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In Vitro Evaluation of the Bioactive Potential of Commercial Pepper Essential Oils

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Abstract

This study analyzed five essential oils derived from plants that, despite sharing the common "pepper", belong to distinct genera and botanical families, which are increasingly recognized for their multifunctional bioactivities, including antioxidant, neuroprotective, and antimicrobial properties. In particular, five commercially available essential oils obtained from *Pimenta dioica*, *Piper nigrum*, *Schinus molle*, *Schinus terebinthifolia*, and *Zanthoxylum armatum* were chemically characterized and systematically evaluated for their biological potential. Gas chromatography–mass spectrometry analysis revealed distinct phytochemical profiles dominated by phenylpropanoids, monoterpenes, or oxygenated monoterpenes, which were further discriminated by multivariate statistical analysis. The essential oils were assessed in vitro for antioxidant capacity (DPPH and TEAC assays), anti-arthritic activity (protein denaturation inhibition), neuroprotective effects (acetylcholinesterase, butyrylcholinesterase, and tyrosinase inhibition), and antibiofilm activity against clinically relevant Gram-positive and Gram-negative bacteria. All oils exhibited measurable antioxidant and enzyme inhibitory activities, with *P. dioica* and *P. nigrum* showing the most balanced redox and neuroprotective profiles. Significant antibiofilm effects were observed during biofilm formation, while mature biofilms displayed strain- and oil-dependent susceptibility, highlighting differences between biomass reduction and metabolic inhibition. Overall, the results demonstrate that pepper-derived essential oils possess complementary and multi-target bioactivities strongly linked to their chemical composition, supporting their potential application as natural agents in food, pharmaceutical, and biomedical fields.

Keywords: pepper; essential oil; composition; antibiofilm; neuroprotective; antioxidant; multivariate analysis

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1. Introduction

Essential oils are complex mixtures of volatile secondary metabolites extracted from various parts of aromatic plants [1]. Their chemical composition, which determines specific biological activities, is influenced by plant species, genotype, geographical origin, and extraction conditions [2]. Essential oils demonstrate a broad spectrum of biological properties, including antioxidant, anti-inflammatory, neuroprotective, and antimicrobial activities, with significant relevance in agri-food, environmental, and clinical-therapeutic

contexts. Their antioxidant capacity is highly relevant, as oxidative stress contributes to the pathogenesis of several diseases, such as cardiovascular, neurodegenerative, and inflammatory disorders [3]. Their anti-arthritic activity is also particularly significant, as arthritis is one of the most prevalent inflammatory diseases characterized by protein denaturation and inflammatory processes [4,5]. In vitro assays can effectively screen compounds for anti-arthritic and anti-inflammatory potential. Essential oils are also recognized for their neuroprotective effects through the inhibition of the enzymes involved in neurodegenerative diseases. These pathologies are associated with neurotransmitter deficiencies and enzyme dysfunction, and the inhibition of key enzymes such as acetylcholinesterase and butyrylcholinesterase is a well-established pharmacological strategy to increase brain acetylcholine levels and improve cognitive function [6]. Anti-tyrosinase activity is also relevant, as tyrosinase is involved in neurodegenerative processes [7,8]. From a microbiological perspective, essential oils are potent biofilm-inhibitory agents, which is critical given the role of bacterial biofilms in chronic infections and their contribution to reduced antibiotic efficacy, with resistance up to 1000 times greater than that of planktonic cells [9].

This study analyzed five essential oils derived from plants that, despite sharing the common "pepper", belong to distinct genera and botanical families. This distinction is crucial because it determines distinct phytochemical profiles and, consequently, biological activities. The plant species investigated included *Pimenta dioica* (L.) Merr. (Myrtaceae) *Piper nigrum* L. (Piperaceae), *Schinus molle* L. (Anacardiaceae), *Schinus terebinthifolia* Raddi (Acacardiaceae), and *Zanthoxylum armatum* DC. (Rutaceae).

P. dioica (allspice, Jamaica pepper) possesses a broad profile of biological activities, supporting its folk use for antioxidant and antimicrobial applications [10]. The essential oil of *P. nigrum* (black pepper) is recognized for its antimicrobial, antioxidant, anti-inflammatory, and antibiofilm properties [11]. The essential oil of *S. molle* (false pepper) is traditionally used for its medicinal properties, including antibacterial, antioxidant, and anti-inflammatory activity [12,13], and has demonstrated efficacy against several Gram-positive and Gram-negative bacteria [8]. The essential oil of *S. terebinthifolia* (Brazilian pink pepper) shows significant antimicrobial and antioxidant activities [14]. Previous studies have also highlighted its anti-inflammatory and anti-rheumatic potential, indicating a possible role in arthritic conditions [15]. Its antibacterial efficacy has been demonstrated against multidrug-resistant strains of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* [16]. The essential oil of *Z. armatum* (Sichuan Green pepper) demonstrates significantly important antioxidant and antimicrobial properties [17]. Recent research has also suggested its anti-inflammatory potential and activity against bacterial pathogens, making it an attractive candidate for antibiofilm studies [18,19].

The objective of this study was to characterize in detail the chemical composition of the essential oils of *P. nigrum*, *S. terebinthifolia*, *Z. armatum*, *S. molle*, and *P. dioica*, and to systematically evaluate their antioxidant, in vitro anti-arthritic, neuroprotective (AChE, BChE, and tyrosinase inhibition), and antibiofilm potential on a panel of clinically relevant bacterial pathogens. Although the study is primarily based on in vitro assays, which, while providing valuable insights, may not fully replicate in vivo conditions, its findings could support the development of new therapeutic and food strategies based on natural products.

2. Results and Discussion

This study systematically evaluated the biological potential of five essential oils (EOs) from pepper-related species: *Pimenta dioica*, *Piper nigrum*, *Schinus terebinthifolia*, *Schinus molle*, and *Zanthoxylum armatum*. The assessment characterized their chemical profiles as well as their antioxidant, anti-arthritic, neuroprotective, and antibiofilm activities. The results demonstrated distinct activity patterns among the oils, corresponding to their unique

phytochemical compositions. These findings are examined in the context of potential therapeutic applications and the underlying mechanisms of action, with particular attention to the structure-activity relationships that influence their biological effects.

2.1. Chemical Composition and Multivariate Analysis

Table 1 reports the percent composition of the five EOs.

Table 1. Percent composition of the EOs of *P. dioica* (PD), *P. nigrum* (PN), *S. molle* (SM), *S. terebinthifolius* (ST) and *Z. armatum* (ZA).

