusses their practical relevance in *in vivo*, semi-field, and open-field applications. By combining pathogen-oriented analysis, mechanistic understanding, and applied perspectives, this review aims to bridge the gap between laboratory research and sustainable disease management in agriculture.

The present review is organized into a series of thematically structured sections to provide a comprehensive overview of plant EOs and their biological activity. It opens with a general introduction to plant EOs, with particular emphasis on their diverse biological properties. This is followed by an analysis of conventional chemical strategies for controlling plant pathogenic fungi, including commonly used pesticides and their associated ecological and health-related drawbacks. The subsequent section focuses on plant EOs, detailing the principal botanical families that produce them, including *Lamiaceae*, *Verbenaceae*, *Magnoliaceae*, *Rutaceae*, *Myrtaceae*, and *Lauraceae*. A dedicated section addresses the antimicrobial activity of EOs, with a discussion of their antifungal mechanisms and potential synergistic interactions, including some relevant studies illustrating the practical applications of these oils.

2. Conventional Chemical Control of Plant Pathogenic Fungi

Fungi and fungus-like organisms cause more plant diseases than any other pathogen group, with over 8000 infectious species identified. Their roles in plant and human illness, their importance in biotechnology and pharmaceuticals, and their function as decomposers have gained extensive global research attention. Several historical famines and periods of severe human hardship have been linked to destructive fungal pathogens of major crops [7].

Traditional chemical control strategies typically depend on compounds that are toxic to target pathogens and operate through specific modes of action, such as commercial fungicides and antibiotics. In an ideal scenario, these chemicals should provide effective control while remaining safe for non-target species and causing minimal disruption to the environment, beneficial microorganisms, and soil ecosystems.

Synthetic fungicides have long been the primary tool for suppressing fungal diseases. Growing awareness of the health and environmental risks of pesticides, along with the loss of key active ingredients, has spurred the search for safer natural alternatives for postharvest disease control [8]. To reduce the likelihood of resistance developing from repeated use of the same chemical class, it has been recommended to rotate fungicides with different modes of action. From the 1980s onward, concerns escalated regarding the hazards posed by agricultural pesticides due to their potential toxicity to humans, beneficial soil organisms, and wildlife, as well as contamination of food crops. These risks arise from dermal absorption, inhalation, or ingestion of pesticide residues. Table 1 outlines the major

types of chemical pesticides, as well as their principal plant disease targets, mechanisms of action, and potential adverse effects on human health and the environment.

Table 1. Common chemical pesticides for common plant diseases and their negative impacts.

Chemical Pesticide ^(a)	Common Target Plant Diseases/Pathogens	Mechanism of Antimicrobial Effect (Mode of Action)	Collateral Negative Impact on Human Health	Collateral Negative Impact on the Environment	References
Chlorothalonil (e.g., Bravo, Daconil, and Echo)	Effective against various fungal plant diseases, including blights, mildews, rusts, and leaf spots.	Multi-site inhibitor interfering with fungal enzymes.	May be associated with respiratory irritation, skin sensitization, endocrine disruption, and potential carcinogenic risks.	Highly toxic to aquatic animals and persistent in soil and water.	[9,10]
Mancozeb (e.g., Penncozeb, Trimanoc, and Vondozeb)	Controls blight, rust, leaf spot, and scabs across a wide range of fruits, vegetables, nuts, and field crops.	Multi-site inhibitor that disrupts multiple enzymes.	Causes several health problems, mainly hepatic, renal and genotoxic.	Moderately persistent, toxic to aquatic organisms, and capable of affecting soil microflora.	[11–14]
Azoxystrobin (e.g., Amistar, Heritage, and Ortiva)	Rusts, mildews, blights, and fruit rots.	Inhibits mitochondrial respiration.	Exhibits low to moderate toxicity and can induce allergic dermatological reactions.	Toxic to non-target organisms, and persistent in soil, affecting microbes and causing oxidative stress in aquatic life.	[15,16]
Propiconazole (e.g., Tilt, Banner Maxx II, and Patch Pro)	Rusts, powdery mildew, leaf spot, and anthracnose.	Inhibits ergosterol synthesis (DMI fungicide).	May disrupt hormones, cause skin irritation, and affect reproductive health.	Persistent in soil and toxic to aquatic invertebrates and fish.	[17,18]
Copper-based fungicides (e.g., copper hydroxide, copper sulfate, copper oxychloride, copper oxide, and copper octanoate)	Wide range of plant diseases: downy mildew, powdery mildew, blight, leaf spot and bacterial diseases such as fire blight.	Cause cell membrane disruption, produce toxic reactive oxygen species (ROS), and interfere with vital enzymes and nucleic acids.	Causes skin and eye irritation, makes it dangerous to swallow, and might affect the liver or kidneys.	Persists in soil, accumulates in waterways, and toxic to beneficial soil microbes.	[19–21]
Sulfur fungicides (e.g., elemental and lime sulfur)	Powdery mildew on fruit trees (fruits), and other broad-spectrum effect against rusts, molds and scabs.	Disrupts fungal respiration.	Hinders lung function and causes respiratory symptoms in children and may also cause skin and eye irritation.	Excessive application acidifies soil, harms beneficial arthropods, and pollutes air.	[22,23]
Captan (e.g., Orthocide, Merpan, and Captan 50 W)	Scabs, blights, brown rot, powdery mildew, and gray mold on fruits, vegetables, and ornamentals.	Multi-site disruptor affecting enzymes and cell membranes.	Leads to skin and eye irritation and is suspected to cause carcinogenicity.	Toxic to aquatic organisms, and breaks down into more persistent metabolites.	[24–26]
Carbendazim/Benomyl (e.g., Samartha, Agrocit, and Benex)	Controls powdery mildew, leaf spot, anthracnose, scabs, and Fusarium wilt.	Inhibits fungal cell division.	Has potential teratogenic effects and causes reproductive toxicity.	Persistent, and toxic to earthworms and aquatic life, with rapid resistance development.	[27,28]

^(a) The names between brackets correspond to the trademarks of active ingredient.

Mitigating the dangers associated with pesticide use requires careful handling and improved agricultural practices. Environmental concerns have focused on sulfur- and copper-based fungicides, which can harm many organisms when washed into soil or aquatic ecosystems. Many countries have launched farmer training programs aimed at reducing excessive pesticide application and enhancing the production of key crops.

In recent years, there has been growing interest in plant-derived extracts as natural alternatives to synthetic pesticides [3,8]. This trend is largely driven by concerns over environmental contamination and the increasing appearance of pesticide-resistant pests, insects, and fungal pathogens.

Overall, plant EO-based strategies differ fundamentally from chemical fungicides. EOs contain diverse bioactive compounds that target multiple sites in pathogens, such as membranes, enzymes, and signaling pathways. In contrast, chemical fungicides usually act on a single, specific target. This multi-target action of EOs lowers the risk of resistance,

whereas pathogens can more easily develop resistance to single-target fungicides. From a sustainability perspective, EOs come from renewable plant sources and are generally biodegradable and pose low environmental and health risks. Although their effectiveness can vary with formulations and conditions, EO-based approaches conceptually support integrated pest management and sustainable agriculture better than conventional chemicals.

3. Plant Essential Oils

EOs are aromatic substances synthesized by many plant species. They are believed to contribute to the plant's natural defense system by providing protection against pathogenic microbes [29–33]. Over time, these oils have been examined in pharmacological research and subsequently evaluated extensively for their antimicrobial properties.

A variety of laboratory techniques have been employed to assess the antimicrobial activity of EOs. Commonly used approaches include agar diffusion assays, broth or agar dilution methods, and tests that measure activity in the vapor phase [34].

Additional antimicrobial evaluation methods include the following:

1. Phenol coefficient (kill-time) assays:

These tests compare the effectiveness of a compound to that of phenol after a 15 min exposure period.

2. Determination of lethal exposure time:

This method measures how long a test substance must be in contact with contaminated silk threads to completely eliminate the target organism.

3. LD₅₀ measurement and growth-curve analysis:

Growth curves are generated to identify the concentration of a compound that suppresses growth in 50% of the microbial population.

4. Poisoned food method:

Here, the delay or reduction in microbial growth is assessed when the organism is cultured on media containing inhibitory substances.

5. Spore-germination tests:

Commonly used for fungi, this test evaluates inhibition during early germination, particularly in short-exposure scenarios.

6. Metabolic CO₂ output monitoring:

This technique detects microbial activity—such as yeast growth—by tracking CO₂ production or by using color-changing indicators like sulfur salts in supplemented milk or tetrazolium dyes (e.g., TTC or INT).

4. Common Bioactive EOs Used as Natural Defenders Against Plant Diseases

4.1. Family Lamiaceae

4.1.1. Sage

Sage is a prominent genus within the *Lamiaceae* family, which includes around 900 species distributed across temperate, subtropical, and tropical regions worldwide, with notable concentrations in the Mediterranean, Central Asia, Central and South America, and southern Africa. Among these, *Salvia officinalis* L., *S. fruticosa* Mill., and *S. divinorum* Epling & Játiva, are the most recognized species, widely used in both traditional and modern medicine.

Sage EO exhibits significant antimicrobial activity against several key plant pathogenic fungi responsible for postharvest and field decay. Studies have demonstrated its potent inhibitory effects on mycelial growth and spore germination of fungi such as *Botrytis cinerea* (gray mold), *Aspergillus niger* (black mold), and *Fusarium oxysporum* (vascular wilt). Its antifungal properties are primarily attributed to its high content of bioactive monoterpenes, including 1,8-cineole, camphor, and α -thujone, which disrupt microbial cell membranes and metabolic processes. These findings highlight the potential of sage oil as a natural biocontrol agent for managing plant diseases and reducing reliance on synthetic fungicides [35,36].

4.1.2. Oregano

Oregano is often referred to as wild marjoram, and it is closely related to *Origanum majorana* L., commonly known as sweet marjoram. Over centuries, numerous varieties and strains have been selected for their distinctive aromas and other desirable traits. The leaves are widely used as a culinary ingredient and are often more aromatic when dried than when fresh. Oregano is known for its warm, slightly bitter flavor, though its intensity can differ across cultivars.

Pavarino et al. [37] documented the antifungal and antibacterial effects of oregano EO on a broad range of plant pathogens. These include *Penicillium purpurogenum*, *Staphylococcus aureus* and *Salmonella enterica*. Its primary fungicidal action is attributed to the high concentration of phenolic compounds, especially carvacrol and thymol, which compromise the integrity and function of microbial cell membranes [38].

4.1.3. Thyme

Thyme is commonly represented by *Thymus vulgaris* L. It belongs to the genus *Thymus* within the *Lamiaceae* family and shares close botanical ties with oregano (*Origanum* spp.).

T. capitatus (L.) Cav. EO exhibited antifungal properties in stored food products, inhibiting the growth of *Botrytis cinerea* and *Monilinia fructicola* [39]. *T. vulgaris* EO has also demonstrated activity against several postharvest fungal pathogens, including *B. cinerea*, *Penicillium italicum*, *P. citrophthora*, and *Rhizopus stolonifer* [30]. The dominant bioactive components, thymol and carvacrol, are responsible for severe membrane damage, leading to cellular leakage and death in fungal cells. Thyme EO is considered a suitable candidate for controlling both field and postharvest diseases [40].

4.1.4. Marjoram

In a study on *Marjorana hortensis* L. (*Lamiaceae*), known commonly as marjoram, Camele et al. [30] reported that *Marjorana hortensis* EO exhibited antifungal activity against *P. citrophthora* and *R. stolonifera*. *M. hortensis* EO also suppressed *Colletotrichum acutatum* and *B. cinerea*. Its efficacy is largely attributed to its complex chemical profile, where oxygenated monoterpenes like α -pinene, limonene, linalool and terpinene-4-ol act synergistically to disrupt fungal membranes and cellular processes [41]. These findings support the potential of marjoram EO as plant-based alternative for disease management [42].

4.2. Family Verbenaceae

Vervain

Verbena officinalis L., or vervain, has a long history in folk medicine and has recently attracted attention for its potential anticancer properties. Despite its broad use, the underlying mechanisms responsible for its pharmacological activity remain poorly understood.

Elshafie et al. [32] recently demonstrated the antimicrobial effectiveness of thyme and vervain EOs, showing that it significantly reduced brown rot lesions in peaches caused by *Monilinia laxa*, *M. fructicola*, and *M. fructigena* under in vivo conditions.

Other research, including Camele et al. [30], has indicated the inhibitory action of vervain EO against significant agents of crop decay, such as *Fusarium graminearum*, *Aspergillus ochraceus*, and *Alternaria alternata*. Its antifungal mechanism is linked to its major volatile constituents, including citral, geraniol, and limonene, which interfere with fungal cellular integrity and enzymatic activity [43].

4.3. Family Magnoliaceae

Magnolia

The genus *Magnolia* comprises roughly 210 species of flowering plants within the Magnoliaceae family. These ancient trees have a long history of traditional use, with both their flowers and their EOs valued across many cultures.

EO extracted from *M. liliflora* Desr. has demonstrated strong antifungal activity in vitro and in vivo against a range of plant pathogens, including *B. cinerea*, *Colletotrichum capsici*, *Fusarium oxysporum*, *F. solani*, *Phytophthora capsici*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* [44]. Its effectiveness against *P. capsici* in vivo has been attributed to several key constituents, such as β -caryophyllene, α -terpineol, α -humulene, farnesene, β -pinene, and caryophyllene oxide [44].

4.4. Family Rutaceae

Lemon (*Citrus limon*)

The *Rutaceae* family, or citrus family, is a major source of EO due to oil glands in its fruit peels, leaves, and flowers.

Citrus limon (L.) Osbeck (lemon) EO, mainly obtained by cold-pressing the peel, is rich in monoterpenes, especially limonene (up to 70%), with β -pinene, γ -terpinene, and citral [45]. The oil exhibits antioxidant, antimicrobial, and anti-inflammatory activity, making it valuable in pharmaceuticals, cosmetics, and food [46]. Its composition varies with cultivar, origin, and extraction method, emphasizing the need for standardization [45]. Limonene contributes to aroma, and also has cytoprotective and chemopreventive effects, supporting its use in aromatherapy and natural preservatives [47].

4.5. Family Myrtaceae

Eucalyptus

Eucalyptus (*Eucalyptus* spp. L'Hér.) EO is widely recognized for its potent and antimicrobial properties against some plant pathogenic fungi [48]. Studies have reported that its strong inhibitory effects against major decay agents are attributed to its high concentration of 1,8-cineole (eucalyptol), which damages fungal membranes and disrupts cellular respiration [49].

4.6. Family Lauraceae

Cinnamon (*Cinnamomum* spp.)

Cinnamon (*Cinnamomum* spp. Schaeff.) EO is one of the most important natural antimicrobial agents, exhibiting potent fungicidal activity against several plant-decaying pathogens, especially against postharvest and soil-borne fungi, including *Botrytis cinerea*, *Rhizoctonia solani*, and *Penicillium expansum* (blue mold). The dominant bioactive component, cinnamaldehyde, is primarily responsible for this strong inhibitory effect, which can damage the fungal cell wall and membrane, leading to cellular leakage and death [50,51].

5. Antimicrobial Activity of Plant EOs

Plant-derived EOs have gained significant attention for their broad antimicrobial potential, notably their antibacterial, antifungal and antiviral activity. For instance, a

study screening 21 different plant EOs found that 19 exhibited inhibitory activity against at least one of six bacterial species, including Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*, with oils such as cinnamon, clove, lime, geranium and rosemary being the most effective [52]. Further work demonstrated strong antibacterial performance of oregano, thyme, clove-bud, garlic and other EOs: in one screening across 31 oils, the most potent against *S. aureus* were oregano, garlic, pelargonium, cinnamon and thyme, and against *E. coli*, the ranking was oregano, thyme, wild thyme, clove-bud and peppermint [53]. Mechanistic studies suggest that these oils act primarily via disruption of microbial plasma membranes, leakage of ions and cellular contents, and inhibition of respiration and enzyme systems [54–58].

Beyond bacteria, plant EOs show promising antifungal and antiviral activity. Reviews note that EOs such as those from fennel, coriander, anise, geranium, Japanese mint, cinnamon, clove and lemongrass exhibit strong antifungal effects against various yeasts and filamentous fungi (e.g., *Candida albicans*), even at low concentrations [59]. On the antiviral front, oils from certain *Eucalyptus* species demonstrated activity in vitro against human pathogens (e.g., Herpes simplex virus 1, Coxsackie B4, and Hepatitis A virus), with IC₅₀ values around 2–6 µg/mL [60]. These findings indicate that plant EOs are a rich source of antimicrobial agents with multi-kingdom activity (bacteria, fungi, viruses) and could serve as key ingredients for novel anti-infective products.

A wide range of EOs have demonstrated strong antifungal activity against major postharvest pathogens, including *Botrytis cinerea* [61–63], species of *Aspergillus* [64–66], *Fusarium* spp. [67,68], *Penicillium* spp. [69], *Rhizopus stolonifer* [70,71], and *Colletotrichum gloeosporioides* [72]. Their effectiveness is largely attributed to the suppression of mycelial development as well as the prevention of spore germination. This mode of action implies that disease progression can be limited by hindering both the initial infection process and the subsequent expansion of fungal mycelia beyond the primary infection point [73].

Clove EO, derived from *Syzygium aromaticum* (L.) Merr. & Perry, has demonstrated notable bioactivity, largely due to its major monoterpene, eugenol [74]. This EO has been shown to inhibit the growth of several postharvest pathogens on apples, including *Botrytis cinerea*, *Monilinia fructigena*, *Penicillium expansum*, and *Phlyctema vagabunda*. Similarly, carvacrol, a phenolic compound abundant in oregano (*O. vulgare*) EO, has been reported to strongly suppress mycelial growth of *Neofabraea alba* on apple fruits [75].

Other Mediterranean EOs, such as those obtained from basil (*Ocimum basilicum* L.), fennel (*Foeniculum vulgare* Mill.), lavender (*Lavandula officinalis* Mill.), marjoram (*O. majorana* L.), peppermint (*Mentha piperita* L.), rosemary (*Rosmarinus officinalis* Spenn.), sage (*Salvia officinalis* L.), savory (*Satureja montana* L.), thyme (*T. vulgaris* L.), oregano, and wild mint (*Mentha arvensis* L.), have also shown considerable antifungal potential, in some cases outperforming conventional chemical treatments, particularly against *B. cinerea* and *P. expansum* during postharvest storage of apples [3].

The observed differences in EO effectiveness are primarily attributable to the individual antifungal properties of their chemical constituents, as well as to synergistic interactions among these compounds [3,75]. Some studies suggest that potential phytotoxic effects of EO treatments may arise from the same active molecules responsible for antifungal activity. Additionally, the duration of storage can influence EO efficacy, indicating that EO-based treatments may be most effective over short storage periods or may require repeated applications tailored to the specific fruit cultivar [3]. Selection of an appropriate EO for postharvest use should therefore consider fruit type, storage duration, and the intended control of decay.

Application methods also play a key role in maximizing antifungal performance. Both dipping and spraying techniques have been investigated for delivering EOs onto fruits

and vegetables, demonstrating improvements in postharvest disease management [76–78]. Combining EO treatments with other postharvest strategies has been shown to enhance overall efficacy against spoilage organisms [73,79,80].

Specific EOs, including thyme (*T. capitatus*), spearmint (*M. spicata* L.), and anise (*Pimpinella anisum* L.), have exhibited inhibition control effects on *M. fructicola* development [65,66]. Lemon myrtle (*Backhousia citriodora* F.Muell.) EO has also displayed strong antifungal activity against this pathogen, largely due to its major constituent, citral, which has been identified as a potent fungitoxic agent with potential applications in topical pharmaceuticals and postharvest disease control [39].

Although the antimicrobial activity of many plant EOs is based on controlled in vitro or greenhouse experiments, the direct quantitative comparison of application doses with synthetic fungicides is not the real target at present. These chemical products are highly optimized formulations designed for field use and are effective at low concentrations but have negative impacts on non-target organisms as well as soil and human health, and lead to pathogen resistance development. When synthetic fungicides are prohibited, EOs represent not only alternatives but leading candidates for sustainable crop protection, supporting the reduction in chemical residues, protection of beneficial organisms, and consumer demand for healthy food.

6. Antimicrobial Action of EO-Constituents Against Plant Pathogens

The potent antimicrobial properties of plant EOs against phytopathogens are primarily attributed to a diverse array of secondary metabolites, predominantly belonging to the terpenoid and phenylpropanoid chemical families [39]. These bioactive constituents, which often act in a synergistic way within the whole oil, target fundamental structures and processes in fungal and bacterial cells, leading to growth inhibition, Spore-germination failure, and cell death.

The most common bioactive constituents can be categorized into the following based on their chemical class and primary mode of:

6.1. Phenolic Compounds

Phenolic compounds such as carvacrol and thymol (abundant in oregano and thyme oils) [31,33], and the aldehyde cinnamaldehyde (from cinnamon oil) [38], are among the most powerful compounds. Their antimicrobial action is largely non-specific and concentration-dependent. Their hydroxyl (-OH) or aldehyde (-CHO) groups impart a strong lipophilic character, enabling them to integrate into and disrupt the microbial plasma membrane. This integration increases membrane permeability, leading to the leakage of vital cellular ions (e.g., K^+ , H^+) and ATP, collapse of the proton motive force, and ultimately, cytolysis.

6.2. Monoterpene Hydrocarbons

Monoterpene hydrocarbons like limonene and pinenes contribute to membrane fluidity and disruption, but are generally less potent than their oxygenated derivatives [41]. In contrast, oxygenated monoterpenes, including alcohols (e.g., linalool, terpinen-4-ol in marjoram), aldehydes (e.g., citral in vervain), and ketones (e.g., camphor in sage), exhibit stronger activity. Their mechanism extends beyond membrane disruption to include the inhibition of key enzymes, interference with energy metabolism (e.g., respiration), and the disturbance of ion channel function. A particularly widespread and significant oxygenated monoterpene is the ether 1,8-cineole (eucalyptol), the major component of many eucalyptus oils. It is known to cause extensive membrane damage and is also a potent inhibitor of fungal sporulation and mycotoxin production.

The specific action of these compounds causes a series of problems inside plant pathogens. In fungi, the first effect is damage to the cell membrane, which slows or stops hyphal growth and germ tube development. Importantly, many EOs and their active compounds strongly reduce or completely block spore germination, which is a critical step for the start and spread of plant diseases. Furthermore, certain components, like thymol and cinnamaldehyde, can denature proteins and inhibit enzymes critical for cell wall synthesis (e.g., chitin synthase in fungi) or virulence factor production. The efficacy of a given constituent is influenced by its concentration and the presence of other compounds in the oil, which can create synergistic effects.

7. Mechanism of Antifungal Activity and Synergistic Interactions of EOs

Plant EOs have been extensively studied for their potent antimicrobial properties, which originate from their complex mixture of bioactive compounds, including phenolic compounds, monoterpenes, sesquiterpenes, alcohols, aldehydes, and other small volatile molecules [81–84]. These molecules act through multi-target mechanisms, simultaneously affecting multiple cellular structures and pathways within fungal pathogens. EOs target multiple cellular sites, unlike conventional fungicides that act on a single site, reducing the likelihood of resistance.

A central antifungal mechanism of EOs involves disruption of the fungal cell wall and plasma membrane. Lipophilic constituents, such as monoterpenes, phenols, and aldehydes, interact with membrane lipids and embedded proteins, with particular affinity for ergosterol, a sterol that is critical for fungal membrane integrity. This interaction alters membrane fluidity, permeability, and overall stability, leading to the leakage of ions, nucleotides, and proteins from the cytoplasm. The resulting disturbance of cellular homeostasis impairs essential physiological processes, including spore germination, hyphal elongation, and nutrient transport, ultimately causing cell death [54,85].

Beyond membrane-targeted effects, EO components can impair mitochondrial function, uncouple oxidative phosphorylation, and deplete intracellular ATP pools, thereby compromising the energy balance of fungal cells. Simultaneously, many EO constituents induce the accumulation of reactive oxygen species (ROS), which damage proteins, lipids, and nucleic acids, resulting in oxidative stress that further enhances antifungal activity [86]. The simultaneous targeting of multiple pathways—membranes, mitochondria, and oxidative homeostasis—reduces the potential for fungal adaptation or resistance, a common drawback of single-site fungicides.

The antifungal activity of EOs is further enhanced by synergistic interactions among their constituent molecules. Combinations of monoterpenes and phenolic compounds often exhibit inhibitory effects that exceed the additive activity of each individual compound [37]. For example, the monoterpene p-cymene, which on its own has relatively weak antifungal activity, can increase the permeability of the fungal membrane, facilitating the cellular uptake of the phenolic compound carvacrol and thereby amplifying its toxicity. Other phenolic and terpenoid constituents, such as thymol, eugenol, and carvone, act cooperatively, targeting both membrane integrity and enzymatic processes within the fungal cell [33]. In addition, EOs can disrupt cell wall enzymes, leading to fragile, thin, and poorly branched hyphae that limit fungal growth [87,88].

Synergistic effects are also observed when whole EOs are blended. In these mixtures, one oil may primarily disrupt membrane integrity while another interferes with enzymatic activity, mitochondrial respiration, or ROS regulation [89,90]. Notable EO combinations—oregano with thyme, and clove with cinnamon—significantly inhibit human and plant pathogens like *Candida albicans* and *Aspergillus* spp., often cutting individual MICs by over 50% [91]. Such combinations allow for the use of lower concentrations,

reducing potential phytotoxicity, volatility, and organoleptic effects, while maintaining strong antifungal efficacy.

The biochemical composition of EOs is a key determinant of their antifungal potency. *Origanum vulgare* ssp. *hirtum* EO, for instance, exhibits a chemotype dominated by the phenolic compounds carvacrol and thymol. This oil demonstrated strong inhibitory activity against *Monilinia* spp. (*M. laxa*, *M. fructigena*, and *M. fructicola*) at concentrations of 250–1000 ppm in vitro on PDA plates containing 0.2% Tween-20, without inducing phytotoxic effects or hemolysis [31,92]. Beyond oregano, Mediterranean aromatic plants such as thyme (*Thymus vulgaris*) and vervain (*Verbena officinalis*) also produce EOs with broad-spectrum antimicrobial properties. These oils have been shown to inhibit multiple bacterial and fungal plant pathogens, including *Aspergillus*, *Fusarium*, *Penicillium*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Clavibacter michiganensis*, *Phytophthora infestans*, and *Sclerotinia sclerotiorum*. In particular, *O. vulgare* and *O. syriacum* display notable activity against *Botrytis cinerea*, confirming their potential in postharvest disease management [30,37,76,77].

The antifungal potential of EOs is not limited to in vitro conditions. In vivo studies have demonstrated that thyme and vervain EOs effectively reduce disease severity in postharvest peach fruits. Thyme EO, characterized by high o-cymene content (56.2%), and vervain EO, rich in citral (44.5%) and isobornyl formate (45.4%), significantly reduced the diameters of lesions caused by *Monilinia* species when applied at 250–500 ppm (thyme) and 500–1000 ppm (vervain), without causing phytotoxic effects [32].

The activity of individual EO constituents has also been extensively evaluated. Studies show that citral exhibits fungicidal activity against *P. citrophthora*, while carvacrol and thymol primarily act as fungistatic agents against *P. citrophthora* and *R. stolonifer*. Thymol additionally inhibits *P. italicum*, and complete inhibition of *B. cinerea* is achieved with citral and carvacrol at 250 ppm and thymol at 150 ppm [93]. Further investigations on *Monilinia* infections in peach fruits confirmed that emulsions of carvacrol and thymol at 150–500 ppm strongly reduce lesion development, demonstrating the practical in vivo antifungal efficacy of these compounds [33].

These findings show that EOs' broad antifungal activity stems from multiple bioactive molecules targeting membranes, enzymes, mitochondria, and oxidative balance. This multi-target mechanism underlies their effectiveness against major plant pathogens such as *Botrytis cinerea*, *Alternaria* spp., and *Fusarium* spp., while minimizing the risk of resistance development. Although complete mycelial inhibition requires high in vitro EO concentrations (200–500 ppm), modern formulations—nanoencapsulation, emulsions, and EO-based coatings—improve stability, prolong bioactivity, and lower practical doses. By combining chemical profiling, mechanistic understanding, and empirical evaluations of both whole oils and their individual constituents, EOs can be effectively harnessed as sustainable antifungal agents in crop protection.

Recent research under open-field and semi-field conditions (in vivo trials) has increasingly demonstrated the practical efficacy of plant EOs for controlling economically significant plant-decaying pathogens. For instance, in open-field trials against tomato late blight (*Phytophthora infestans*), thyme and cinnamon EOs applied as foliar sprays significantly reduced disease severity, showcasing performance comparable to some synthetic fungicides [88]. Similarly, semi-field studies on strawberry anthracnose (*Colletotrichum* spp.) demonstrated that clove and lemongrass oil formulations effectively suppressed fruit rot, highlighting their potential in high-value-crop protection. These studies show that EOs both inhibit pathogens and induce systemic resistance, providing multi-modal defense even under high natural-pathogen pressure.

To address inherent limitations of EOs—such as volatility, photodegradation, and poor water solubility—advanced formulation strategies are essential for enhancing field performance. Nanoemulsions and encapsulation techniques have shown particular promise. For example, encapsulated thyme or garlic oil nanoemulsions demonstrate improved stability, prolonged leaf persistence, and enhanced efficacy against pathogens like *Botrytis cinerea* in grapevines and *Alternaria solani* in potatoes [94].

These formulations increase practical utility by enabling controlled release, protecting active compounds from degradation, and improving coverage and uptake, effectively translating in vitro activity into reliable disease management under real-world conditions. Although comprehensive economic assessments are limited, evidence suggests that well-formulated, sustainably sourced plant EOs can provide a competitive and environmentally friendly alternative—or complement—to conventional synthetic fungicides.

Overall, the antifungal activity of plant EOs and their bioactive constituents results from a coordinated, multi-target mode of action (Figure 1). Key mechanisms include disruption of fungal cell membrane integrity and permeability, leading to leakage of ions and vital cellular components; impairment of mitochondrial function and energy metabolism; induction of oxidative stress through reactive oxygen species (ROS) accumulation; and inhibition of essential enzymatic processes involved in cell wall synthesis, virulence, spore germination, and hyphal development. These effects often act simultaneously and are frequently enhanced by synergistic interactions among individual constituents or between blended EOs. This combined biochemical action disrupts fungal cell balance, slows pathogen growth, and lowers the risk of resistance, thereby supporting the broad-spectrum effectiveness of EOs against plant-decaying and postharvest pathogens.

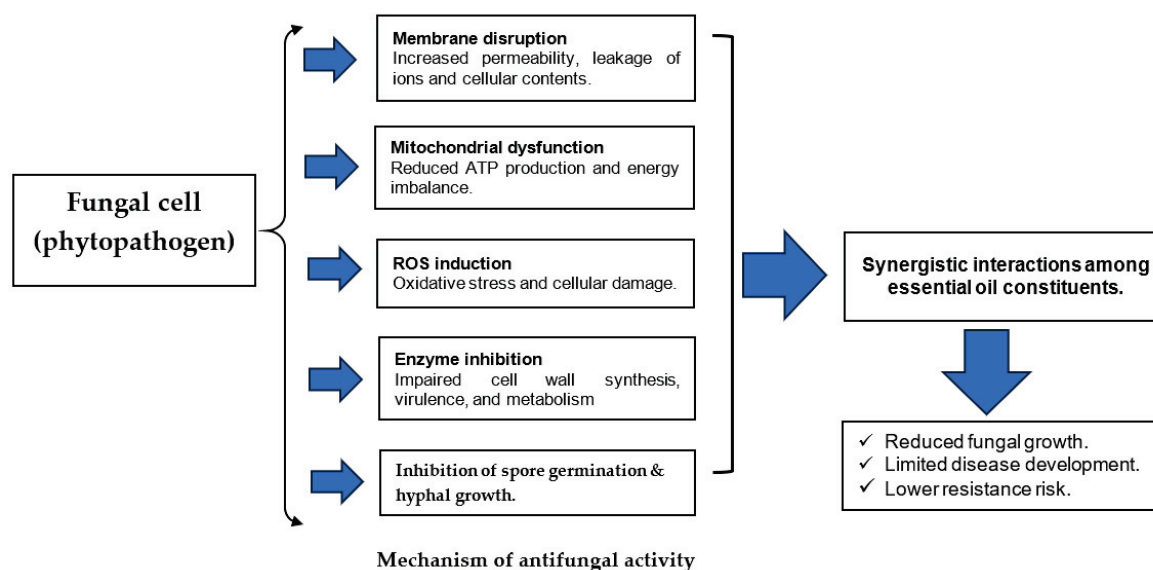


Figure 1. Schematic diagram illustrating the main mechanisms of antifungal action of essential oils.