		Amount (%)					RI [a]	RI [b]	Identification [c]
RT		**PD**	**PN**	**SM**	**ST**	**ZA**			
12.29	Tricyclene	-	-	0.75	-	-	921	922	1,2
12.86	α-Thujene	-	1.38	0.68	0.37	-	929	928	1,2,3
13.33	α-Pinene	0.08	12.47	7.11	16.56	0.19	935	933	1,2,3
14.11	Camphene	0.03	0.25	4.58	-	-	946	944	1,2,3
16.08	Sabinene	-	20.23	12.46	1.86	0.77	973	973	1,2,3
17.57	β-Myrcene	-	1.52	15.35	16.38	2.38	993	995	1,2,3
18.37	α-Phellandrene	0.07	1.09	18.65	15.98	0.15	1004	1007	1,2,3
18.78	Cyclofenchene	-	6.96	-	0.33	-	1009	886	1,2
19.14	α-Terpinene	-	-	0.15	7.72	-	1014	1014	1,2,3
19.84	*p*-Cymene	0.08	0.57	9.18	13.06	-	1024	1022	1,2,3
20.16	D-Limonene	-	17.12	16.94	0.51	-	1028	1020	1,2,3
20.21	*p*-Mentha-1(7).8-diene	-	-	-	-	24.5	1029	1018	1,2
22.25	γ-Terpinene	-	-	0.13	0.97	0.30	1057	1053	1,2,3
24.36	Terpinolene	-	0.37	-	0.21	0.29	1086	1084	1,2
25.52	Linalool	0.19	0.31	-	0.31	52.64	1102	1100	1,2,3
27.37	Octanoic acid. methyl ester	-	-	0.49	-	-	1127	1128	1,2
29.65	α-Phellandren-8-ol	-	-	-	0.84	-	1159	1166	1,2
30.76	Terpinen-4-ol	-	0.35	0.42	0.33	1.08	1175	1160	1,2,3
31.32	*p*-Cymen-8-ol	-	-	-	0.95	-	1183	1183	1,2,3
31.82	α-Terpineol	-	-	-	-	0.24	1190	1198	1,2,3
32.55	*cis*-Sabinol	-	-	0.30	0.27	-	1200	1148	1,2
32.87	Eucarvone	-	-	-	0.2	-	1205	1222	1,2
36.08	Piperitone	-	-	-	-	0.18	1252	1255	1,2
36.49	*cis*-Geraniol	-	-	-	-	0.13	1258	1255	1,2,3
37.37	Phellandral	-	-	-	0.54	0.18	1271	1271	1,2
38.24	Bornyl acetate	-	-	0.67	-	-	1284	1280	1,2,3
38.37	Safrole	0.11	-	-	-	-	1285	1287	1,2,3
39.82	Carvacrol	-	-	-	0.31	-	1307	1300	1,2,3
41.68	δ-EIemene	-	2.00	-	-	-	1336	1340	1,2
43.03	Citronellyl acetate	-	-	-	0.61	-	1357	1352	1,2
44.00	Copaene	-	3.92	-	1.23	-	1372	1376	1,2
44.01	Eugenol	85.26	-	-	-	-	1372	1374	1,2,3
44.95	Methyl cinnamate	-	-	-	-	16.05	1387	1380	1,2
44.98	β-Cubebene	-	0.30	-	-	-	1387	1389	1,2

Table 1. *Cont.*

		Amount (%)					RI [a]	RI [b]	Identification [c]
RT		**PD**	**PN**	**SM**	**ST**	**ZA**			
45.16	β-Elemene	-	0.33	-	-	-	1390	1387	1,2,3
46.66	Caryophyllene	9.50	24.53	2.10	0.26	0.53	1414	1414	1,2,3
48.74	Humulene	2.58	1.21	-	7.66	-	1448	1447	1,2,3
50.47	γ-Amorphene	-	0.27	-	-	-	1477	1483	1,2
50.59	Germacrene D	-	-	-	0.68	-	1478	1482	1,2,3
51.42	Bicyclogermacrene	-	-	1.87	0.28	-	1492	1494	1,2,3
51.75	α-Muurolene	-	0.52	0.86	0.25	-	1498	1499	1,2,3
52.40	β-Bisabolene	-	1.48	-	-	-	1509	1509	1,2,3
52.51	γ-Cadinene	-	-	0.82	1.05	-	1511	1512	1,2,3
53.15	Cadina-1(10).4-diene	-	1.21	3.45	0.98	-	1522	1524	1,2
54.69	Elemol	-	-	-	0.24	-	1548	1547	1,2
56.11	Spathulenol	-	-	0.54	-	-	1573	1575	1,2
56.39	Caryophyllene oxide	1.63	1.60	-	0.38	-	1578	1584	1,2,3
60.41	15-Hydroxy-α-muurolene	-	-	-	0.38	-	1650	1645	1,2
66.33	Benzyl Benzoate	0.46	-	-	-	-	1762	1762	1,2
75.86	Camphorene	-	-	1.77	-	-	1947	1944	1,2
	Total								
	Monoterpene hydrocarbons	0.26	62.31	85.98	73.95	28.58			
	Oxygenated monoterpenes	0.19	0.31	1.39	4.36	54.45			
	Sesquiterpene hydrocarbons	12.08	35.77	9.1	12.1	0.53			
	Oxygenated sesquiterpenes	1.63	1.6	-	1.29	-			
	Phenylpropanoids	85.26	-	-	-	16.05			
	Others	0.57	-	2.26	-	-			
	N° of compounds identified	11	23	22	31	16			

[a] The Kovats retention indices determined relative to a series of *n*-alkanes (C_{10}–C_{35}) on the apolar DB-5; [b] Kovats index for each compound; [c] Identification method: 1 = comparison of the Kovats retention indices with published data, 2 = comparison of mass spectra with those listed in the NIST 02 and Wiley 275 libraries and with published data, and 3 = identification confirmed by matching the fragmentation pattern of the sample with that of the corresponding authentic standard analyzed under the same conditions; - = absent. RT: Retention Time.

P. dioica EO was characterized by a predominance of phenylpropanoids, particularly a high concentration of eugenol (85.26%). The profile of *P. dioica* was consistent with the literature, which described eugenol content ranging from 65.82% to 74.71% [20]. *P. nigrum* EO primarily contains monoterpene hydrocarbons (62.31%) and sesquiterpene hydrocarbons (35.77%), with sabinene (20.23%), D-limonene (17.12%), and caryophyllene (24.53%) as major constituents. Also in literature reports, *P. nigrum* EOs were characterized by β-caryophyllene as the main component (23.49%), followed by limonene (18.68%), 3-carene (22.2%), and α-pinene (4.03%) [21]. Similarly, Bagheri et al. [22] reported comparable levels of β-caryophyllene (18.64%), limonene (14.95%) and sabinene (13.19%).

S. molle EO is dominated by monoterpene hydrocarbons, especially α-phellandrene (18.65%), D-limonene (16.94%), β-myrcene (15.35%), and sabinene (12.46%). Also, *S. terebinthifolia* EO is rich in monoterpene hydrocarbons (73.95%), especially α-pinene (16.56%), β-myrcene (16.38%), α-phellandrene (15.98%), and p-cymene (13.06%). The profile of *S. molle* EO was partially consistent with the findings of Díaz et al. [23], who reported a monoterpene-rich oil (58.9%), but with a more substantial sesquiterpene fraction (29.9%).

Also, the *S. tereninthifolia* EO was consistent with studies on fruit-derived EOs, as reported by da Silva Dannenberg et al. [24], who identified limonene (24%), α-phellandrene (21.1%) and α-pinene (16.9%) as the predominant components.

Z. armatum EO exhibits elevated levels of oxygenated monoterpenes, particularly linalool (53.64%), and a significant amount of methyl cinnamate (16.05%). This linalool predominance aligns with Guleria et al. [25], who identified linalool as the primary component at 30.58%

The PCA (Principal Component Analysis) and the hierarchical clustering heatmap together offer a comprehensive understanding of the chemical relationships among the pepper species (Table 2, Figures 1 and 2). Both analyses were conducted considering 25 main components as variables (linalool, methyl cinnamate, *p*-Mentha-1(7),8-diene, α-pinene, terpinen-4-ol, α-terpinene, *p*-cymene, γ-cadinene, β-myrcene, α-phellandrene, α-terpinene, bicyclogermacrene, camphene, camphor, cadina-1(10),4-diene, D-limonene, sabinene, caryophyllene oxide, eugenol, caryophyllene, cyclofenchene, β-elemene, δ-elemene, α-thujene, and copaene) and the five different essential oils as observations. Both analyses emphasize the contribution of individual variables (chemical compounds) to the primary components and how these contributions shape the clustering of the samples based on their chemical composition.