8. Future Research Perspectives on the Bio-Applications of EOs

Future research on the biological applications of plant EOs should focus on overcoming limitations related to stability, volatility, and delivery while simultaneously enhancing their efficacy and safety in both agricultural and biomedical contexts. Advances in nanoencapsulation, emulsification technologies, and polymer-based coatings offer promising strategies to improve EO persistence, control release rates, and reduce phytotoxicity, enabling more reliable and effective field applications. Integrating chemical profiling with omics technologies—including genomics, transcriptomics, and metabolomics—will help elucidate precise molecular targets, resistance mechanisms, and pathways affected by EO

constituents. Further studies should investigate synergistic interactions between EOs, their individual compounds, and conventional antifungal or antibacterial agents, potentially reducing required doses and mitigating resistance development. Expanding in vivo evaluations and field trials remains essential to validate laboratory findings under real environmental conditions, particularly concerning postharvest disease management and crop protection. Comprehensive safety assessments involving toxicology, environmental impact, and non-target effects will also guide regulatory acceptance.

Although comprehensive economic analyses remain limited, existing evidence suggests that plant EOs—especially when locally sourced and appropriately formulated—may represent a competitive, cost-effective, and environmentally sustainable alternative or complement to synthetic fungicides. Their broader adoption has the potential to reduce chemical residues, lower environmental toxicity, and support farm-level circular economies; however, long-term cost–benefit evaluations addressing yield stability, formulation scalability, and production consistency are still required.

Finally, systematic exploration of underutilized aromatic plants spices, region-specific chemotypes, and sustainable extraction technologies could expand the diversity and availability of bioactive EO compounds. Collectively, these research directions will facilitate the development of robust, eco-friendly EO-based biopesticides and therapeutic agents with enhanced practical applicability.

9. Conclusions

Plant EOs have emerged as promising, eco-friendly alternatives to synthetic pesticides for managing a broad spectrum of phytopathogenic fungi. As evidenced in this review, a wide range of EOs and their formulations, such as oregano, thyme, vervain, magnolia, lemon, eucalyptus and cinnamon, along with their bioactive constituents (e.g., carvacrol, thymol, linalool, and citral), demonstrate potent antifungal activity both in vitro and in vivo. Their mechanisms, which can interfere with essential fungal processes, enable effective inhibition of mycelial growth and spore germination, often at low concentrations. These findings support their incorporation into practical applications such as seed treatments or soil amendments, thereby protecting crops and reducing postharvest losses. However, translating this promising efficacy to open-field conditions requires addressing critical challenges related to formulation, stability, and optimal application dosage. In summary, the novelty of this review lies in its targeted focus on plant-decaying and postharvest pathogens, its integrated discussion of antifungal mechanisms and synergistic interactions of EO constituents, and its emphasis on formulation strategies and field applicability. By linking chemical composition, biological activity, and practical deployment, this work extends beyond descriptive surveys and provides a comprehensive framework for the development of EO-based tools in sustainable crop protection and postharvest disease management. Future research should, therefore, focus on these aspects, particularly for high-impact fungal pathogens, to enable the effective integration of EOs as reliable tools in organic and integrated pest management programs.

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Essential Oils as Alternative Green Broad-Spectrum Biocides

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Abstract: Natural compounds from plants represent suitable options to replace synthetic biocides when employed against microorganisms in various applications. Essential oils (EOs) have attracted increased interest due to their biocompatible and rather innocuous nature, and complex biological activity (fungicide, biocide and anti-inflammatory, antioxidant, immunomodulatory action, etc.). EOs are complex mixtures of derived metabolites with high volatility obtained from various vegetal parts and employed to a great extent in different healthcare (natural cures, nutrition, phyto- and aromatherapy, spices) and cosmetics applications (perfumery, personal and beauty care), as well as in cleaning products, agriculture and pest control, food conservation and active packaging, or even for restauration and preservation of cultural artifacts. EOs can act in synergy with other compounds, organic and synthetic as well, when employed in different complex formulations. This review will illustrate the employment of EOs in different applications based on some of the most recent reports in a systematic and comprehensive, though not exhaustive, manner. Some critical assessments will also be included, as well as some perspectives in this regard.

Keywords: essential oils; biofilm; healthcare applications; pest control; food packaging systems; cultural heritage protection

1. Introduction

Essential oils (EOs) represent concentrated hydrophobic mixtures of natural organic compounds comprising mainly terpenes, terpenoids, and phenylpropanoids isolated from many vegetal parts of plants (e.g., flowers—rose, chamomile, jasmine, lavender; leaves—peppermint, rosemary, thyme, patchouli; fruits—lemon; roots—vetiver, elecampane; seeds—fennel, anise, cumin, nutmeg; grasses—lemongrass; rhizomes—ginger; bulbs—garlic;) and wood species (e.g., wood as whole—cedarwood, sandalwood; barks—cinnamon, sassafras; leaves—eucalyptus; peels—lemon, lime, orange, tangerine, grapefruit, bergamot; natural resins—frankincense, myrrh; berries—juniper; tree blossoms—ylang-ylang; flowers—clove, orange; heartwood—agarwood) [1].

It is well known that plant extracts usually consist of dozens of organic compounds and only one of them actually has biocide activity [2]. For example, cinnamaldehyde is the biologic active compound in cinnamon oil, thymol in thyme oil, linalool in coriander oil, citral in lemongrass extract, etc. The structures of the main biologic active compound in EOs extracted from different plants are presented in Figure 1.

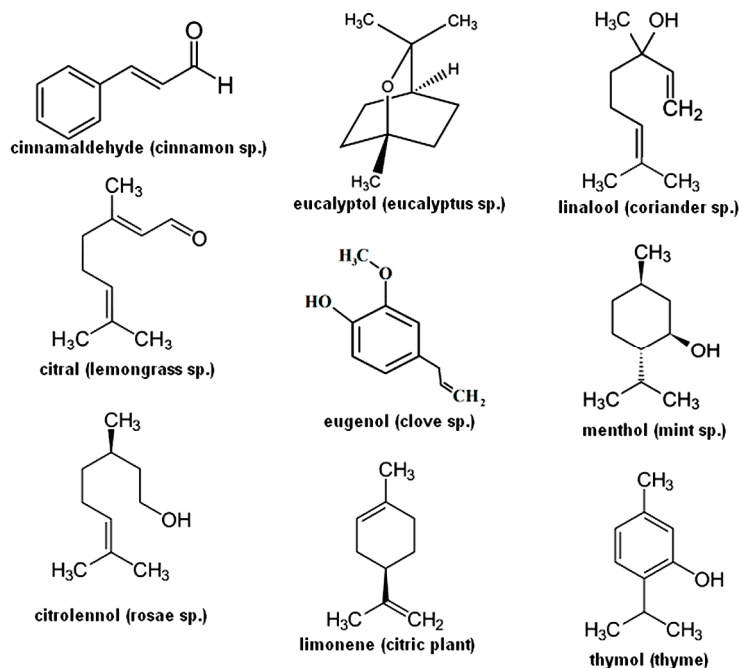
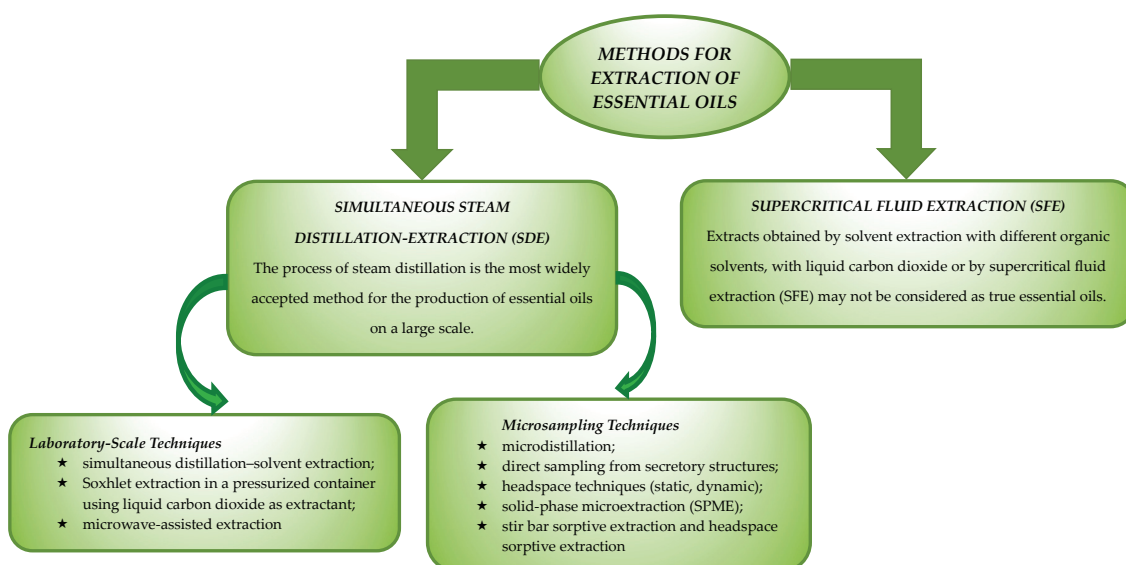


Figure 1. Chemical structures of main components in EOs from different plants.

Some species have high versatility and, therefore, we find them used in a wide range of applications. For example, elecampane (*Inula helenium*), a native species from Eastern Europe, is mostly used in the treatment of respiratory and urinary infections, some digestive syndromes, and also for skin diseases [3]. At the same time, it has been shown that chemical components from *Inula helenium* root essential oil, namely sesquiterpene lactones, alantolactone, and isoalantolactone, possess a very complex biological activity that includes antiproliferative, anti-inflammatory, and antioxidant properties, as well as the capacity to activate enzyme-assisted detoxification, as previously reported [3–7].

Due to their complex chemical composition and consequently diverse properties, different distillation methods can be employed for the extraction of essential oils from plants, as illustrated in Scheme 1.



Scheme 1. Methods for extraction of EOs (adapted after [8]).

The most applied technique is hydro-distillation, a traditional method for extraction, in which the appropriate vegetal material is usually boiled in water [9]. Thus, high-boiling-point organic compounds are effectively separated from their respective mixtures. The oil is vaporized at the boiling point of water or lower, and the resulting hot vapors are further cooled down and condensed until the formation of a biphasic liquid. This method of extraction is preferred considering aspects such as economic (low-cost technique, simple instrumentation) and environmental benefits (it is free from organic solvents) as well.

One of the main applications of essential oils is their use as natural, environmentally friendly alternative biocides. A biocide exerts its effects directly on the target microorganisms, as well as indirectly by preventing the formation and/or destroying the biofilm—the sole mediator of all interactions between support and the surrounding microbiota. The Eos' biocide activity is a sum of complex phenomena, among which preventing the formation of the biofilm (or even the biofilm degradation) in an effective manner has a primary role in regard to fighting against microorganisms. Thus, EOs have been used as biocides to protect the food surface [10], or for medical and health purposes (aromatherapy and natural cures, cleaning products, cosmetics, and personal care) [11,12], as well as in agriculture as herbicides and for pest control [13], and even for the protection and conservation of cultural heritage [14]. In the first mentioned case, the EOs are included as additives in biodegradable films and coatings for active food packaging [15], which can provide a long shelf life and preserve the good quality of food products during storage by preventing inherent degradation phenomena which may occur under different detrimental circumstances (e.g., proliferation of microorganisms on biofilm, exposure to chemical contaminants, moisture or light conditions). Eos' specific action in such applications is related to their significant antimicrobial activity (conferred mainly by terpenoids and phenol compounds from their composition) and is manifested as a complex phenomenon, including molecular mechanisms and intermolecular interactions which have been comprehensively reviewed and discussed [16]. The beneficial effects of EOs are also related to their pro-oxidant effects at the cellular level [17].

EOs represent an effective biological strategy applied in order to prevent biofilm occurrence, and their action is manifested through the regulation of proteins coded directly by genes implicated in motility, quorum sensing (QS, known as the cell-to-cell signaling process) [18], and exo-polymer substance (EPS) matrices [10]. Nevertheless, chemical strategies using different biocides (oxidizing, non-oxidizing) and disinfectants are efficient ways to remove biofilms [19]. Such chemical diversity is strongly related to the multifarious interactions, which may add to the mechanical stability of the biofilm, making it possible to employ complex approaches (chemical and mechanical treatments acting in synergy) in order to eliminate the biofilm [20].

EOs' ability to impair the formation of a biofilm has attracted more and more interest considering that they can effectively contribute to reducing the dose of antibiotics [21].

The worldwide interest in replacing synthetic biocides with highly specialized natural ones in various applications, from pest control in agriculture to antimicrobial food packaging, and from healthcare to protection of the cultural heritage, has significantly increased. A solid indication of this interest is the number of articles published in the last decade (although only 16 review articles), as summarized in Figure 2.

Therefore, this review will illustrate the general effort to limit or even to eliminate synthetic biocides from different applications based on some of the most recent reports. It will also provide an appraisal and up-to-date background of the topic and a comprehensive, though not exhaustive, range of applications where EOs have been successfully replacing synthetic biocides. Some critical assessments will also be included, mainly concerning new and complex approaches in the field, as well as some of the EO limitations (e.g., their photochemical and oxidative stability, selectivity, etc.). At the same time, some perspectives in this regard will be presented in order to optimize future research strategies.

ARTICLES ON ESSENTIAL OILS APPLICATIONS
(DATA FROM ISI WEB OF SCIENCE)

9 articles (2015–2023)	7 articles (2015–2023)	4 articles (2019–2023)	37 articles (2013–2023)	8 articles (2013–2022)
essential oils as biocides for wood products	essential oils as biocides for agriculture	essential oils as biocides for cultural heritage protection	essential oils as biocides, membranes and thin layers	essential oils as biocides and active food packaging

Figure 2. A survey of Web of Science Core Collection related to the articles published in the period 2013–2023 on the addressed topic.

2. Biofilm-Mediated Specific Interactions Between Microbiota and EOs Biocides

Although the microbiota is a widely unexplored ecosystem, it is of particular importance in human health and diseases, and the modulation of this complex is of the utmost consideration and a biomedical priority. In recent years, the huge problem of antibiotic resistance has been tackled with numerous antibacterial alternatives that have also responded to people's demand for natural therapeutic products that have no side effects, such as dysbiosis and cyto/hepatotoxicity. Energy metabolism, infection, nutritional status, immunity, behavior, and response to stress form the normal physiology of the host, which is controlled by the microbiota [22]. Homeostasis at the level of the intestinal mucosa and beyond is provided by a well-balanced intestinal microbiota [23].

The mechanisms responsible for the beneficial or harmful influences of the microbiota on the host are still largely undefined, even though its composition and physiology have been intensively researched. Investigation of high-throughput human multi-omic data, such as metagenomic and metabolomic data, together with measures of host physiology and mechanistic experiments in humans, animals, and cells evidences the intricate signaling pathways between the host and different microbiota species [24].

Usually, a higher diversity of the microbiota correlates with a state of health, while a lower bacterial diversity has been observed in people with metabolic disorders, type 1 and 2 diabetes, psoriatic arthritis, celiac disease, atopic eczema, arterial stiffness, and obesity. The microbiota is in a continuous dynamic and it undergoes alterations many times in a human life. It varies greatly from one individual to another, is susceptible to exogenous and endogenous changes, and has an additional impact on the health of the host [25]. The microbiota is influenced by several molecular factors: (1) host-derived factors, which include specific microribonucleic acids, and nonspecific molecules, such as antimicrobial peptides, mucus, immunoglobulin A, and hormones [26]; (2) microbial factors, such as lipopolysaccharides, metabolites, short chain fatty acids, and quorum sensing signaling molecules [27]; (3) dietary molecules (i.e., fats, sugars, proteins); (4) medications (mainly antibiotics, but any drugs could impact the gut microbiota, including natural products utilized in traditional medicine, prophylaxis, or immune stimulation); and (5) various nano- and microstructure particles, which could reach the gastrointestinal tract accidentally by ingestion or by means of oral therapy [28]. The diet is the most important modeling agent for gut microbiota [29].

Different edible (or not edible) substances such as essential oils (EOs) and plant extracts which could be ingested in various situations induce changes into the composition of the gut microbiota, and its modulation [30,31] (Li et al. 2018; Unusan 2020). At the same time, the gut microbiota is impacted by various micro- and nanoparticles currently present in the environment, dust, air, and water, as well as medication and self-care products (mouthwashes, tooth cream, sunscreen, etc.) (Xie et al. 2022).

Complementary and alternative medicine engages drugs derived from plants for therapeutic purposes, used by approximately 75% of the world's population, especially against metabolic diseases in which the microbiota plays an important role, such as obesity [32].

Essential oils obtained from fruits, aromatic plants, and spices are used to improve human health due to their multiple biological activities [31]. For example, gastrointestinal

infection, inflammation, metabolic diseases, and carcinogenesis are the main targets of fruit EO therapy for healthy intestinal functions in humans [29]. EOs are usually colorless liquids at room temperature obtained by plant extraction with water or organic solvent and they are highly volatile plant metabolites [1,31]. EOs are complex mixtures of up to 200 different organic compounds in low amounts (<1%), such as hydrocarbons and their oxygenated derivatives. The interaction between fruit EOs and the gastrointestinal microbiota (GM) involves the modification of the intestinal environment through the microbial metabolism of the bioactive compounds in the EOs or through the compounds excreted by the host. These changes influence the composition and density of the microbiota, but interferences with inevitable impact on the health of the host can also be generated, such as altering the ability of the microbiota to synthesize certain metabolites important for the host (i.e., SCFA and vitamins) [33,34].

Complex omics platforms have been used to investigate the effects of EO bioactives on host microbiota and metabolism, leading to the recent development of metabolomic analysis. The specific interactions between fruit EOs and the host microbiota are currently studied in stool microbiota with free bacteria or blood samples, and not on mucosal biopsies with adherent bacteria that cover the entire microbiota which may make results inaccurate. Different regions of the gut (i.e., adhered to gastrointestinal mucosal proteins and epithelial cells) and outside the gut (i.e., central nervous system, systemic immune system, blood metabolites, etc.) are currently being investigated in various animal models to monitor microbiota–EO interferences [35].

Several plants and fruit peels such as pine, citrus, thyme, cannabis, hops, etc., contain terpenes, aromatic compounds with pharmacological and therapeutic properties, namely anti-inflammatory, antioxidant, antibacterial, and gastroprotective activity [36]. The citrus family of fruits, such as sweet oranges, grapefruits and lemons, contains d-Limonene, an active component that improves metabolic parameters and modulates the intestinal microbiota [37].

Polyphenols are a large class of bioactive phytochemicals present in most fruits and are consumed as antioxidants [38]. They are secondary metabolites with over 10,000 structural variants, and they include flavonoids (flavonols, flavanones, flavanols, flavones, isoflavones, and anthocyanidins) and nonflavonoids (phenolic acids, stilbenes, coumarins, xanthenes, lignans, and curcuminoids). Some of them (flavanones, flavonols, isoflavone, anthocyanins, proanthocyanidins, and resveratrol) are studied for their significant influence on GI tract function, impacting on insulin signaling, downregulation of oxidative stress, gut bacteria modulation, improvement of endothelial dysfunction, modulation of intestinal absorption, and metabolism [39].

Generally, inter-individual variation in the microbiota pattern and metabolism is important for the health benefits of phytochemicals because the bioactivity of polyphenols can be influenced by their microbial metabolism [40]. Phytoestrogens (stilbenes, coumestans, isoflavones, ellagitannins, and lignans) are polyphenols found in numerous plants, such as soy beans, flaxseed and other seeds, cereals, vegetables, fruits, tea, chocolate, etc. These compounds have a chemical structure similar to human estrogens, but they can exert both estrogenic and antiestrogenic effects.

Isoflavones, ellagitannins, and lignans are metabolized by intestinal bacteria to produce equol, urolithins, and enterolignans, having more estrogenic/antiestrogenic and antioxidant activity in comparison with their precursors. In addition, these metabolites have anti-inflammatory, antiproliferative, and apoptosis-inducing effects and exhibit efficiency against certain diseases, such as cancer, cardiovascular disease, and osteoporosis, as well as menopausal symptoms [41,42]. Ursolic acid is a natural pentacyclic triterpenoid, present in fruit peels (apples, prunes, bilberries, cranberries, hawthorn) and in many herbs and spices (rosemary, thyme, basil, oregano, peppermint, lavender), with anticancer, antidiabetic, antiarrhythmic, anti-hyperlipidemic and antihypercholesterolemic, antimicrobial, hepatoprotective, and hepatoregenerative properties [43,44].

Essential oils (EOs) have received increased attention because of their multiple biological attributes like antifungal, antiviral, anti-inflammatory, antimicrobial, antioxidant, wound healing, anti-parasitic, anti-mutagenic, anti-quorum sensing, antibiofilm, and immune-modulatory activities, and their pharmacological effects against resistant pathogens and increased efficiency against multidrug-resistant and biofilm embedded microorganisms [45].

Natural products extracted from plants offer better biocompatibility and fewer side effects on the human body in comparison with synthetic antibiotics. They have been largely employed to treat infections caused by resistant microorganisms and, therefore, they may represent potential substitutes to conventional drugs [46–49].

EOs are complex mixtures of volatile secondary metabolites obtained from various parts of the plant, such as flowers, fruits, buds, rhizomes, seeds, leaves, stems, twigs, roots, bark, and wood, and they are extensively applied as adjuvants for healthiness purposes [50]. EOs consist of a large diversity of aromatic oily liquids (approximately 3000 EOs are known nowadays) with a versatile composition, including aldehydes, alcohols, ethers or oxides, ketones, esters, amines, amides, phenols, heterocycles, and mainly terpenes obtained by steam or water distillation [51,52]. EOs are also used as raw materials in various fields, such as perfumes, cosmetics, aromatherapy, phototherapy, spices, household cleaning products, beauty and personal care, and nutrition, or natural health treatments. Moreover, EOs may improve the efficiency of some drugs against different microbes [21].

The progress of novel antimicrobial and anti-pathogenic methodologies can be stimulated by the introduction of essential oils as an eco-friendly alternative to antibiotics, acting by mediating the attenuation of bacterial virulence without interfering with microbial development and aiming to regulate bacterial mechanisms in relation to the expression of virulence factors. The strategy applied against bacterial virulence when treating infections is based on generating bacterial pathogens that are more reactive to the host immune system and stopping the infection process caused by bacteria. Prevention is expected with the prevalence of multidrug-resistant bacterial pathogens with no occurrence of novel microbes with high resistance. Used in sub-minimum inhibitory concentrations (MICs), agents acting against virulence can interrupt the bacterial infection without inhibiting bacterial vitality or even killing the bacteria. The production of antibiotics, virulence factors of pathogens, exo-enzymes, and resistance are regulated by the QS system. Therefore, QS (the bacterial communication system) seems to be a promising target for new anti-virulence agents [53–55].

Due to their complex composition, multiple mechanisms that probably act synergistically are involved in EOs' biological effects [10]. Currently, about 300 of the 3000 known EOs are used commercially, and the most commonly used include lemon, peppermint, citronella, eucalyptus, mint, and orange oil. Most essential oils modify cytoplasmic membranes, leading to the release of lipo-polysaccharides due to their hydrophobic constituents. Essential oils affect the fluidity, permeability, and function of the proteins involved in membrane transport, and the composition of fatty acids in the cytoplasmic membrane as well. Processes such as the formation and dispersal of biofilms can be genetically regulated when using specific essential oils. Several traits, such as cell morphology, the structure and composition of the cell wall, cell division, cellular respiration, ion transport, and the energy balance of the bacterial cell, are influenced by different essential oils. In relation to the concerning medical aspects, EOs aim to accomplish two therapeutic targets: (1) prevention of biofilm formation and production of virulence factor and (2) eradication of already established biofilms [56].

2.1. Applications of Essential Oils Biocides as Antibiofilm Agents

Literature reports have claimed that 60% up to 85% of all microbial contaminations are associated with biofilms grown on natural, intact, or damaged tissues (skin, mucosa, endothelial cells, teeth surface) or artificial devices (catheters—central venous, peritoneal, or urinary; dentistry materials; heart valves; intrauterine contraceptive devices; contact

lenses; other implants) [57]. In their natural environment, the biofilm structure is favorable to bacteria because it offers safety against harmful conditions, enhances the competition for accessible nutritive substances in a specific zone, boosts the acquirement of novel phenotypic characteristics by gene transfer, and increases interactions in the metabolism of different microbial agents [58].

Biofilm formation is a complex process usually developed in four steps: reversible attachment, followed by irreversible attachment, cell division, and EPS excretion. As the biofilm matures, new bacterial species may be introduced before critical mass is reached and cells are released. Bacteria induce biofilm formation and communicate via quorum sensing [59]. Quorum sensing (QS) permits bacteria to synchronize and control gene expression through the formation and detection of extracellular chemicals called auto-inducers [60]. The cumulative regulation of genetic elements as numbers generates several phenotypic changes which aid the bacteria to develop certain activities to adjust to changes in the environment [61].

Certain bacteria and many strains have developed resistance to nearly all currently used antibiotics and chemotherapy agents, termed multi-drug resistance (MDR) [62]. MDR can be defined as non-susceptibility to at least one agent in three or more antimicrobial categories [63]. MDR bacteria and biofilms are ubiquitous phenomena and represent one of the significant hazards to worldwide healthiness with several deleterious medical and economic consequences [64]. The antibiotic resistance has emerged and developed due to the excessive use and misuse of antibiotics, unsuitable and incorrect prescribing, and large-scale utilization in agricultural applications [65].

Biofilms can have both desirable and damaging effects. While the industry has experienced both the positive and negative aspects of biofilm development, clinically, the devastating consequences of biofilms have been observed. During food processing, product contamination can take place as a result of biofilm appearance by spoilage and the occurrence of bacterial pathogens on food contact surfaces, which lowers products' shelf-life and causes human diseases [66]. A large number of human infections like cystic fibrosis and otitis media are caused by pathogenic bacteria in medical institutions, particularly on artificial devices like catheters [57].

Sessile cells within biofilms present increased protection in the environment because the barrier properties of the sludge matrix hinder the entry of different agents resistant against microbes. The stationary phase dormant zones in biofilms protect bacterial cells, but many antibiotics can penetrate the EPS since antibiotics mainly act through the disruption of microbial processes [67–69]. The increased antibiotic resistance of sessile cells compared with planktonic cells generates clinical issues. Sessile cells within a biofilm can be 10–10,000 times more resistant to antibacterial agents than their free-floating counterparts [70].

An important clinical aspect is the fact that bacterial biofilms cause chronic infections due to their increased tolerance to antibiotics and disinfectant chemicals as well as increased resistance to the host immune system, including phagocytic white blood cells and other components of the body's defense system [71,72]. Many biofilms are present in a variety of microbial infections, including dental infections [73], periodontitis [74], lung infections resulting from cystic fibrosis and facial filling [75], chronic wounds [76], ear inflammation [77], implant-associated infections [78], chronic rhino-sinusitis [79], contamination in intensive care units [80], contact lens infections [81], and human gastrointestinal tract infections [82].

The absence of novel efficient agents against microbes causes the occurrence of multidrug-resistant microorganisms to be regarded as a global challenge. Alternative treatments of infections caused by microorganisms resistant to traditional therapies are highly necessary. Nowadays, natural antimicrobials have attracted scientific interest due to their function in several microorganism control issues [83]. Natural products from plants can be synthesized as a part of their defense system, and these compounds could provide a valuable source of new drug molecules [84].

Different essential oils were investigated in vitro for their anti-QS activities in *Chromobacterium violaceum* at sub-MICs [85]. Peppermint, lavender, cinnamon, and clove oils exhibited an appreciable inhibition of violacein production. At sub-MICs, clove oil inhibited violacein production in the treated bacteria with no relevant vitality loss of bacterial cells up to 0.12%. The reduction in the formation of violacein in bacteria is due to the anti-QS activity of clove oil and not to its antimicrobial properties. At concentrations higher than 0.16%, the reduction in violacein formation is due to the anti-QS activity of clove oil and its antibacterial properties as well. The anti-QS activity was not exhibited by the major constituent of clove oil, namely eugenol.

Clove oil [86], peppermint oil, and menthol [87] in sub-MICs were tested in two in vitro studies to decrease biofilm formation in *Pseudomonas aeruginosa*. The reduction in various virulence factors (elastase, proteases, pyocyanin, chitinase) in sub-MICs proved that the inhibition of biofilm formation is a QS-regulated process. The production of virulence factors, exopolysaccharide production, and swarming motility were highly inhibited by all compounds under study [86,87]. The native AHL (N-acyl homoserine lactone autoinducer) production was significantly reduced by up to 56% in *Pseudomonas aeruginosa* cells by clove oil (at 1.6%) [86].

The biofilms of two species of *Vibrio*, i.e., *V. parahaemolyticus* ATCC 17802 and *V. alginolyticus* ATCC 33787, were eradicated by 56% and 55%, respectively, using *Petroselinum crispum* EO at a concentration of 40 mg/mL. *Ocimum basilicum* EO was proved to inhibit biofilms of *V. parahaemolyticus* to 55%, and *V. vulnificus* and *V. cholerae* non O1 to 87.45%, at a concentration of 50 mg/mL [88]. Another study [89] investigated the effect of *Mentha spicata* EO on two *Vibrio* spp., and it was found that the percentage of inhibited biofilm production was 11.6% for *V. alginolyticus* and 40% for *V. vulnificus* using a concentration of 92 µg/mL.

Bay oil, clove oil, pimento berry oil, and eugenol were tested for their antibiofilm activity [90]. All three EOs and eugenol inhibited biofilm formation in concentrations of 0.005% to 99.7% compared to the control. The vitality of the bacteria was reduced by 20% at a concentration higher than 0.01%.

Different EOs were screened for their antibiofilm activity against uropathogenic *Escherichia coli* in an in vitro study [91]. Carvacrol and thymol per se, as well as essential oils containing both of them in different proportions, namely oregano oil and thyme oil, strongly inhibited the biofilm formation of *Escherichia coli* in sub-MICs (at 0.01%). The reductions in biofilm mass were 88.9%, 86.1%, 94.5%, 94.5% for oregano oil, thyme oil, carvacrol, and thymol, respectively, over the control.

The evaluation of differential gene expression of biofilm-borne bacterial cells in *Staphylococcus aureus* ATCC 29213 at a tea tree oil concentration of 1 mg/mL and an incubation time of 60 min was investigated [92]. An in vitro study [93] investigated the biofilm inhibition activity of cinnamaldehyde (CA) and two different cinnamon oils of *Cinnamomum zeylanicum* (EOCz) and *Cinnamomum cassia* (EOCc). In sub-MICs, EOCc inhibited the biofilm formation of *Escherichia coli* (sub-MIC: 0.12 mg/mL) and *Pseudomonas aeruginosa* (sub-MIC: 0.06 mg/mL) to 100%, without disturbing bacterial cell growth. The biofilm inhibitory effect of cinnamaldehyde was mainly attributed to its cytotoxic activity, and not to its anti-QS effect, as confirmed by cell viability studies.