Table 2. Loadings of the significant variables on the first three principal components from data analysis.

Variable (Compound)	PC1 Loading	PC2 Loading	PC3 Loading
Linalool	−0.1176	0.3068	−0.0908
Methyl cinnamate	0.0590	0.2502	0.1901
p-Mentha-1(7),8-diene	0.1996	0.1514	−0.2746
α-Pinene	−0.1148	0.2904	−0.1877
Terpinen-4-ol	0.3024	0.1331	0.0455
α-Terpinene	0.3001	0.1600	0.0142
p-Cymene	−0.2522	0.2121	−0.0264
γ-Cadinene	0.1670	0.0380	0.3336
β-Myrcene	0.2848	0.1343	0.1400
α-Phellandrene	−0.0358	0.2871	−0.2478
α-Terpinene	−0.0331	−0.2651	−0.2115
Bicyclogermacrene	−0.0341	−0.2651	−0.2099
Camphene	0.0187	−0.1626	−0.2776
Camphor	−0.2572	0.2081	−0.0421
Cadina-1(10),4-diene	−0.2126	0.2258	0.0657
D-Limonene	−0.0833	−0.1169	0.1861
Sabinene	−0.0331	−0.2651	−0.2115
Caryophyllene oxide	−0.2880	0.1804	−0.2252
Eugenol	0.1005	0.0308	0.4230
Caryophyllene	0.2421	0.1479	−0.2252
Cyclofenchene	−0.2572	0.2081	−0.0421
β-Elemene	0.2963	0.1299	0.1147
δ-Elemene	0.1829	0.2424	0.1962
α-Thujene	−0.2617	0.0840	−0.2697

Table 2. *Cont.*

Variable (Compound)	PC1 Loading	PC2 Loading	PC3 Loading
Copaene	0.2114	0.1387	0.0000
Eigenvalue	8.6118	8.4627	4.6706
Total variance (%)	34.44%	33.85%	18.68%

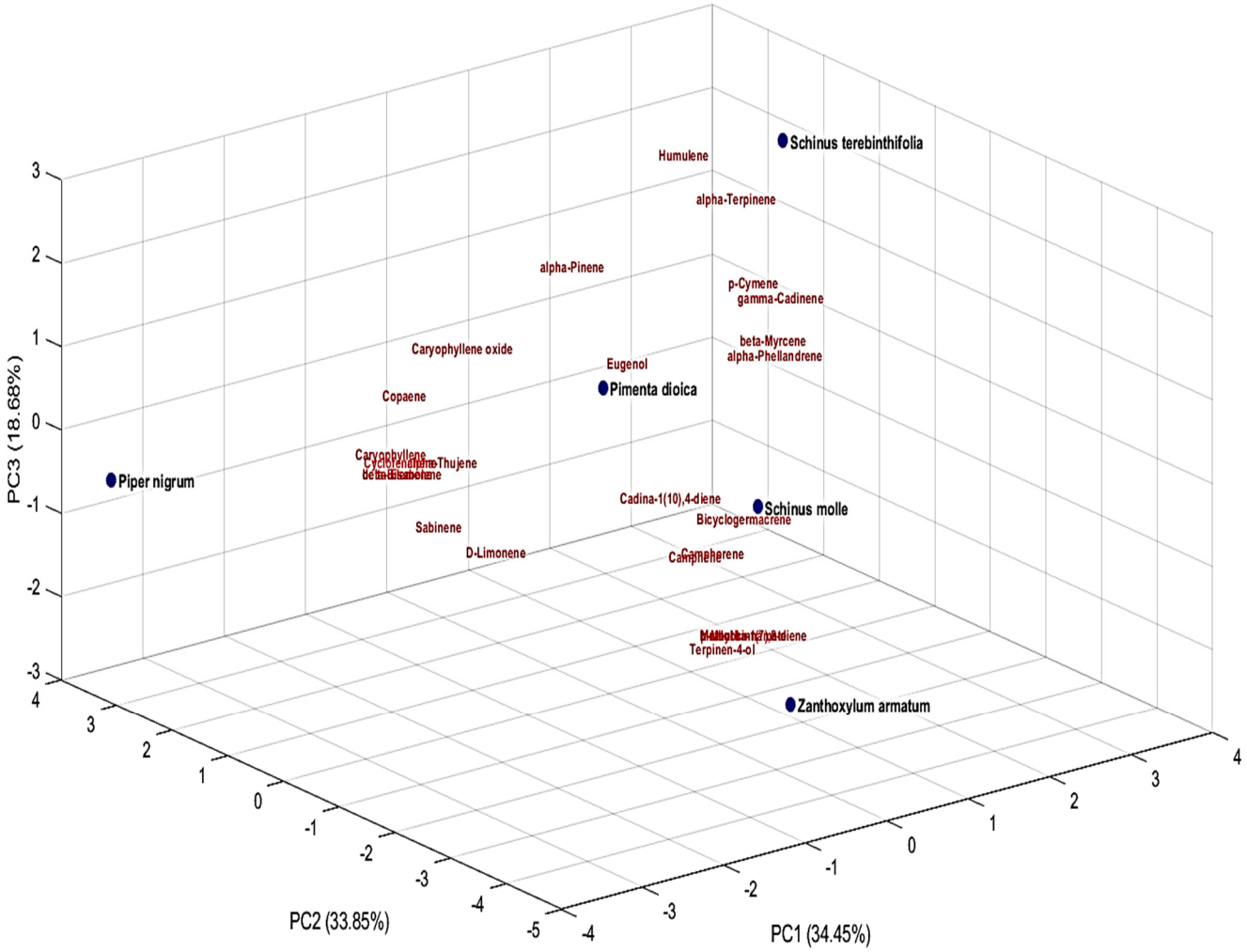

Figure 1. Biplot obtained by principal component analysis (PCA) of the five EOs based on their chemical profiles, in the three-dimensional space. The vectors shown are the eigenvectors of the covariance matrix, representing the contribution of volatile compounds, including linalool, methyl cinnamate, *p*-mentha-1(7),8-diene, α-pinene, terpinen-4-ol, α-terpinene, *p*-cymene, γ-cadinene, β-myrcene, α-phellandrene, bicyclogermacrene, camphene, camphor, cadina-1(10),4-diene, D-limonene, sabinene, caryophyllene oxide, eugenol, caryophyllene, cyclofenchene, β-elemene, δ-elemene, α-thujene, and copaene, to the first three principal components.

The three-dimensional Principal Component Analysis (PCA) biplot provides a multivariate overview of the chemical differentiation among samples and explain 86.98% of the total variance.

The Principal Component 1 (PC1), which explains 34.45% of the total variance, is primarily influenced by compounds such as α-pinene, α-terpinene, *p*-cymene, and β-myrcene, all of which contribute positively to this component. These compounds are abundant in *Schinus* EOs, which explains the clear separation of *S. molle* and *S. terebinthifolia*

along PC1. Conversely, compounds like caryophyllene oxide and eugenol contribute negatively to PC1, reflecting their higher abundance in species *P. dioica* and *Z. armatum*.

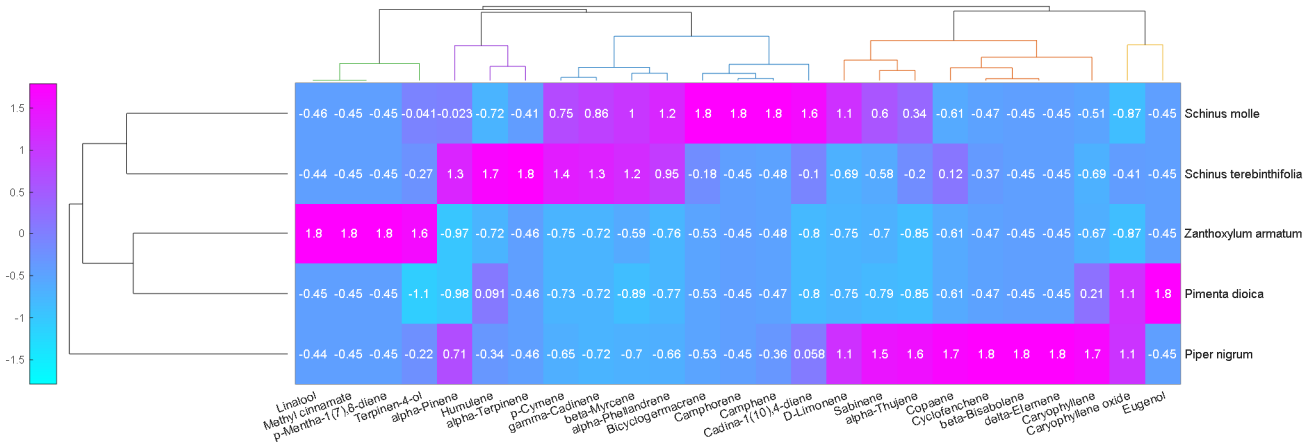

Figure 2. Heatmap showing the standardized abundances of volatile compounds across the five EOs. Each column represents a specific volatile compound, and color intensity reflects the compound abundance.

The Principal Component 2 (PC2), accounting for 33.85% of the variance, highlights the dominance of sesquiterpenes, with compounds such as caryophyllene oxide, cyclofenchene, and copane contributing positively to the component. These sesquiterpenes are notably higher in *P. nigrum*, which is distinctly separated along PC2. On the contrary, D-limonene and sabinene, which are more abundant in *Schinus* species, contribute negatively to PC2.

The Principal Component 3 (PC3, 18.88% of variance) is heavily influenced by oxygenated compounds, particularly eugenol and methyl cinnamate, both of which contribute positively to this component. Their higher concentrations in *P. dioica* and *Z. armatum* result in their separation along PC3. Meanwhile, hydrocarbons such as α-pinene and camphene contribute negatively to PC3, thus associating the *Schinus* species with the opposite end of the spectrum.

These contributions are mirrored in the hierarchical clustering heatmap, where the heatmap values indicate the standardized abundance of these compounds across species.

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

The hierarchical clustering heatmap reveals three main clusters based on chemical composition:

1. Cluster I: Comprises *S. molle* and *S. terebinthifolia*, which are characterized by high concentrations of monoterpenes like α-pinene, α-terpinene, and β-myrcene (as seen in both the dendrogram and PCA plots). This cluster is clearly separated from the others due to the abundance of these compounds, which contribute positively to PC1 and negatively to PC3.
2. Cluster II: *P. nigrum*, which is distinguished by high levels of caryophyllene and cyclofenchene. These compounds contribute positively to PC2, thus placing *P. nigrum*

apart from the other species in both the heatmap and PCA analysis. This species has a unique chemical profile dominated by sesquiterpenes.

3. Cluster III: *Z. armatum* and *P. dioica* form a separate group based on their distinctive chemical makeup, with eugenol and methyl cinnamate playing a key role in the differentiation along PC3.

The chemical compositions revealed by both PCA and the heatmap corroborate the cluster structure. The separation between *Schinus* species, *P. nigrum*, and the combination of *Z. armatum* and *P. dioica* is chemically driven, particularly by the specific volatile compounds that dominate each group. The clustering patterns reflect how these compounds, such as monoterpenes and sesquiterpenes, contribute differently to each principal component, confirming the taxonomic distinction between these species.

Thus, the PCA and hierarchical clustering analyses demonstrate that the chemical profiles of the "pepper" species are a key factor in determining their grouping. The chemical compounds contributing to each principal component (PC1, PC2, and PC3) have a clear and positive/negative impact on the clustering, directly aligning the species into distinct groups. The PCA results, with the contribution of each variable to the components, align with the clustering patterns observed in the heatmap, confirming the utility of these methods for distinguishing between species based on their volatile compound profiles.

This analysis integrates the individual contributions of each variable to the principal components and clearly links these to the clustering patterns observed in both PCA and hierarchical clustering methods. The use of compound loadings and score plots, together with the heatmap, offers a robust framework for understanding the chemical variability among the pepper species.

2.2. Biological Activity

2.2.1. Antioxidant Activity

All essential oils demonstrated measurable antioxidant capacity, with significant differences observed across assays, highlighting their potential to mitigate oxidative stress-related conditions (Table 3).

Table 3. Biochemical characterization of the EOs.

	Anti-Arthritic Activity (IC_{50}, µg)	AChE Inhibition (%)	BChE Inhibition (%)	TEAC [µM(TE)/g]	DPPH (IC_{50}, µg/mL DPPH)	Tyrosinase Inhibition (DOPA) (IC_{50} µg)
PD	$6.59^{b} \pm 1.02$	86.9^{d}	68.3^{c}	$38.29^{c} \pm 6.1$	$0.13^{a} \pm 0.02$	$2.56^{a} \pm 0.48$
PN	$8.67^{b} \pm 1.05$	65.2^{c}	57.8^{c}	$5.07^{a} \pm 0.3$	$0.42^{b} \pm 0.05$	$2.65^{a} \pm 0.63$
SM	$12.27^{d} \pm 0.95$	68.9^{c}	-	$2.79^{a} \pm 0.1$	$0.31^{b} \pm 0.08$	$2.6^{a} \pm 0.7$
ST	$4.78^{a} \pm 1.1$	69.4^{c}	99.2^{d}	$3.87^{a} \pm 1.02$	$3.74^{c} \pm 0.13$	$6.37^{b} \pm 1.02$
ZA	$3.82^{a} \pm 0.2$	33.4^{b}	-	$7.77^{a} \pm 1.58$	$5.09^{d} \pm 0.24$	-

Results are the average of three independent experiments (±SD). Data were statistically analyzed through GraphPad PRISM 10.5.0 software. Different letters over the lines indicate statistically significant differences ($p < 0.001$) according to one-way ANOVA followed by Tukey's post-hoc test. $p < 0.001$. PD: *P. dioica*; PN: *P. nigrum*; SM: *S. molle*; ST: *S. terebenthifolia*; ZA: *Z. armatum*. For the anti-arthritic activity, IC_{50} represents the concentration of sample (in µg) required to inhibit 50% of BSA denaturation; for the DPPH test, IC_{50} represents the concentration of sample (in µg) required to inhibit 1 mL of DPPH at 50%; for tyrosinase inhibition test, IC_{50} represents the concentration (in µg) required to inhibit the enzyme tyrosinase (10 U/mL) at 50%.