The EO from *Melaleuca bracteata* (MBEO) leaves was investigated for its anti-QS, antibiofilm, and anti-virulence factor activity in *Chromobacterium violaceum* [94]. MBEO (MIC: 10‰); used at sub-MICs of 0.625, 1.25, 2.5, and 5.0‰, it inhibited the production of biofilm mass, violacein, and different virulence factors, respectively. The viability of bacterial cells was not affected. The swarming motility of *Chromobacterium violaceum* cells was meaningfully disturbed at 5. Concentrations of 5.0, 2.5, and 0.625 of MBEO led to a significant decrease in the concentration of the auto-inducer QS signal in *Chromobacterium violaceum* cells, comparative to the control.

Five EOs and four major active compounds were tested concerning their antibiofilm activities in antibiotic-resistant *Staphylococcus aureus* strains [95]. Eugenol and thyme oil

revealed a concentration-dependent biofilm inhibition. At concentrations of 0.2% and 12.8% eugenol, the reduction in biofilm formation was found to be 19.4% and 91.6%, respectively. Thyme oil exhibited a reduced antibiofilm activity, comparative to eugenol. The number of bacterial colonies was significantly reduced by thyme oil and eugenol in the highest concentration tested (12.8%). At the same time, the biofilm matrix was destroyed. Microscopy analysis revealed that the effect of both antibiofilm agents is due to their biocidal activity.

Cassia oil and the polypeptide antibiotic colistin were tested for their ability to kill *Pseudomonas aeruginosa* in planktonic cells and in biofilms [96]. Most of the *Pseudomonas aeruginosa* in liquid culture and biofilm was eradicated by cassia oil in concentrations of 0.2–0.4%, while a concentration higher than 100 µg/mL of colistin killed the bacteria inside the biofilm.

The eradication of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in biofilms was demonstrated by oregano oil after 1 h of incubation at concentrations of 0.4 and 1.0 mg/mL, respectively [97]. Oregano oil effectively reduced the bacterial burden by 25–49-fold in comparison to the control, after bacterial inoculation formed early-stage biofilms.

The activity of tea tree EO, *Lavandula angustifolia* (lavender essential oil) (LEO), *Melissa officinalis* (*Melissa* essential oil or lemon balm-MEO), and linalool, linalyl acetate, α -terpineol, terpinen-4-ol on biofilms formed by *S. aureus* and *E. coli* reference strains demonstrated that MEO, α -terpineol, and terpinen-4-ol showed a higher antibiofilm effect than LEO and its major components, i.e., linalool and linalyl acetate [98]. The tests proved that the *E. coli* biofilm was more susceptible than the *S. aureus* biofilms to the action of EOs, especially to TTO, which destroyed it after 1 h exposure to a 0.78% concentration, contrary to the opinion stating that Gram-negative microorganisms are more resistant to EOs. In comparison with LEO and TTO, the MEO effect is more dependent on the action time. The in vivo tests on biomedical surfaces of urinary catheters and tracheal tubes showed that TTO and terpinen-4-ol used at 2xMIC caused visible biofilm eradication, while increased concentrations were required to eradicate the microbial biofilm on surgical mesh [98]. Another study on the antimicrobial action of TTO against *S. aureus* clinical strains in different growth phases, including stationary phase and biofilms, found that the minimum biofilm eradication concentration was usually 2x CMI, lower than 1% v/v. The inhibition of biofilm took place in 15 min at a TTO concentration >1% v/v [99].

The antifungal efficiency of the EO of *Mentha piperita* against *C. albicans* and *C. dubliniensis* biofilm was tested in a study which revealed that biofilm formation was inhibited at a maximum concentration of 2 µL/mL in a dose-dependent manner. This effect is due to the increased concentration of this EO in menthol, which can be incorporated into the fungal cell membrane; the phenolic monoterpene, bearing a hydroxyl group on the phenolic ring, also exhibits an antimicrobial effect due to the cytoplasmic membrane disruption [100].

Eighty-three EOs were screened for their antibiofilm activity [101], and some EOs, namely bay (*Pimenta racemosa*), cade (*Juniperus oxycedrus*), cedarwood (*Calocedrus decurrens*), frankincense (*Boswellia carterii*), lovage root (*Levisticum officinale*), oregano (*Origanum vulgare*), sandalwood (*Santalum album*), thyme red (*Thymus vulgaris*), and Vetiver Haiti (*Cymbopogon martini*) inhibited the *S. aureus* biofilm at a 0.01% (v/v) concentration. Others, like black pepper (*Piper nigrum*), cananga (*Cananga odorata*), and myrrh (*Commiphora myrrha*) oils exhibited a strong antibiofilm activity at a sub-MIC. One active compound was *cis*-nerolidol (0.01% (v/v)), which proved to be more efficient than *trans*-nerolidol contained in the three EOs, which inhibited more than 80% versus 45% of the *S. aureus* biofilm growth.

The antibiofilm activity of essential oil from *Satureja hortensis* was tested on *Candida*, *Staphylococcus*, and periodontal bacteria biofilms. The antimicrobial effect of carvacrol inhibited *Candida* and *Staphylococcus* biofilms at 0.03% and 0.06% concentrations. The growth inhibitory effect against periodontal bacteria and the antibiofilm effect in sub-MIC concentration were registered [102]. *S. epidermidis* is one of the main nosocomial agents of indwelling medical devices' biofilm associated infections (BAIs). Farnesol produced a significant destruction of *S. epidermidis* biofilm structure and an important reduction in

biofilm thickness [103]. The EOs from *Ferula asafetida* and *Dorema aucheri* (belonging to *Apiaceae* family) were tested for their activity against *P. aeruginosa* biofilm formation using 25 µg/mL concentrations. *Ferula* EO decreased pigmentogenesis, protease, and biofilm development, while *Dorema* EO affected only pyoverdine and elastase production [104]. The EOs of *Cinnamomum burmannii* and *Massoia aromatic* are another source of antibiofilm agents, proving to inhibit both *P. aeruginosa* and *S. aureus* biofilms. The efficiency of these EOs is based on the presence of cinnamic aldehyde and massoia lactone, respectively, in their structure [105]. Biofilms formed by *S. aureus* and *P. aeruginosa* respiratory isolates and reference strains were inhibited in both initial phases as well as maturation by the EOs of *Eucalyptus smithii* and *Juniperus communis* [106].

Several EOs from the *Lamiaceae* and *Apiaceae* families (*Ammi visnaga*, *Ammoides verticillata*, *Artemisia arborescens*, *Dittrichia graveolens*, *Lavandula dentate*, *Lavandula multifida*, *Mentha piperita*, *Origanum vulgare*, *Rosmarinus ericalyx*, *Thymbra capitata*), rich in oxygenated monoterpenes (mostly alcohols, such as thymol, carvacrol, and linalool), were investigated for their antibiofilm effect. The EOs from *T. capitata* and *O. glandulosum* (0.75–1.5%) inhibited *E. faecalis* biofilms, similar to those extracted from *A. verticillata* and *L. multifida* (1.50–3.00%). The administration of EOs proved to be more efficient than the administration of the main component itself [107]. The EO from *Baccharis psiadioides* (*Asteraceae* family) is known for its antipyretic and anti-inflammatory properties, and as a snake bite antidote. At the same time, it has been proved to exhibit antimicrobial and antibiofilm action on 13 resistant *E. faecalis* strains [108].

Eugenol, a major compound in clove (*S. aromaticum*) EO, acts by disrupting cellular membrane permeability, and citral, containing geranial (*trans*-citral, citral A) and neral (*cis*-citral, citral B), found in the citrus plants leaves and fruits, affects both the cytoplasmic/outer membrane as well as the stress response mediated by the sigma factor RpoS in *E. coli* [109]. The preformed biofilms of different *Candida* spp., excepting *C. glabrata*, were inhibited by the *Thymbra capitata* EO at 2xMIC, probably due to the high content in phenols (carvacrol) [110]. Ten terpenes, the main components of EOs (carvacrol, citral, eucalyptol, eugenol, farnesol, geraniol, linalool, menthol, γ -terpinene, and thymol), were tested on different *Candida* strains (*C. albicans*, *C. parapsilosis*, *C. glabrata*). The best results were obtained using carvacrol against *C. albicans*, *C. glabrata*, and *C. parapsilosis* biofilms, but good results were recorded for geraniol and thymol [111].

The antibiofilm effect of *Citrus limon* and *Zingiber officinale* EOs were investigated, and it has been shown that they can be used against biofilms of *Klebsiella ornithinolytica*, *K. oxytoca* and *K. terrigena* [112]. Dual-species biofilms produced by *L. monocytogenes* SZMC 21307 and *E. coli* SZMC 0582 were eradicated by their treatment with *Cinnamomum zeylanicum* EO at concentrations of 1 mg/mL [113]. Using *Origanum majorana* EO and *Thymus vulgaris* EO, the inhibitory effect was detected at 0.5 mg/mL and 1 mg/mL concentration, respectively. These values were much lower than those recorded in the eradication of monoculture biofilms. All studied EOs decreased biofilm formation but at concentrations higher than those required for monospecific biofilms eradication.

A comprehensive study on the synergistic effect of EO and antibiotics proved that the association of EOs with antibiotics was beneficial [114]. EOs of *Cinnamomum zeylanicum*, *Mentha piperita*, *Origanum vulgare*, and *Thymus vulgaris* were associated with norfloxacin, oxacillin, and gentamicin, and their combined activity was investigated on bacterial biofilms produced by *S. aureus*, *S. epidermidis* IG4, and *E. faecalis*. The results showed that all EOs had a synergistic effect. The advantages of combined therapy are obvious: the decrease in antibiotic doses and implicitly reducing the resistance to antimicrobial drugs.

Essential oils from 21 plants were investigated for their activity against 20 fluconazole-resistant strains of *Candida albicans* fungus. The oils of *Cymbopogon martini* exhibited strong inhibitory activity with MIC₅₀ in the 90–100 µg/mL range due to their main constituents, citral and cinnamaldehyde, respectively. The test oils exhibited remarkably synergy with fluconazole or amphotericin B and were more effective for the inhibition of azole- and amphotericin B-resistant strains than fluconazole and amphotericin B [115].

Antiquorum sensing and antibiofilm activity for seed extract from *Trigonella foenum-graecum* L. (Fenugreek) were reported, and sub-MICs were tested in *Pseudomonas aeruginosa* strains [116]. *T. foenum-graecum* seed extract inhibited the AHL regulated virulence factors to a large extent, including protease, LasB elastase, pyocyanin production, chitinase, EPS, and swarming motility. *T. foenum-graecum* seed extract decreased the biofilm forming ability of the pathogens in tested strains in high levels. Caffeine, the major compound of *T. foenum-graecum* extract, reduced the biofilm formation and occurrence of QS regulated virulence factors at a 200 µg/mL concentration level [116].

2.2. Essential Oils Biocides in Dentistry

Dental plaque biofilm plays an essential role in oral pathology, the etiology of dental caries, but also in the contamination of dental materials surfaces, such as those used in implant and prosthetic rehabilitation (implants, impression materials, alloys for prosthetic use, etc.).

Chemical substances as medications with both antibacterial and antibiofilm activities are not biologically friendly to the dental and peri-radicular tissues. In the recent years, research on the use of natural products for root canal disinfection and removing the smear layer and the microbial biofilms formed in the mouth has gained importance [117]. *E. faecalis* is commonly recovered from teeth with persistent endodontic infections, creating biofilms attached to the canal walls or located in isthmuses and ramifications from where are difficult to eliminate by current substances, such as sodium hypochlorite and chlorhexidine [118]. Chloroform solutions of eucalyptus and orange EOs associated with cetrimide at concentrations varying from 0.05% to 0.3% reduced the biofilm by 70–85%. The two EOs enhanced the efficiency of cetrimide, which effectively eradicated the biofilms in lower doses, the synergic effect probably being due to lipophilic compounds (e.g., terpenoids or phenolics). The *Melaleuca alternifolia* EO presents an antibacterial effect and is very effective against oral *S. mutans* biofilm, decreasing the gingival bleeding index. Mouthwashes with this EO decreased the total oral bacteria counts. The EO was used at 5% concentration without side effects [119]. Carvacrol and oregano oil are known for their effect on *Staphylococcus* strains. They showed in vitro effects on staphylococcal biofilms, and the biofilm inhibitory concentrations had 2–4x CMI values. Thymol is used in mouthwash, with anti-plaque effects due to the biofilm matrix destabilizing effect [120].

The use of EOs (menthol, thymol, and eucalyptol) for oral health proved to be beneficial by preventing the biofilm formation in patients with prostheses [121]. In several cases, these EOs were more efficient than cetyl-pyridinium chloride [122]. EOs can be used daily, in the long-term, for reducing the supragingival plaque and gingivitis [123]. EOs prevented plaque-like biofilm development for 7 h after mouthwash, presenting a possible alternative to chlorhexidine for the pre-surgical rinse or after periodontitis treatments [124]. Mouthwash containing EOs rich in eucalyptol, methyl salicylate, menthol and thymol, combined with ethanol at high dilution, exhibited an increased antibiofilm activity. The nonalcoholic mixture of EOs tested on *Aggregatibacter actinomycetemcomitans* strains had better anti-planktonic behavior [125].

A promising approach for the treatment and prevention of caries consisted of combinations of these EOs with xylitol in mouth rinse against *S. mutans*-derived biofilms [126]. EOs from *Mentha piperita* and *Rosmarinus officinalis* proved to be effective against *S. mutans*, one of the main agents of dental caries. *Mentha piperita* EO (having a menthol concentration below 3.6%) was more effective than rosemary oil (containing piperitone as the main component) and chlorhexidine (at 4000 and 8000 ppm). The use of toothpaste blended with EOs indicated that lower concentrations of the EOs were more effective than chlorhexidine [127]. The association of chlorhexidine with EO is indicated for better antibiofilm activity in oral treatment [128]. Other alternatives to chlorhexidine were represented by eugenol and citral at subinhibitory concentrations, when they influenced biofilm formation and the virulence of methicillin-susceptible *S. aureus*, MRSA, and *L. monocytogenes* strains, with a low-risk for selecting resistance [129].

The effects of EOs obtained from twenty medicinal and aromatic species on biofilms produced in vitro by different microbial strains were compared with the results recorded with nystatin and chlorhexidine digluconate. The *Aloysia gratissima* and *Coriandrum* spp. EOs strongly inhibited *C. albicans*, *Fusobacterium nucleatum*, *P. gingivalis*, *S. mitis*, and *S. sanguis*. The *C. articulatus* EO inhibited *F. nucleatum* and *P. gingivalis* biofilms. *A. gratissima* inhibited the *S. mitis* biofilm more intensively than chlorhexidine [130]. The antibiofilm efficacy of *Cymbopogon martini* and *Thymus zygis* EOs was tested on the multispecific biofilms of *S. mitis*, *S. sanguinis*, and *E. faecalis* in the root canals of extracted teeth. The addition of an oil-based irrigant to 1.5% sodium hypochlorite proved to be more efficient against biofilm development [131]. A good antibiofilm and anti-caries effect, comparable to that of chlorhexidine (0.12%), was observed for a mouth rinse containing *Matricaria chamomilla* L. EO. The antibiofilm effect has been evaluated as a decrease in Colony-forming units (CFUs) for total *S. mutans*, *S. sobrinus* and *Lactobacillus* sp., and the anti-caries effect has been studied as the effect on enamel demineralization compared to phosphate-buffered saline solution. Mouthwash containing *Matricaria chamomilla* EO produced a mineral loss reduction of 39.4%, which was very close to that of chlorhexidine (47.4%) [132]. The *Citrus limonum* and *C. aurantium* EOs exhibited an antibiofilm effect comparable to 0.2% chlorhexidine but lower than 1% sodium hypochlorite on multi-specific biofilms formed by *C. albicans*, *E. faecalis*, and *E. coli* [133].