In both TEAC and DPPH assays, *P. dioica*, *S. molle*, and *P. nigrum* EOs exhibited the strongest radical-scavenging activity, as indicated by low IC_{50} values in the DPPH assay (0.13 µg/mL, 0.31 µg/mL, and 0.42 µg/mL, respectively). *S. terebinthifolia* and *Z. armatum* EOs showed moderate antioxidant effects, reflected by higher IC_{50} values and greater

variability. These results align with the established understanding that EOs, which are rich in phenolic compounds and terpenoids, serve as significant sources of natural antioxidants [26]. Notably, discrepancies between TEAC and DPPH assay results, particularly for certain oils, emphasize the complementary nature of these methods in evaluating antioxidant mechanisms. For example, the EO of *S. molle* exhibited the lowest TEAC value [2.79 μM(TE)/g], but demonstrated strong performance in the DPPH assay, indicating that the antioxidant compounds in *S. molle* are especially effective against the DPPH radical, although they are not efficiently detected by the TEAC method. Conversely, *S. terebinthifolia* EO displayed the opposite trend, with moderate TEAC values [3.87 μM(TE)/g] but lower DPPH activity. This variation indicates that the specific antioxidant compounds in each EO interact differently with the radical species used in each assay, reflecting differences in chemical structure and reaction kinetics [27]. *Z. armatum* EO recorded a high TEAC value [7.77 μM(TE)/g] but required a relatively large quantity in the DPPH assay. *P. nigrum* EO demonstrated intermediate TEAC activity [5.07 μM(TE)/g) but excellent DPPH performance. The EO of *P. dioica* achieved the highest performance in both assays, indicating a superior and balanced antioxidant profile, likely due to a broad array of active compounds capable of both hydrogen donation and electron transfer. These findings confirm that the two assays evaluate complementary aspects of antioxidant activity: TEAC assesses electron transfer capacity, while DPPH primarily measures free radical scavenging through hydrogen transfer [28]. The distinct chemical composition of each EO determines its reactivity in the two evaluation systems, underscoring the need to employ both methods for comprehensive antioxidant characterization. The strong antioxidant capacity observed in *P. dioica* and *P. nigrum* EOs is consistent with previous research on *Pimenta* and *Piper* species, which are recognized for their significant antioxidant properties. *P. nigrum* EO contains components such as (E)-caryophyllene, limonene, and sabinene, which contribute to its DPPH radical scavenging activity [29]. Other *Piper* species also exhibit strong antioxidant activities linked to their phenolic content and diverse chemical composition [30]. The EO of *Pimenta racemosa* var. *recemosa* (Mill.) J. W. Moore, predominated by eugenol (70.87%), β-myrcene (12.88%) and D-limonene (8.35%), exhibits an interesting antioxidant activity [31]. Similarly, *S. molle* EO has been documented for its antioxidant effects, with its composition, often rich in monoterpenes and sesquiterpenes, contributing to its radical-scavenging capabilities [32]. *S. terebinthifolia* EO also demonstrated antioxidant potential, with studies confirming its capacity in various assays [14].

The antioxidant properties of *Z. armatum* EO, like other species of this genus [33], are well established and attributed to their diverse phytochemicals, including volatile oils, flavonoids, and phenols, which enable it to act as a free radical scavenger [34]. The observed antioxidant activities are particularly relevant given their implications for microbial persistence, chronic inflammation, and neurodegenerative conditions. Oxidative stress, defined as an imbalance between reactive oxygen species and antioxidant defenses, is a common pathogenic factor [35]. It contributes to cellular damage and disease progression in various disorders, including neurodegenerative and inflammatory conditions [36]. The consistent antioxidant activity across all samples supports the presence of redox-active compounds and highlights the potential of these essential oils to modulate oxidative stress, which is relevant to microbial persistence and to chronic inflammatory and neurodegenerative conditions. Additionally, EOs, through their antioxidant properties, may mitigate oxidative stress, which is often implicated in bacterial virulence and antimicrobial resistance mechanisms [37]. Their ability to effectively scavenge free radicals and reduce oxidative burden indicates them as promising natural agents for modulating these conditions, either independently or in combination with existing therapies. The comprehensive evaluation

using both DPPH and TEAC assays provides a robust characterization of their antioxidant profiles, which is essential for understanding their potential therapeutic applications.

2.2.2. In Vitro Anti-Arthritic Activity

In vitro anti-arthritic activity, assessed by inhibition of protein denaturation, demonstrated significant variation among the tested essential oils (Table 3), with IC_{50} values ranging from 3.82 to 12.27 µg/mL. Protein denaturation initiates the production of autoantigens and immune responses that contribute to inflammation and joint damage [38]. *Z. armatum* EO exhibited the strongest activity, with the lowest IC_{50} value (3.82 ± 0.2 µg/mL).

The EO of *S. terebinthifolia* also demonstrated notable effects (IC_{50} = 4.78 ± 1.1 µg/mL), indicating substantial interference with denaturation processes involved in inflammation. *P. dioica* EO displayed moderate activity (IC_{50} = 6.59 ± 1.02 µg/mL), while *P. nigrum* EO was less effective (IC_{50} = 8.67 ± 1.05 µg/mL). *S. molle* was the least effective, requiring the highest concentration (IC_{50} = 12.27 ± 0.95 µg/mL) to achieve 50% inhibition. The capacity of these EOs to prevent protein denaturation indicates a potential protective effect against the pathogenesis of arthritis [39]. The pronounced anti-arthritic effect of *Z. armatum* EO aligns with its traditional use in folk medicine for treating inflammation. Previous studies further corroborate its anti-inflammatory properties [40]. *S. terebinthifolia* has also been documented to possess anti-inflammatory effects [41]. *P. nigrum* is also traditionally employed for the management of rheumatism and muscle pain [42]. A direct correlation between anti-arthritic activity and antioxidant capacity was not observed. Although *P. dioica* EO demonstrated superior antioxidant performance, its anti-arthritic activity remained moderate. In contrast, the EO of *Z. armatum* exhibited the strongest anti-arthritic effect despite possessing only intermediate antioxidant values. This discrepancy suggests that the mechanisms underlying protein denaturation inhibition may involve bioactive compounds distinct from, or acting synergistically with, classical antioxidant molecules. Evidence suggests that anti-denaturing activity can result from direct interactions between specific components and binding sites on proteins, particularly those rich in tyrosine, threonine, and lysine, which stabilize protein structure and prevent denaturation. These findings underscore the complex, multi-target nature of essential oils and highlight the necessity for further investigation into their phytochemical composition and molecular mechanisms. The observed anti-arthritic effects, especially from *Z. armatum* and *S. terebinthifolia*, support their potential to modulate inflammation-related pathways and justify further in vivo and clinical studies to identify novel anti-inflammatory and anti-arthritic agents [43].

2.2.3. Cholinesterase Inhibitory Activity

The cholinesterase inhibition profiles of the tested EOs demonstrated considerable variability, indicating distinct neuroprotective patterns relevant for those interested in potential approaches for managing neurodegenerative disorders such as Alzheimer's disease. Acetylcholinesterase inhibition is a primary therapeutic strategy for these conditions, designed to increase acetylcholine levels in the brain [44], while butyrylcholinesterase activity is also relevant, especially in later stages of neurodegeneration [45]. In the AChE assay, all EOs except *Z. armatum* demonstrated marked inhibitory activity at the tested concentration, with the 0.05 mM galantamine reference yielding 47.23% inhibition. Notably, *P. dioica* EO showed the highest inhibition (86.9%), followed by the EOs of *S. terebinthifolia* (69.4%), *S. molle* (68.9%), and *P. nigrum* (65.2%). This strong AChE inhibition by *Piper* species aligns with previous research that reports that various *Piper* EOs also display anticholinesterase activity [46,47]. Similarly, essential oils from other *Pimenta* species, such as *P. racemosa*, have demonstrated a significant inhibitory effect [31]. In contrast, the EO of *Z. armatum* exhibited only 33.4% inhibition, suggesting a phytochemical profile less well suited to the AChE

active site. Many essential oils are recognized for their AChE inhibitory actions, commonly attributed to their terpene constituents [48].

The inhibition of BChE resulted in a more selective inhibition among the samples. *S. terebinthifolia* EO showed the strongest BChE inhibition (99.2%), far surpassing the reference galantamine (49.54% at 0.05 mM). *P. dioica* and *P. nigrum* EOs also had measurable effects (68.3% and 57.8%), indicating moderate to good inhibition. The EOs of *Z. armatum* and *S. molle* were ineffective, showing no detectable BChE inhibition. This selectivity likely reflects differences in enzyme affinity and bioactive constituents, as the gorge structure and catalytic residues of BChE differ from those of AChE. The strong BChE inhibition exerted by *S. terebinthifolia* EO is notable, since selective BChE inhibitors are under investigation for later-stage Alzheimer's disease when AChE declines. Together, the dual cholinesterase inhibition by *S. terebinthifolia* and *P. dioica* EOs is clinically relevant in advanced neurodegeneration, where BChE rises as AChE falls, partially compensating for cholinergic deficits but also contributing to pathological acetylcholine depletion [49]. These findings support the hypothesis that pepper-derived essential oils have several neuroprotective compounds that act by complementary mechanisms, potentially enabling multitarget therapies for cognitive impairment and neurodegenerative diseases.

2.2.4. Tyrosinase Inhibitory Activity

Tyrosinase inhibition further differentiated the studied essential oils, demonstrating a range of activities relevant to hyperpigmentation disorders and neurodegenerative diseases such as Parkinson's disease [44,50]. Tyrosinase, a copper-containing enzyme, catalyzes the rate-limiting steps in melanin biosynthesis. Excessive tyrosinase activity contributes to hyperpigmentation disorders, including melasma and age spots [51]. In the context of neurodegeneration, tyrosinase-mediated oxidation of dopamine generates neurotoxic metabolites that may promote dopaminergic neuronal death [52]. In assays using DOPA as the substrate, *P. dioica*, *S. molle*, and *P. nigrum* EOs exhibited strong inhibitory activity, with notably low IC_{50} values of 2.56 ± 0.48, 2.6 ± 0.7, and 2.65 ± 0.63 µg/mL, respectively (Table 3). These values approach the potency of synthetic tyrosinase inhibitors and are substantially lower than those typically reported for plant extracts [52]. Other *Piper* species, such as *P. cubeba* L., have also demonstrated significant tyrosinase inhibitory activity [53]. In contrast, the EO of *Z. armatum* exhibited no detectable tyrosinase inhibition under the experimental conditions. This result persists despite its strong anti-arthritic and moderate antioxidant activities, indicating that the bioactive compounds responsible for these effects do not interact effectively with the tyrosinase active site. The EO of *S. terebinthifolia* demonstrated a moderate inhibitory effect, with an IC_{50} of 6.37 ± 1.02 µg/mL. The mechanisms underlying the tyrosinase inhibition by these EOs likely involve multiple modes of action, including competitive inhibition at the active site, copper chelation, and the reduction of enzymatically generated quinones via antioxidant activity. Various terpenes and phenolic compounds can contribute to these effects [54]. The pronounced tyrosinase-inhibitory activity observed in the EOs of *P. dioica*, *S. molle*, and *P. nigrum* indicates potential applications in dermatology, cosmetics, and neuroprotection. Additionally, the dual ability of certain EOs, particularly *P. dioica* and *P. nigrum*, to inhibit both cholinesterases and tyrosinase highlights their multifaceted neuroprotective potential. This supports further investigation of these EOs as multi-target therapeutic agents for cognitive impairment, oxidative stress, and neurological diseases [55]. The distinct behaviors exhibited by *S. molle* and *S. terebenthifolia* EOs are noteworthy, with *S. terebenthifolia* displaying substantially weaker tyrosinase inhibitory activity. This difference may be attributed to variations in the synergistic interactions among their components.

2.3. Antibiofilm Activity

The need to identify novel antibiofilm agents is driven by the resistance of bacterial biofilms to conventional antimicrobial therapies [56]. In this study, the antibiofilm potential of the five essential oils was evaluated, revealing varied yet promising effects against clinically relevant bacterial pathogens. Several oils exhibited broad-spectrum efficacy, while others displayed strain-specific susceptibility.

Inhibition of Biofilm Formation and Mature Biofilms

The crystal violet assay, performed after the evaluation of the minimum inhibitory concentration (Table 4), identified distinct patterns of biofilm inhibition among bacterial strains.

Table 4. Minimum inhibitory concentration of the essential oils (µL/mL).

	AB	EC	KP	LM	PA	SA
P. dioica	34.0 ^{a} ± 2.0	34.0 ^{a} ± 2.0	34.0 ^{a} ± 2.0	32.0 ± 2.0 ^{a}	32.0 ^{a} ± 2.0	32.0 ^{a} ± 2.0
P. nigrum	38.0 ^{a} ± 2.0	36.0 ^{a} ± 2.0	34.0 ^{a} ± 2.0	36.0 ^{a} ± 2.0	36.0 ^{a} ± 2.0	36.0 ^{b} ± 2.0
S. molle	38.0 ^{a} ±2.0	38.0 ^{a} ± 2.0	42.0 ^{b} ± 2.0	44.0 ^{b} ± 2.0	>50 ^{b}	>50 ^{c}
S. terebinthifolia	38.0 ^{a} ± 2.0	38.0 ^{a} ± 2.0	36.0 ^{a} ± 2.0	34.0 ^{a} ± 2.0	36.0 ^{a} ± 2.0	38.0 ^{b} ± 2.0
Z. armatum	38.0 ^{a} ± 2.0	36.0 ± 2.0 ^{a}	38.0 ^{a} ± 2.0	38.0 ^{a} ± 2.0	36.0 ^{a} ± 2.0	36.0 ^{b} ± 2.0
Tetracycline	31.0 ± 1.0	31.0 ± 1.0	30.0 ± 1.0	30.0 ± 1.0	30.0 ± 1.0	26.0 ± 1.0

Values are means ± SD of three measurements, means within each column with different letters differ significantly ($p \leq 0.05$). AB = *Acinetobacter baumannii*; EC = *Escherichia coli*; KP = *Klebsiella pneumoniae*; LM = *Listeria monocytogenes*; PA = *Pseudomonas aeruginosa*; SA = *Staphylococcus aureus*.

Results are shown in Tables 5–8.

Table 5. Effect of the essential oils on biofilm adhesion.