The EOs from *A. gratissima*, *Baccharis dracunculifolia*, *C. sativum*, and *Lippiasidoides* demonstrated a potent inhibitory activity on *S. mutans* biofilm, probably due to the presence of compounds such as thymol, carvacrol, and *trans*-nerolidol [134]. Mouthwashes containing *Citrus hystrix* leaf EO alone or in combination with chlorhexidine inhibited the periodontopathogenic bacteria and *S. sanguinis* and *S. mutans* biofilms [135]. The *B. dracunculifolia* EO reduced the growth rate of *S. mutans* biofilm at the same level as triclosan (one week of use) [136]. EO from *Curcuma longa* inhibited the growth, acid production, and *S. mutans* adherence to saliva-coated hydroxyapatite beads and biofilm development [137]. The *Melaleuca alternifolia* (tree), *Eucalyptus radiata* (eucalyptus), *Lavandula officinalis* (lavandula), and *Rosmarinus officinalis* (rosmarinus) EOs significantly inhibited adhesion of *S. mutans* (>50%). Tea tree oil and manuka oil significantly inhibited the adhesion of *P. gingivalis* [138]. EOs from *Coriandrum sativum* exhibited an inhibitory activity against *C. albicans* oral isolates from patients with periodontal disease, similar to nystatin, suggesting its promising potential for the prophylaxis and treatment of oral candidiasis [139]. Due to its major components (decanal and *trans*-2-decenal), *C. sativum* EO could bind membrane ergosterol, similarly to nystatin and amphotericin B. The *C. sativum* EO also inhibited the proteolytic activity of *C. albicans* and affected the normal morphology of yeast cells (at 156.0 to 312.50 mg/mL concentration), probably by affecting membrane permeability due to the presence of mono- and sesquiterpene hydrocarbons [140].

Eugenol (90.2%), eugenol acetate (6.5%), and β -caryophyllene (1.3%), the major components of *Syzygium aromaticum* EO showed a significant inhibition of *S. aureus* biofilm production at a concentration of 0.106 mg/mL [141]. At the same concentration of *Cinnamomum zeylanicum* EO, the biofilm formation of *S. aureus* was significantly reduced, probably due to its components: cinnamaldehyde (86.5%), benzaldehyde (4.2%), cineole (1.7%), cinnamic acid (1.5%), and eugenol (0.1%). Besides the examples presented above, Table 1 summarizes the most used essential oils and their components with QS and activity against biofilms formation, reduction in virulence factor, and eradication of biofilms tested against numerous other types of bacteria.

Table 1. EOs with antibiofilm activity.

EOs	Plant Species	Bacteria	Antibiofilm Activity	Ref.
Clove oil, tea tree oil, cinnamon oil	<i>Syzygium aromaticum</i> , <i>Melaleuca alternifolia</i> , <i>Cinnamomum zeylanicum</i>	SA, PA	IBF	[142]
Thymol, carvacrol, eugenol	-	ST	BE	[143]
Oregano oil, <i>T. zygis</i> oil, rosemary oil	<i>Origanum majorana</i> , <i>Thymus zygis</i> , <i>Rosmarinus officinalis</i>	EC	IBF	[144]
Cinnamon oil, eugenol	<i>Cinnamomum zeylanicum</i>	SA, PA	IBF, BE	[145]
<i>Achillea biebersteinii</i> oil	<i>Achillea biebersteinii</i>	SA, EC, PA, SE	IBF	[146]
<i>Mentha pulegium</i> oil	<i>Mentha pulegium</i>	AB	IBF	[147]
<i>Cymbopogon citratus</i> oil	<i>Cymbopogon citratus</i>	SM, CA	IBF	[28]
<i>Calamintha nepeta</i> , <i>Foeniculum vulgare</i> , <i>Ridolfia segetum</i> oils	<i>Calamintha nepeta</i> , <i>Foeniculum vulgare</i> , <i>Ridolfia segetum</i>	PA	IBF	[11]
Lemongrass oil	<i>Cymbopogon flexuosus</i>	SA, CA	BE, IVBGE	[148]
<i>Origanum majorana</i> , <i>Rosmarinus officinalis</i> , <i>Thymus zygis</i> oils	<i>Origanum majorana</i> , <i>Rosmarinus officinalis</i> , <i>Thymus zygis</i>	SA	IBF, BE	[149]
<i>Plectranthus barbatus</i> oil	<i>Plectranthus barbatus</i>	PA, CV	IBF, VI, IVBGE	[150]
<i>Cupressus sempervirens</i> oil	<i>Cupressus sempervirens</i>	KP	IBF, BE	[151]
Thyme, oregano, and cinnamon oils	-	<i>Acinetobacter</i> , <i>Sphingomonas</i> , <i>Stenotrophomonas</i>	IBF	[46]
Clove oil	-	<i>Aspergillus niger</i>	IBF	[152]
Eucalyptus oil	<i>Eucalyptus globolus</i>	SA	IBF, BE	[153]
<i>Lippia sidoides</i> oil	<i>Lippia sidoides</i>	SA	BE	[154]
Citrus oil	<i>Citrus reticulata</i>	PA	IBF	[155]
<i>Pogostemon heyneanus</i> , <i>Cinnamomum tamala</i> oils	<i>Pogostemon heyneanus</i> , <i>Cinnamomum tamala</i>	SA	IVBGE	[156]
<i>Thymbra spicata</i> oil	<i>Thymbra spicata</i>	PA	IBF, BE	[157]
Spearmint oil	-	<i>Hafnia alvei</i>	IVBGE	[158]
<i>Thymus vulgaris</i> oil	<i>Thymus vulgaris</i>	PF	IVBGE	[159]
<i>Pimpinella anisum</i> , <i>Cinnamomum zeylanicum</i> , <i>Syzygium aromaticum</i> , <i>Cuminum cyminum</i> oils	<i>Pimpinella anisum</i> , <i>Cinnamomum zeylanicum</i> , <i>Syzygium aromaticum</i> , <i>Cuminum cyminum</i>	SA, SE, EF, EC, PA, CA, KP, <i>Streptococcus pyogenes</i> , <i>Aeromonas hydrophila</i> , <i>Proteus mirabilis</i>	IBF, BE	[160]
Carvacrol, eugenol	-	<i>Pectobacterium</i>	IVBGE	[161]

Abbreviations: *Staphylococcus aureus* (SA), *Escherichia coli* (EC), *Pseudomonas aeruginosa* (PA), *Chromobacterium violaceum* (CV), *Staphylococcus epidermis* (SE), *Acinetobacter baumannii* (AB), *Streptococcus mutans* (SM), *Candida albicans* (CA), *Candida dubliniensis* (CD), *Pseudomonas putida* (PP), *Listeria monocytogenes* (LM), *Enterococcus faecalis* (EF), *Salmonella typhimurium* (ST), *Klebsiella pneumonia* (KP), *Pseudomonas fluorescens* (PF); BE = biofilm eradication, IBF = inhibition of biofilm formation, IVBGE = inhibition of virulence factor- and biofilm-related gene expression, RVF = reduction in virulence factors, VI = violacein inhibition.

3. Essential Oils as Green Biocides in Agriculture and for Pest Control

Essential oils as well as their corresponding constituents and derivatives are effectively used as antimicrobial reagents in various applications (cosmetics, pharmaceuticals, health-care, agriculture, food processing, and conservation etc.), exerting biocide activity against a wide variety of pathogenic vectors [162–165]. These are effective agents against fungi,

viruses, bacteria, and insects [166–168] given their mainly significant antioxidant properties and action through variable active mechanisms. In relation to their above-mentioned specific activity, essential oils have attracted increasing interest as innovative, environmentally friendly solutions as alternative feasible ways to better protect and preserve wood-based products used in structural applications. Such an approach is beneficial as it involves compounds derived from renewable resources, with some of essential oils exhibiting multi-task action in relation to both environmental and health safe concerns. The use of essential oils as natural pesticides represents a valid alternative, even considering their high volatility which implies a variation in biologically active compounds with the concentration level in the treated wood [169]. Table 2 exemplifies some corresponding activities as efficient biocides for different essential oils, as presented in the literature data.

Table 2. Biocide activity of some EOs in wood protection and preservation.

Vegetal Source of Essential Oils	Biocide Activity	Reference
Cinnamon, cassia	active against termites; fungicide (similar to wood tar oil)	[162]
<i>Origanum vulgare</i> (oregano); <i>Acorus calamus</i> (sweet flag); <i>Syzygium aromaticum</i> (cloves- flower buds); <i>Satureja hortensis</i> (summer savory); <i>Betula</i> tree species (birch)	fungicide: active against fungal organisms such as <i>Coniophora puteana</i> , <i>Trametes versicolor</i> mildews: <i>Aspergillus niger</i> , <i>Penicillium brevicompactum</i> (tests on wood specimens from beech tree species)	[165]
<i>Anethum graveolens</i> (dill weed); <i>Salvia rosmarinus</i> (rosemary); <i>Cymbopogon</i> species (lemongrass); <i>Geranium</i> species (geranium), <i>Melaleuca alternifolia</i> (tea tree)	efficient up to 100% as termites' repellent (testing activity against <i>Reticulitermes flavipes</i> —Kollar)	[163]
Coniferous species belonging to the family <i>Cupressaceae</i> (namely, juniper, and cypress); tea tree (<i>Melaleuca alternifolia</i>); <i>Eucalyptus</i> species from the myrtle family <i>Myrtaceae</i> ; softwood species <i>Cupressus nootkatensis</i> (yellow cedar)	fungicide, insecticide	[170–173]
<i>Cinnamomum cassia</i> (cinnamon); <i>Geranium</i> species, <i>Lavandula</i> species (lavender), <i>Origanum vulgare</i> (oregano); <i>Thymus vulgaris</i> (thyme)	fungicide	[174]
Trees and shrubs from <i>Meliaceae</i> family (mahogany family), e.g., <i>Melia azedarach</i> (white cedar)	activity against viruses and insects	[175]
Cloves—aromatic flower buds of the tree <i>Syzygium aromaticum</i> ; thyme— <i>Thymus vulgaris</i> ; mint— <i>Mentha</i> species; lemongrass— <i>Cymbopogon</i> species; cinnamon— <i>Cinnamomum cassia</i> ; rosemary— <i>Salvia rosmarinus</i> ; oregano— <i>Origanum vulgare</i>	activity against viruses and insects	[176]
Vetiver—a perennial bunchgrass (<i>Chrysopogon zizanioides</i>) patchouli—a bushy perennial herb (<i>Pogostemon cablin</i>) orange (<i>Citrus sinensis</i>)—from which oil is extracted as a by-product of orange juice production by centrifugation	active against termites (<i>Coptotermes formosanus</i>)	[177–179]
<i>Trachyspermum ammi</i> (ajowan or thymol seeds); <i>Anethum graveolens</i> (dill weed); <i>Geranium</i> species (geranium); <i>Cymbopogon</i> species (lemongrass); <i>Salvia rosmarinus</i> (rosemary); <i>Melaleuca alternifolia</i> (tea tree); <i>Thymus vulgaris</i> (thyme)	active against mildews: <i>Aspergillus niger</i> , <i>Penicillium chrysogenum</i> , <i>Trichoderma viride</i> active against decay fungi: <i>Gloeophyllum traheum</i> , <i>Postia placenta</i> , <i>Trametes versicolor</i>	[180,181]
<i>Asteraceae</i> shrub (<i>Artemisia monosperma</i>); coniferous tree Mediterranean cypress (<i>Cupressus sempervirens</i>); lemon tree (<i>Citrus limon</i>); coniferous tree from cypress family (<i>Thuja occidentalis</i>); Peruvian pepper tree (<i>Schinus molle</i>); rose geranium shrub (<i>Pelargonium graveolens</i>)	efficient fungicide activity against fungi-degrading wood	[182]

Table 2. Cont.

Vegetal Source of Essential Oils	Biocide Activity	Reference
Formosan juniper (<i>Juniperus formosana</i>)—extracts from leaves are highly efficient antifungal agents; fruits are also an effective source of compounds with fungal activity	against decay fungi	[183]
<i>Cinnamomum osmophloeum</i> Kaneh	100% antifungal efficiency (<i>Coriolus versicolor</i> and <i>Laetiporus sulphureus</i>)	[184]
Flax seeds— <i>Cinnamomum cassia</i> (cinnamon bark); <i>Citrus sinensis</i> (citrus peels); tung tree <i>Vernicia fordii</i> (tung seeds); <i>Aleurites moluccana</i> (nut of kukui tree); non-edible olive oil (called lampante olive oil; here extracted from Istrian white olives variety)	bactericide, fungicide, and insecticide (termites, nematodes)	[185–189]

Essential oils extracted from woody-type plant species, such as coniferous species belonging to the family *Cupressaceae* (namely, juniper, and cypress), tea tree (*Melaleuca alternifolia*), *Eucalyptus* species from the myrtle family *Myrtaceae*, or softwood species *Cupressus nootkatensis* (yellow cedar) presented efficient fungicide and insecticide activity in experimental trials [170–173]. Active compounds from essential oils derived from thyme, *Geranium* species, *Lavandula* species (lavender), cinnamon, and oregano present effective biocide action against wood decay caused by different degrading molds [182]. Compounds such as thymol, carvone or carvacrol as aldehyde- and ketone- type forms, or those with phenolic structure, as well as terpenes and their ester forms present in volatile oils, are very efficient in protecting wood surfaces towards basidiomycete-type microorganisms that cause rotting [190,191]. Other compounds presenting essential oils, including geraniol, thymol, carvone, citronellol, and borneol, to a large extent inhibit the germination and development processes for mold spores.

Antiviral and insect repellent activities were noticed for essential oils extracted from *Meliaceae* or mahogany family [175]. The same effective action as biocide agents was observed for volatile oils derived from well-known healing plants (e.g., cloves—aromatic flower buds of the tree *Syzygium aromaticum*, thyme—*Thymus vulgaris*, mint—*Mentha* species, lemongrass—*Cymbopogon* species, cinnamon—*Cinnamomum cassia*, rosemary—*Salvia rosmarinus*, oregano—*Origanum vulgare*) [176]. Essential oils from cassia and cinnamon species, as well as their correspondent extracts, exhibited specific anti-termite and antifungal activity. The same effect was noticed when using wood tar oil when applied in order to conserve wood characteristics, namely, to protect ships against rotting processes, as water repellent in roofing applications, and as antibacterial agents in healthcare applications [162].

Essential oils extracted from vetiver, a perennial bunchgrass (*Chrysopogon zizanioides*) [177], patchouli—a bushy perennial herb (*Paogostemoncablin*) [178], as well as orange oil—produced by cells within the rind of an orange fruit (*Citrus sinensis* fruit) and extracted as a by-product of orange juice production by centrifugation [179] and cinnamaldehyde compound from cinnamon oil [192] were tested and confirmed to be active agents against termites (*Coptotermes formosanus*), with various results. Essential oils can also be used as fumigants, and the effectiveness of such substances extracted from different vegetal species (e.g., dill weed *Anethum graveolens*, rosemary *Salvia rosmarinus*, lemongrass *Cymbopogon* species, geranium *Geranium* species, tea tree *Melaleuca alternifolia*) is being tested and assessed in experimental trials as repellents against subterranean termites (*Reticulitermes flavipes*—Kollar), wherein insect mortality ranged between 95 and 100% in both formulations [163].

Essential oils can be effectively applied for wood impregnation in order to inhibit mildew activity, as indicated in experiments performed on pine wood specimens that were impregnated with steam-distilled extracts derived from plants including geranium

Geranium species, lemongrass *Cymbopogon* species, rosemary, ajowan, tea tree *Melaleuca alternifolia*, dill weed, and thyme *Thymus vulgaris*. All these had positive results against *Penicillium chrysogenum*, *Trichoderma viride*, and *Aspergillus niger* microorganisms [180], using both treatment solution (when a protection up to 20 weeks was evidenced using extracts from dill weed, geranium and thyme) and fumigation (when vapors from dill weed were efficient at the same extent as solution). The same plant extracts were applied in tests for the inhibition of decay fungi such as *Trametes versicolor*, *Postia placenta*, and *Gloeophyllum traheum* [181], when the most effective was that derived from thyme.