Adhesion Inhibition (%)	PD (µL/mL)		PN (µL/mL)		SM (µL/mL)		ST (µL/mL)		ZA (µg/mL)	
	10	20	10	20	10	20	10	20	10	20
AB	54.68 ^{c} ± 2.12	87.61 ^{d} ± 1.77	53.99 ^{c} ± 1.13	66.25 ^{c} ± 2.01	0.00 ± 0.00	28.06 ^{a} ± 0.98	13.93 ^{a} ± 0.95	63.96 ^{c} ± 1.34	70.52 ^{c} ± 1.24	71.01 ^{c} ± 2.07
EC	73.07 ^{c} ± 1.57	87.87 ^{d} ± 1.05	59.82 ^{c} ± 1.67	60.27 ^{c} ± 2.14	36.04 ^{b} ± 1.46	54.68 ^{b} ± 1.57	55.24 ^{c} ± 3.01	76.70 ^{c} ± 1.13	80.18 ^{d} ± 1.13	81.22 ^{d} ± 2.12
KP	75.01 ^{c} ± 1.33	86.36 ^{d} ± 1.12	35.9 ^{b} ± 2.04	72.73 ^{c} ± 3.22	0.00 ± 0.00	32.21 ^{b} ± 1.02	71.67 ^{c} ± 1.67	86.80 ^{d} ± 2.04	67.44 ^{c} ± 1.57	71.78 ^{c} ± 3.44
LM	68.45 ^{c} ± 2.54	81.60 ^{c} ± 1.76	51.81 ^{c} ± 3.44	72.87 ^{c} ± 3.46	0.00 ± 0.00	14.22 ^{a} ± 0.88	57.78 ^{c} ± 2.21	78.12 ^{c} ± 2.24	68.10 ^{c} ± 2.13	72.27 ^{c} ± 3.67
PA	78.02 ^{c} ± 2.57	92.43 ^{d} ± 1.54	60.35 ^{c} ± 2.67	70.97 ^{c} ± 2.96	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	72.22 ^{c} ± 1.67	73.99 ^{c} ± 3.67	77.2 ^{c} ± 2.57
SA	65.02 ^{c} ± 2.07	84.36 ^{d} ± 1.43	62.98 ^{c} ± 3.02	66.10 ^{c} ± 2.78	0.00 ± 0.00	0.00 ± 0.00	33.0 ^{b} ± 2.67	52.58 ^{c} ± 1.67	69.19 ^{c} ± 3.04	69.7 ^{c} ± 1.67

The analysis was performed by the crystal violet (CV) assay. Results were compared with the control (untreated bacteria), for which an inhibition = 0 was assumed. Results are expressed as percentages and represent the average of three independent experiments (±SD). Different letters over the lines indicate statistically significant differences ($p < 0.05$) according to ANOVA followed by Dunnett's multiple comparison test. PD = *Pimenta dioica*; PN = *P. nigrum*; SM = *Schinus molle;* ST = *Schinus terebinthifolia*; ZA = *Zanthoxylum armatum*. AB = *Acinetobacter baumannii*; EC = *Escherichia coli*; KP = *Klebsiella pneumoniae*; LM = *Listeria monocytogenes*; PA = *Pseudomonas aeruginosa*; SA = *Staphylococcus aureus*.

Table 6. Effect of the essential oils on mature biofilm.

Inhibition of Mature Biofilm (%)	PD (µL/mL)		PN (µL/mL)		SM (µL/mL)		ST (µL/mL)		ZA (µL/mL)	
	10	20	10	20	10	20	10	20	10	20
AB	0.00 ± 0.00	42.88 ^{b} ± 2.24	33.59 ^{b} ± 1.67	45.70 ^{b} ± 1.34	0.00 ± 0.00	40.13 ^{b} ± 1.34	0.00 ± 0.00	44.21 ^{b} ± 2.24	0.00 ± 0.00	37.73 ^{b} ± 1.13
EC	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	47.92 ^{b} ± 2.08	0.00 ± 0.00	13.99 ^{a} ± 0.07	0.00 ± 0.00	0.00 ± 0.00
KP	0.00 ± 0.00	37.17 ^{b} ± 1.34	13.28 ^{a} ± 0.42	16.04 ^{a} ± 0.76	8.80 ^{a} ± 0.11	36.04 ^{b} ± 1.57	29.11 ^{b} ± 1.07	34.48 ^{b} ± 1.57	18.98 ^{a} ± 0.98	19.49 ^{a} ± 1.03
LM	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.0 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
PA	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	35.02 ^{b} ± 1.34	0.00 ± 0.00	71.98 ^{c} ± 3.13	0.00 ± 0.00	54.38 ^{c} 1.34	45.95 ^{b} ± 2.13	53.70 ^{c} ± 2.67
SA	0.00 ± 0.00	0.00 ± 0.00	42.01 ^{b} ± 2.04	44.85 ^{b} ± 2.86	0.00 ± 0.00	52.20 ^{c} ± 2.24	0.00 ± 0.00	44.43 ^{b} ± 2.02	40.33 ^{b} ± 1.57	60.09 ^{c} ± 3.67

Results were compared with the control (untreated bacteria), for which an inhibition = 0 was assumed. Results are expressed as percentages and represent the average of three independent experiments ± SD. Different letters over the lines indicate statistically significant differences ($p < 0.05$) according to ANOVA followed by Dunnett's multiple comparison test. PD = *Pimenta dioica*; PN = *P. nigrum*; SM = *Schinus molle;* ST = *Schinus terebinthifolia*; ZA = *Zanthoxylum armatum*. AB = *Acinetobacter baumannii*; EC = *Escherichia coli*; KP = *Klebsiella pneumoniae*; LM = *Listeria monocytogenes*; PA = *Pseudomonas aeruginosa*; SA = *Staphylococcus aureus*.

Table 7. Effect of the essential oils on sessile cells metabolism in immature biofilm.

Inhibition of Adhesion (%)	PD (µL/mL)		PN (µL/mL)		SM (µL/mL)		ST (µL/mL)		ZA (µL/mL)	
	10	20	10	20	10	20	10	20	10	20
AB	63.97 ^{c} ± 2.33	87.37 ^{d} ± 2.24	57.26 ^{c} ± 1.35	88.49 ^{d} ± 1.88	40.15 ^{b} ± 1.12	63.97 ^{c} ± 1.97	0.00 ± 0.00	35.24 ^{b} ± 1.34	73.58 ^{c} ± 2.12	74.6 ^{c} ± 2.25
EC	81.57 ^{c} ± 1.13	88.89 ^{d} ± 2.01	26.87 ^{b} ± 0.87	68.2 ^{c} ± 2.65	26.32 ^{b} ± 2.24	50.92 ^{b} ± 2.54	0.00 ± 0.00	13.81 ^{a} ± 0.77	17.05 ^{a} ± 0.98	34.72 ^{b} ± 1.88
KP	79.29 ^{c} ± 2.01	83.1 ^{d} ± 2.06	0.00 ± 0.00	24.56 ^{a} ± 2.34	6.92 ^{a} ± 0.55	25.58 ^{b} ± 1.04	0.00 ± 0.00	0.00 ± 0.00	22.10 ^{a} ± 1.56	41.80 ^{b} ± 1.65
LM	76.72 ^{c} ± 2.22	81.19 ^{d} ± 1.34	0.00 ± 0.00	39.75 ^{b} ± 1.15	0.00 ± 0.00	32.54 ^{b} ± 2.12	0.00 ± 0.00	0.00 ± 0.00	49.71 ^{b} ± 1.45	58.60 ^{c} ± 2.56
PA	72.77 ^{c} ± 1.34	82.06 ^{d} ± 1.13	0.00 ± 0.00	56.89 ^{c} ± 2.21	0.00 ± 0.00	37.19 ^{b} ± 2.13	0.00 ± 0.00	28.00 ^{b} ± 1.44	54.69 ^{c} ± 2.01	66.1 ^{c} ± 1.44
SA	81.37 ^{d} ± 2.14	82.88 ^{d} ± 2.22	67.23 ^{c} ± 2.43	72.67 ^{c} ± 2.24	36.74 ^{b} ± 0.78	47.68 ^{b} ± 2.12	31.17 ^{b} ± 2.11	69.27 ^{c} ± 2.08	74.91 ^{c} ± 2.22	75.31 ^{c} ± 1.21

The analysis was performed by the crystal violet (CV) assay. Results, expressed as a percentage, were compared with the control (untreated bacteria), for which an inhibition = 0 was assumed. Results are expressed as percentages and represent the average of three independent experiments ± SD. Different letters over the lines indicate statistically significant differences ($p < 0.05$) according to ANOVA followed by Dunnett's multiple comparison test. PD = *Pimenta dioica*; PN = *P. nigrum*; SM = *Schinus molle;* ST = *Schinus terebinthifolia*; ZA = *Zanthoxylum armatum*. AB = *Acinetobacter baumannii*; EC = *Escherichia coli*; KP = *Klebsiella pneumoniae*; LM = *Listeria monocytogenes*; PA = *Pseudomonas aeruginosa*; SA = *Staphylococcus aureus*.