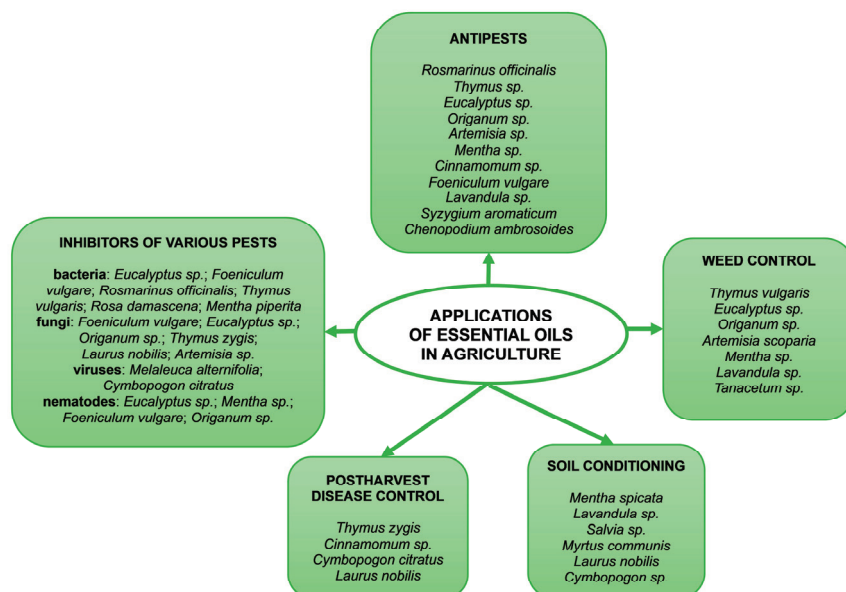
An important antifungal activity was evidenced for essential oils from clove, sweet flag, and oregano in experiments testing the protection of beech wood samples against molds and decay microorganisms (*Aspergillus niger*, *Penicillium brevicompactum*, respectively, *Coniophora puteana*, and *Trametes versicolor*), while no efficiency was noticed for oils extracted from savory and birch [165].

Another experimental study [182] proved the effectiveness of essential oils extracted from specific plants from Egypt in testing antibiofilm activity against wood rotting microorganisms, namely *Hexagoniaapiaria* and *Ganoderma lucidum*. Plant extracts containing geranium oil, eugenol, and cinnamaldehyde compounds presented significant fungicide activity when tested against biofilm formation caused by fungal microorganisms such as *Trichoderma harzianum*, *Coniophora puteana*, *Ophiostoma floccosum*, *Ophiostoma piceae*, *Oligoporus placenta*, *Androdia xanthan*, *Shaeropsis sapinea*, and *Leptographium procerum* [193].

Essential oils extracted from *Juniperus formosana* (leaf, fruits) were tested as antifungal agents against different wood degrading molds [183], with the most effective being the extract obtained from leaves which contain compounds with significant fungicide action, such as α -cadinol and elemol. The same enhanced effect against fungal biofilm formation was observed when using essential oil extracted from *Cinnamomum osmophloeum* Kaneh, which contains cinnamaldehyde as the most effective fungicide compound [184]. Significant action as antifungal active agent was proved for essential oil compounds such as eugenol and isoeugenol, as well as α -methyl cinnamaldehyde and (*E*)-2-methylcinnamic acid [194]. Considering the chemical structure of compounds that constitute the EOs, it has been proven that reagents which contain functional groups such aldehyde or carboxylic acid, a conjugated double bond, and an alkyl chain pendant to the aromatic ring are involved up to a large extent in the modulation action of EOs as effective fungicide agents. A relevant example is given by the phenylpropene-type compounds, which exhibit enhanced fungicide action (*Laetiporus betulina* and *Laetiporus sulphureus*) mainly due to their structural characteristics, namely methyl functional groups linked to the benzene rings in the *ortho* position.

Overall, essential oils extracted from almost any part of selected vegetal species, trees, or plants have proved to exhibit a variable effective activity against bacteria, fungi, and insects (termites, nematodes). Such effectiveness has been confirmed for EOs derived from flax seeds or linseed *Linum usitatissimum* [185], cinnamon bark *Cinnamomum cassia* [186], citrus peels from orange fruits *Citrus sinensis* [187], tung seeds from tung-oil tree *Vernicia fordii*, and kukui nut (*Aleurites moluccana* or candle nut species) [195], as well as for some by-products, such as lampante oil [189], which results from Istrian white olives during their processing for the production of virgin oil.

Essential oils offer also significant potential for agricultural purposes (e.g., protection of stored products, livestock, bees, and crops), since most of them are non-harmful in toxicity to the surrounding environment, including animals [196,197]. Some examples of plants with biocide activity in agriculture applications are schematically presented in Scheme 2.



Scheme 2. Some examples of plants with biocide activity in agriculture applications through essential oils from their chemical constituents (adapted from [196]).

Despite the considerable advantages of EOs, these also have some disadvantages, such as relatively slow action; reduced effectiveness with time duration; high quantities are required and applied for an increased efficacy (e.g., for weed control that may cause harmful damage to the environment and microbial communities in the soil, they can exert a negative effect on crops, and even partly their destruction). Increased volatility is another significant problem raised when using EOs, and an effective method to control this issue as well as the release properties is EO microencapsulation. This method can provide an effective production of natural biocides which successfully mimic the chemical compartmentalization in plants by protecting essential oils from degradation [198]. The microencapsulation strategy also has beneficial effects in relation to the chemical stability (e.g., oxidative stability, thermostability), shelf-life, and biological activity of EOs [198–204].

4. Essential Oils for Antimicrobial Food Packaging Systems

In recent decades, research interest has been remarkably focused on the use of natural products of natural origin (animal—chitosan, propolis; vegetal—essential oils; microbial—lysozyme, nisin, etc.) that have biocide properties in most various applications: cosmetics and pharmaceuticals, food preservation and packaging, biomedical engineering, etc. [205–207]. Among them, essential oils (EOs) showed high effectiveness against a wide range of bacteria and were significantly safer than their synthetic counterparts, which made them especially suitable for food preservation and packaging, despite their intrinsic disadvantages (complex composition that depends on many parameters—species and cultivar, cultivation area, time of harvesting, storage and transportation; they are highly volatile, liposoluble, and insoluble in water; instable upon exposure to UV-vis radiation when undergoing photochemical and oxidative degradation). Therefore, the pre-processing of EOs is required in order to limit their volatility and instability, but to maintain or increase their biologic activity, ability to interact with food, and dispersibility. Thus, the (micro/nano) encapsulation of EOs [208] or their incorporation in various blends and composite formulations, such as basil oil–chitosan–PVA [209], starch–chitosan–oregano oil [210], or chitosan–gelatin–orange peel oil [211], has been successfully employed.

The antibacterial activity of EOs is of special interest when it comes to their application in food processing and preservation. The most relevant feature in line with this is EOs' lipophilic character associated with their hydrophobicity. This allows them to adhere to the phospho-lipidic membrane of bacterial cells and alter its properties as to make it more

permeable and, subsequently, cause leakages of ions and other cytoplasmatic elements and metabolites [52]. After a certain loss of volume, bacterial cell death occurs.

Interestingly, EOs can act on a single target or multiple targets. For example, *trans*-cinnamaldehyde has showed an inhibitory effect on the growth of *Escherichia coli* and *Salmonella typhimurium* by accessing the periplasm and penetrating into cells [51]. It was demonstrated that EOs' antibacterial activity strongly depends on their composition. The highest activity was shown by EOs containing mainly aldehydes and phenols (cinnamaldehyde, citral, carvacrol, eugenol, thymol—it seems that the position of the –OH group on the benzene ring does not affect the biocide activity of phenol-containing EOs), EOs containing terpene alcohols displayed medium activity, while those containing ketones or esters proved to be much less active [212].

Another characteristic of concern is that EOs' activity against Gram-positive bacteria is higher than for Gram-negative bacteria. This is related to the EOs' chemical composition and the reduced sensitivity of Gram-negative bacteria biofilm, which explains the high effectiveness of EOs in inhibiting the growth of bacteria such as *Salmonella* spp., *E. coli*, and *Listeria monocytogenes* [52]. At the same time, combining two or more EOs may be beneficial if they act synergistically; otherwise, they can neutralize each other's effect [213]. They also interact with food preservatives and may intercede in conservation procedures. Some of the following factors have been considered as potential synergists when EOs were employed in food processing and packaging: low pH and oxygen levels (vacuum), medium temperature and high hydrostatic pressure, low water activity, chelators, etc. For example, low oxygen levels (vacuum packaging) have a limiting effect on bacteria metabolism and prevent the chemo-oxidative degradation of EOs (oregano and thyme EOs showed significantly increased activity against *S. typhimurium* and *S. aureus*, while clove and coriander showed completely biocidal activity on *A. hydrophila*) [213].

Some applications of the most well-known EOs are systematically presented in Table 3.

Table 3. Essential oils in food packaging applications.

EO/Main Active Compound/Plant	Formulation	Application	Observations	Ref.
Cinnamon (cinnamaldehyde)	sodium alginate film	ham	Cinnamon migrates through membrane faster than winter-mint.	[214]
<i>Cinnamomum cassia</i> (Chinese cinnamon)	solution	fruits and vegetables preservation	Active against <i>Stenotrophomonas maltophilia</i> and <i>Bacillus subtilis</i> , and three <i>Penicillium</i> spp., <i>S. maltophilia</i> , and <i>B. subtilis</i> on bread, carrots, potatoes, sweet potatoes, and apples (testing in situ).	[215]
Cinnaon, clove (cinnamaldehyde, eugenol)	tapioca starch-PHA film	bread	High amount of clove EO is required. Cinnamon film is bactericide; fit for baking.	[216]
Clove (eugenol)	gelatin-chitosan film	fish	Bactericide effects.	[217]
Oregano (carvacrol)	edible films in whey protein isolate formulation	wrapping for Queso Blanco cheese	The edible films strongly limited the oxidation of lipids and hampered the growth of yeasts and mildews.	[218]
Lemon (citral), lemon verbena	chitosan coating	fish (rainbow trout) packaging	Active against <i>Psychrotrophic</i> bacteria and <i>Enterobacteriaceae</i> .	[219]
Bergamot, oregano	hydroxypropyl methylcellulose films	edible coatings for fresh 'Formosa' plum	Effective for storage in low concentrations (2–5% EOs).	[220]

Table 3. Cont.

EO/Main Active Compound/Plant	Formulation	Application	Observations	Ref.
Cinnamon, mustard	zein-based coatings	preservation of cherry tomatoes	Populations of <i>S. typhimurium</i> decreased; decrease in the tensile strength of coatings; no ascorbic acid formed during storage.	[221]
Oregano	polypropylene (PP) and polyethylene terephthalate (PET) film	antimicrobial (<i>E. coli</i>) packaging films for cheese	Packaging film with oregano EO in different concentrations (0, 4, 6 and 8%) were active against two <i>Escherichia coli</i> O157:H7 strains.	[222]
Lemon	chitosan coating	strawberry—control fruit fungal decay	Chitosan–lemon oil coatings had a negative impact on fruit’s aroma profile.	[223]
Basil (eugenol)	PVA/chitosan active films	food packaging	Homogenous transparent films with strong interaction between components, UV-vis and water resistant, and radical scavenging against DPPH.	[209]
Black cumin	PET film assembled with antimicrobial chitosan and alginate coatings incorporating EO	antimicrobial packaging film for chicken meat	Active against <i>Staphylococcus aureus</i> and <i>Escherichia coli</i> .	[224]
Mint	chitosan film	food packaging	Biodegradable film incorporated EO and showed improved properties (e.g., tensile strength, opacity, water vapor barrier). Active against <i>Staphylococcus aureus</i> , <i>Salmonella typhimurium</i> , <i>Klebsiella pneumoniae</i> , <i>Bacillus subtilis</i> , and <i>Pseudomonas aeruginosa</i> .	[225]
Clove	chitosan nanoparticles	fungicide; controlled release to extend the shelf life of the fruits and vegetables in fresh state.	Highly active against <i>Aspergillus niger</i> .	[152]
Orange	chitosan/fish gelatin composite films	active preservative packaging	EO presence yielded in films with low mechanical properties (tensile strength, elastic modulus), reduced water solubility, moisture content, and water vapor permeability, but with increased elongation at break and hydrophobicity. Moreover, EO enhanced the inhibitory activity of coatings, being efficient when testing free radicals (DPPH and ABTS) and different pathogens (<i>Staphylococcus aureus</i> and <i>Escherichia coli</i>).	[211]
Pomegranate, <i>Thymus kotschyanus</i> (pulegone)	chitosan–starch composite film	packaging that may extend the shelf life of beef	Antibacterial properties against several spoilage bacteria and <i>Listeria monocytogenes</i> .	[226]
Grass (<i>Cymbopogon flexuosus</i>)	EO microemulsion	prevent the adherence of bacterial species to stainless steel and PP surfaces	Active and efficient when testing microorganisms such as <i>L. monocytogenes</i> , <i>S. aureus</i> , and <i>S. typhimurium</i> .	[227]

Table 3. Cont.

EO/Main Active Compound/Plant	Formulation	Application	Observations	Ref.
Clove (<i>Syzygium aromaticum</i> ; eugenol), cinnamon (<i>Cinnamomum zeylanicum</i> , cinnamaldehyde)	solution	biocide to <i>S. aureus</i> strains separated during milking process applied to cows presenting mastitis	Significantly limited the formation of the biofilm on various surfaces (polystyrene, stainless steel).	[141]
Litsea (<i>Litsea cubeba</i> , citral)	polyvinyl acetate (PVA) film	antimicrobial packaging material	Citral coatings have showed effectiveness as bactericide agents when testing <i>Escherichia coli</i> and <i>Staphylococcus aureus</i> and may be employed in hurdle technology applications.	[228]
Clove	composite films based on PLA/graphene oxide nanosheets and EO	food safety and preservation packaging	Graphene oxide increased the thermo-mechanical and barrier properties of the composite films, while EO improved their flexibility and bactericide effects (<i>Staphylococcus aureus</i> , <i>Escherichia coli</i>).	[229]
Black pepper, ginger	PVA/gum Arabic/chitosan composite films incorporating EO	wound bandages and preservation packaging for food	The films were considerably flexible and thermostable, resistant to break. The incorporation of EOs made films active and efficient when testing <i>Bacillus cereus</i> , <i>Staphylococcus aureus</i> , <i>Escherichia coli</i> , and <i>Salmonella typhimurium</i> .	[230]

Modern Approaches

Novel concepts for the use of EOs in food preservation and active packaging comprise the design of edible coatings that are able to preserve the original characteristics of food. These films consist of complex carbohydrates, proteins, and lipids, which provide satisfactory mechanical properties and good barrier characteristics against water and oxygen. It has been shown that the phenolic components of EOs have the highest bactericide activity, as in the case of carvacrol and thymol, which were able to destroy the outer layer of Gram-negative bacteria, or oregano and rosemary EOs, which have showed a strong antioxidative effect on lipids as demonstrated on various types of cheddar cheese (Gram-negative bacteria—*Escherichia coli*, *Salmonella choleraesuis*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*—and Gram-positive bacteria—*Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Enterococcus faecalis*) [52]. Other coating formulations include sodium caseinate, which significantly increased the barrier effect against water and acted as a matrix when cinnamon and ginger EOs were added to the formulation, thus granting their homogeneous dispersion in bulk [231].

Protection against bacteria is a constant concern along the entire chain of food processing. Therefore, understanding biofilm behavior toward various biocides may offer valuable insights into the effective control of biofilm development on food surfaces. Hence, the hurdle technology seems to be a successful alternative as it wisely combines two or more control methods, such as physical–chemical or chemical–biological, aiming at multiple bacteria targets [232].

Thus, it was reported that EOs have increased the efficiency of other disinfectants and sterilization procedures when used in various combinations: peracetic acid and EOs of *Lippia sidoides*, *Thymus vulgaris*, and *Pimenta pseudochariophyllus* [154]; EOs of *Lippia sidoides* combined with *Thymus vulgaris* or peracetic acid [233]; cold nitrogen plasma and clove oil [234] or thyme oil [235].

It is not possible to advance in active food packaging without considering the employment of nanotechnology in the elimination of pathogens along the food processing chain and maintenance of food characteristics during storage [236]. Recent studies have focused on various nano-carriers that are able to incorporate EOs and ensure their homogeneous distribution, at the same time providing good barrier properties, diffusivity, and preservation of food organoleptic features. EOs have been successfully used in such applications. Nanoemulsions were considered for fruit and vegetable coatings: oregano EO was tested on lettuce against *L. monocytogenes*, *E. coli*, and *S. Typhimurium* [237], and carvacrol and eugenol nanoemulsions were tested on spinach against *E. coli* and *S. enterica* [238]. Other films containing EO nanoemulsions have been proven to be effective against *E. coli*: an edible film based on sodium alginate and thyme EO [239], and a carnauba wax film including lemongrass EO [240]. Even EO-based nanoemulsions have been considered for combined techniques in hurdle technology [241].

Nanoliposomes were considered when nanoliposome-encapsulated thymol and carvacrol proved to be effective against *S. aureus* [242,243], and carvacrol encapsulated in nanoliposomes or in polymeric nanocapsules of Eudragit® showed biocide activity against *S. aureus*, *Listeria monocytogenes*, *E. coli*, and *Salmonella* spp. [244].

Polymeric nanoparticles and nanocapsules have been employed as well, but the nanoparticles are preferred when the active compound needs to be released at slow rates as they are adsorbed at the surface of the nanoparticles, not incorporated inside the particle as in the case of nanocapsules [245]. The following various formulations have been successfully tested on the most common bacteria: zein nanoparticles containing thymol [246], carvacrol-loaded chitosan [247], and chitosan-tripolyphosphate nanoparticles [248].

Nanofibers made of natural (proteins, lipids, carbohydrates) or synthetic polymers and containing EOs can be considered for edible coatings and in active packaging. Thus, cinnamon EO has been encapsulated in β -cyclodextrin proteoliposomes, which were subsequently incorporated into nanofibers of poly (ethylene oxide) PEO [249]. This new biocide packaging material proved to be highly effective against *B. cereus* on beef.

5. Essential Oils Applications in the Conservation of Cultural Heritage

Biodeterioration describes a complex of phenomena that occur at the interface between the micro-/macrobiota and the corresponding substrate, a system of interrelated reactions and interactions based on the metabolic necessities of the living microorganisms. It can affect different materials (organic—wood, leather, natural textiles, paper and papyrus, natural pigments and dyes; inorganic—stone, marble, metals, glass) found in a wide variety of cultural assets, such as buildings and monuments, books, old documents and historical records, paintings on different substrates, murals and cave imprints, clothing, weaponry, stained glass, etc. [14]. The preservation of the historical and cultural heritage of mankind is of the utmost importance, as it provides opportunities for the next generations to further research, study, and understand the achievements of their ancestors and honor their legacy. Moreover, any kind of artifact should be considered for restoration and conservation, and sustainable, environmentally friendly approaches to prevent, limit, or even combat biodeterioration must be taken into consideration for long-term applications [250,251].

Various methods have been employed to fight against the biodeterioration of cultural heritage, benefiting from the advances in analytical investigations. Thus, mechanical methods have shown limited effectiveness against microbial growth. However, laser techniques have yielded satisfactory results when used for surface cleaning [252]. Other physical treatments have been applied to preserve heritage sites and artifacts, such as the following:

- thermal methods have disinfectant effects [253];
- UV irradiation limited the growth of fungi, algae, and bacteria [254];
- gamma radiation granted site sterilization [255];
- atmospheric plasma torches were effective in cleaning historical stones [256].

Nevertheless, chemical and biochemical methods are the most widely used [257], and the effectiveness of biocides of various classes (quaternary ammonium salts, isothiazoles, phenols, aldehydes, etc.) has been tested [258]. Even the employment of inorganic nanoparticles has been considered, and studies have confirmed their effectiveness in preventing the biodeterioration of cultural heritage [259–262].

Despite the advances in the aforementioned methods, a new sustainable, environmentally friendly approach has attracted a lot of interest in recent decades, namely the use of natural biocides. Among them, essential oils (EOs) have proven to be effective biocides for the recovery and conservation of heritage objects, monuments, and artifacts, even though their activity has mainly been tested in the laboratory and for short time intervals.

One major concern derives from their complex composition, as they contain numerous organic molecules aside the main active component (see Figure 1). Therefore, the possible long-term interactions of other compounds in extracts with the heritage substrates must be assessed and addressed.

Another issue of interest is the photochemical stability of EOs and their activity after long-term service under photo-oxidative stress [263]. Temperature, UV-vis radiation, and the presence of oxygen are the main factors affecting their stability, since EOs are prone to degradative processes which result in the loss of biologic activity. It has been demonstrated that some EOs, namely eugenol and thymol, have maintained their biocide activity after exposure to UV-vis radiation under controlled conditions, while cinnamaldehyde showed a significant decrease in its inhibitory activity after UV irradiation [2].

Many EOs are active biocides against fungi, bacteria, and insects, and some of them are highly selective. For example, EOs from *Thymus* and *Mentha* showed an antifungal activity higher than a commercial fungicide selected as reference [264]; carvacrol specifically inhibited *Staphylococcus aureus* and *Staphylococcus epidermidis* [265]; most Lamiaceae-derived EOs are highly effective fungicides [266]; EOs from *Thymus vulgaris* and *Pelargonium graveolens* have limited the growth of *Aspergillus niger*, *Penicillium chrysogenum*, and *Trichoderma viride*, so that they can be used to prevent the mold colonization of wooden artifacts [181].

By applying the principle of synergy, mixtures of EOs have been designed, with consideration of their chemical composition, and tested. Mixtures of EOs from thyme and oregano are the most effective biocides, given their similarity in composition (they both contain terpenes) [267]. EOs from *Citrus aurantium* L. var. *amara* and *Cinnamomum zeylanicum* (Zege emulsion) were effective against mold colonies in paintings [268]. A complex biofilm made of cyanobacteria, chlorophyte, and green algae (*Chlorella*) that colonizes stones surface has been treated with EOs from various plants (basil, cloves, eucalyptus, thyme, pine tree, and tea tree), and the results were compared with those of a commercially available biocide, namely Preventol® RI50 (ammonium quaternary salts). The EO mixtures showed a higher biocide activity than the individual EOs due to their synergic action [269]. A strong antifungal effect has been confirmed for mixed EOs from oregano, lemongrass, and peppermint (mixing ratio = 1:1:1) when tested on historical papers [270]. However, the interactions of EOs in mixtures have to be further investigated because, at this moment, it is difficult to anticipate their overall biocide activity in order to design a specific formulation for an intended application and expect it to act satisfactorily.

Some typical applications of EOs in the conservation of cultural heritage are summarized in Table 4.

Table 4. Some applications of EOs in restoration and preservation of cultural heritage.

Application	EOs/Plant	Observations	Reference
Cement grit, ceramic, marble	EO mainly composed of phenolic monoterpene carvacrol, extracted from <i>Thymus capitata</i> (Malta)	Effective against green algae and cyanobacteria.	[271]
Natural cultural heritage sites: caves	EOs of two <i>Origanum vulgare</i> plants (wild and R)	Active against fungi (phylum <i>Ascomycota</i>): <i>Sordariomycetes</i> (11 isolates), <i>Eurotiomycetes</i> (7 isolates), and <i>Dothideomycetes</i> (1 isolate).	[272]
Limestone and granite blocks from Royal Tombs in Tanis, Egypt	Various EOs (fennel, lemon, marjoram, rosemary, and spearmint)	Active against yeast colonies (<i>Candida albicans</i> , <i>C. lipolytica</i> , <i>Lodderomyces elongisporus</i> , and <i>Saccharomyces cerevisiae</i>).	[273]
Mosaic tesserae (“Casa di Leda”, Greco–Roman site, Solunto, Italy)	EOs from <i>Thymus vulgaris</i> and <i>Origanum vulgare</i>	Effective against bacteria (<i>Bacillus</i>), fungi (<i>Alternaria</i> , <i>Aspergillus</i>), cyanobacteria (<i>Chroococcus</i>), and green algae (<i>Chlorella</i>).	[274]
Outdoors stone from heritage sites in South Korea (Royal Tombs of the Joseon Dynasty) and Laos (Vat Phou temple)	Eugenol isolated from volatile clove extracts	Great antifungal activity.	[275]
Wooden artworks	EOs from <i>Origanum vulgare</i> or <i>Thymus vulgaris</i>	Fungal colonization (<i>Aspergillus flavus</i>) or insect infestation (<i>Anobium punctatum</i>).	[251]
religious artifacts made of wood and stone	EOs from cinnamon bark and leaves of the genus <i>Cinnamom</i>	Phototrophs (<i>Chlorella</i> sp., <i>Chroococcus</i> sp.); microfungus (<i>Torula</i> sp.); brown rot fungus (<i>Coniophora puteana</i>).	[276]
Marble statue restoration (statue of Silvanus, National Archeological Museum of Florence)	EOs (carvacrol, eugenol, cinnamaldehyde and thymol) of <i>Coridothymus capitatus</i> L., <i>Syzygium aromaticum</i> (L.) Merr. and L.M. Perry, <i>Cinnamomum zeylanicum</i> Blume, and <i>Origanum vulgare</i> subsp. <i>Hirtum</i> Link Ietsw	Tested on cyanobacteria, fungi, and lichens.	[277]
Tholu Bommalu typical Indian leather puppets (International Puppets Museum “Antonio Pasqualino”, Palermo, Italy)	EOs with complex composition extracted from <i>Thymus vulgaris</i> and <i>Crithmum maritimum</i>	Excellent inhibitory activity against isolated bacteria (<i>Bacillus</i> , <i>Georgenia</i> , <i>Streptomyces</i> , <i>Ornithinibacillus</i>).	[278]
Cleaning and restoration of artworks	Free EOs, namely <i>Lavandula luisieri</i> and <i>Calamintha nepeta</i> , encapsulated by co-precipitation with β -cyclodextrin	High activity against <i>Aspergillus niger</i> , <i>Cladosporium</i> spp., <i>Penicillium</i> spp., <i>Rhodotorula</i> sp. and <i>Arthrobacter</i> sp. strains, and <i>Aspergillus</i> spp., <i>Epicoccum nigrum</i> , <i>Fusarium</i> spp.	[279]
Preservation of waterlogged archeological wood	EOs from cinnamon bark, wild thyme, and common thyme	Effective against cellulolytic fungal strains: <i>Chaetomium</i> sp., <i>Fusarium</i> sp., <i>Aspergillus japonicus</i> , and <i>Stachybotrys chartarum</i> .	[280]

Table 4. Cont.

Application	EOs/Plant	Observations	Reference
Documents on different supports	EOs of the plants <i>Pimpinella anisum</i> L. (anise), <i>Syzygium aromaticum</i> L. (clove), <i>Cuminum cyminum</i> L. (cumin), <i>Allium sativum</i> L. (garlic), <i>Laurus nobilis</i> L. (laurel), <i>Citrus sinensis</i> (L.) Osbeck (orange sweet), and <i>Origanum vulgare</i> L. (oregano)	Clove, garlic, and oregano oils showed the highest antibacterial activity against <i>Enterobacter agglomerans</i> and <i>Streptomyces</i> sp. Clove and oregano oils were effective against <i>Bacillus</i> sp. EOs of sweet orange and laurel had a negative activity against fungi, while anise and clove were highly effective against fungi. Isolated bacterial strains: <i>Bacillus polymyxa</i> , <i>Bacillus cereus</i> , <i>Bacillus thuringiensis</i> , <i>Bacillus</i> sp., <i>Enterobacter agglomerans</i> , and <i>Streptomyces</i> sp. The fungal strains: <i>Aspergillus niger</i> , <i>Aspergillus clavatus</i> , <i>Penicillium</i> sp., and <i>Fusarium</i> sp.	[281]
Documentary heritage (photographic papers, glass slides, books, and a map made on paper)	Two essential oils of <i>Origanum vulgare</i> L. and <i>Thymus vulgaris</i> L.	Antifungal activity against <i>Scopulariopsis</i> sp. and <i>Fusarium</i> sp. isolated from documentary heritage.	[282]
Historical art craft materials (fibers, woods, dyes, stone)	EO of the wild <i>Thymus capitatus</i> (L.) Hofmegg. and Lk. (northern Sicily)	High antimicrobial activity of the oil detected against <i>Bacillus subtilis</i> , <i>Fusarium oxysporum</i> , and <i>Aspergillus niger</i> .	[283]
Artworks	EOs from tea tree, <i>Calamintha nepeta</i> , and <i>Allium sativum</i> L. extracts	Effective against <i>Bacillus subtilis</i> , <i>Micrococcus luteus</i> , <i>Penicillium chrysogenum</i> , and <i>Aspergillus</i> spp.	[250]
Mural paintings (15th–16th century fresco wall paintings from granite churches located in northern Portugal)	EOs from <i>Rosmarinus officinalis</i> , <i>Foeniculum vulgare</i> , <i>Citrus lemmon</i> , <i>Ocimum basilicum</i> , and <i>Salvia officinalis</i>	Isolated fungal strains: <i>Alternaria alternata</i> , <i>Alternaria tenuissima</i> , TZ _{10.2.2} (possibly <i>Pestalotia</i> sp.) and TZ _{8.2.2} (possibly <i>Penicillium</i> sp.). Basil EO was the only one able to inhibit the growth of all fungal isolates.	[284]

6. Concluding Remarks and Perspectives

The range of various applications where EOs are employed as alternative, green, wide-spectrum biocides (medicine and pharmacy, wood protection, pest control, active food packaging, preservation of cultural heritage) has substantiated the importance of these natural compounds, both theoretically and practically. EOs have the ability to be used in various forms, formulations, and procedures, in indoor and outdoor conditions, and on different (natural and synthetic, organic and inorganic, raw or treated, contemporary or old/ancient) substrates. This behavior has enabled their employment not only against microbiota by the means of biofilms, with the relevance of the biofilm-mediated specific interactions between the microbiota and EOs as biocides has been emphasized in this review, but also against fungi and insects. Some limitations have also been critically reviewed, namely their specificity (EOs activity against Gram-positive bacteria is higher than that of Gram-negative bacteria), photochemical stability, pH, and temperature sensitivity.

Nevertheless, modern approaches have already emerged. The use of combined techniques (chemical, physical, biological) and/or active compounds, employment of mixtures of synthetic and natural biocides working in synergy, multitasking solutions (inhibition and removal of mixed species biofilm), and the incorporation of EOs in nanostructured support materials are only a few of the novel directions in research.

A better understanding of the phenomena involved in the essential processes of biofilm formation (e.g., occurrence, growth, and maturation) will make it easier to identify new solutions and design appropriate materials. On the other hand, the constant concern over the side effects of the EOs (toxicity, leakage, odor release, overdosage) may lead to

materials with improved characteristics and a lower content in EOs, without limiting their biocide activity. In this context, standardized methods to evaluate disinfection efficiency are highly required.

Nanotechnology employment in EO applications is highly relevant since it allows for the efficient transport of EOs to the sites of action, and their controlled release. Still, the information on this subject is scarce and in-depth studies are necessary. For example, the residual effect generated by nano-encapsulated EOs after the extinction of bacteria, and the effects of the remaining nanocarriers from cosmetic products or implantable devices, need to be further investigated.

However, EOs and their applications represent a very active field of research, with promising prospects. The ongoing research in this field will provide information that will enable scientists and engineers to expand the list of EOs and their range of applications. A close collaboration between research groups and industrial facilities will accelerate technology transfer, thereby reducing the time from the laboratory design stage to end-user application.

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