Table 8. Effect of the five essential oils on sessile cells metabolism in mature biofilm.

Inhibition of Mature Biofilm (%)	PD (µL/mL)		PN (µL/mL)		SM (µL/mL)		ST (µL/mL)		ZA (µL/mL)	
	10	20	10	20	10	20	10	20	10	20
AB	65.09 ^{c} ± 2.13	83.64 ^{d} ± 1.12	64.48 ^{c} ± 1.67	72.28 ^{c} ± 1.44	0.00 ± 0.00	63.96 ^{c} ± 2.12	31.16 ^{b} ± 1.12	66.78 ^{c} ± 2.56	55.92 ^{c} ± 3.11	57.69 ^{c} ± 2.13
EC	15.92 ^{a} ± 1.21	47.65 ^{b} ± 1.03	0.00 ± 0.00	38.54 ^{b} ± 2.10	8.57 ^{a} ± 0,67	24.95 ^{b} ± 1.67	0.00 ± 0.00	56.14 ^{c} ± 2.57	0.00 ± 0.00	0.00 ± 0.00

Table 8. *Cont.*

Inhibition of Mature Biofilm (%)	PD (µL/mL)		PN (µL/mL)		SM (µL/mL)		ST (µL/mL)		ZA (µL/mL)	
	10	20	10	20	10	20	10	20	10	20
KP	30.94 ^{b} ± 1.57	66.18 ^{c} ± 2.12	24.38 ^{a} ± 1.1)	49.70 ^{b} ± 1.67	10.39 ^{a} ± 1.03	36.06 ^{b} ± 1.17	8.74 ^{a} ± 0.66	62.64 ^{c} ± 2.33	0.00 ± 0.00	2.13 nd ± 0.02
LM	59.46 ^{c} ± 2.21	64.70 ^{c} ± 2.88	16.94 ^{a} ± 1.44	26.87 ^{b} ± 1.13	8.81 ^{a} ± 0.78	35.32 ^{b} ± 1.04	8.00 ^{a} ± 0.63	50.04 ^{c} ± 2.24	0.00 ± 0.00	1.49 nd ± 0.01
PA	65.46 ^{c} ± 2.12	69.34 ^{c} ± 2.43	23.03 ^{a} ± 1.13	30.42 ^{b} ± 2.01	00.00 ± 0.00	14.44 ^{a} ± 0.44	2.74 nd ± 0.02	52.82 ^{c} ± 2.12	0.00 ± 0.00	0.00 ± 0.00
SA	43.83 ^{b} ± 1.15	63.51 ^{c} ± 2.67	0.00 ± 0.00	50.3 ^{b} ± 0.87	23.62 ^{a} ± 1.67	42.21 ^{b} ± 1.01	10.04 ^{a} ± 0.89	73.85 ^{c} ± 3.13	0.00 ± 0.00	0.00 ± 0.00

The analysis was performed by the crystal violet (CV) assay. Results were compared with the control (untreated bacteria), for which an inhibition = 0 was assumed. Results are expressed as percentages and represent the average of three independent experiments (±SD). Different letters over the lines indicate statistically significant differences ($p < 0.05$) according to ANOVA followed by Dunnett's multiple comparison test. PD = *Pimenta dioica*; PN = *P. nigrum*; SM = *Schinus molle;* ST = *Schinus terebinthifolia*; ZA = *Zanthoxylum armatum*. AB = *Acinetobacter baumannii*; EC = *Escherichia coli*; KP = *Klebsiella pneumoniae*; LM = *Listeria monocytogenes*; PA = *Pseudomonas aeruginosa*; SA = *Staphylococcus aureus*.

2.4. Metabolic Activity of Bacterial Sessile Cells

The MTT assay measures the metabolic activity of sessile cells by quantifying the reduction of tetrazolium salt by bacterial dehydrogenases. This method provided complementary data regarding the impact of essential oils on cellular viability within biofilms. Results of the MTT test are shown in Tables 7 and 8. They corroborated the antibiofilm activity observed with crystal violet staining, demonstrating that the extracts not only reduce biofilm biomass but also can act on bacterial metabolism. *P. dioica* EO at 20 µL/mL exhibited the highest metabolic inhibition during biofilm formation, with inhibition rates of 88.89% for *E. coli*, 87.37% for *A. baumannii*, 83.14% for *K. pneumoniae*, 82.88% for *S. aureus*, 82.06% for *P. aeruginosa*, and 81.19% for *L. monocytogenes*. The EO of *P. nigrum* at 10 µL/mL showed substantial efficacy against *A. baumannii* (88.49%) and *E. coli* (68.22%). *S. terebinthifolia* oil EO at 20 µL/mL resulted in inhibition above 69% for *S. aureus* (69.27%). *Z. armatum* EO displayed dose-dependent activity, with inhibition percentages ranging from 17.05% to 75.31% across strains and concentrations (Table 7). For mature biofilms (Table 8), the EO of *P. dioica* at 20 µL/mL sustained high metabolic inhibition rates of 83.64% for *A. baumannii*, 69.34% for *P. aeruginosa*, 66.18% for *K. pneumoniae*, 64.70% for *L. monocytogenes*, and 63.51% for *S. aureus*. The EO of *S. terebinthifolia* at 20 µL/mL exhibited strong metabolic inhibition, ranging from 50.04% to 73.85%, with the highest efficacy observed against *S. aureus* (73.85%). The EOs of *P. nigrum* and *Z. armatum* generally showed markedly reduced activity, with several inhibition values near zero, particularly against *E. coli*, *K. pneumoniae*, and *L. monocytogenes*. Notably, *S. molle* EO demonstrated moderate metabolic inhibition against *A. baumannii* (63.96%) but showed limited activity against the other strains in mature biofilms. The comparison of crystal violet and MTT assay results indicated that inhibition of cellular metabolism (MTT) was, in some instances, substantially greater than the reduction in biomass (CV). This finding suggests that essential oils exert a strong, direct cytotoxic effect on sessile cells before the biofilm matrix is detached or disrupted. This mechanism is widely associated with essential oil components disrupting membrane integrity and critical cellular processes [56]. Notably, the EO of *P. nigrum* achieved 88.49% metabolic inhibition against the sessile cells within the *A. baumannii* immature biofilm, and 72.28% against the metabolism of sessile cells present in the *A. baumannii* mature biofilm. This EO also showed a significant effect (68.22%) against the metabolism of *E. coli* sessile cells.

The EO of *P. dioica* consistently exhibited the highest antibiofilm activity across all tested strains, including both Gram-negative (*P. aeruginosa*, *A. baumannii*, *E. coli*, *K. pneu-*

moniae) and Gram-positive (*S. aureus, L. monocytogenes*) strains, during both initial biofilm formation (Table 5) and in mature biofilms (Table 6). For *P. aeruginosa*, this EO at 20 µL/mL achieved 92.43% biofilm inhibition, substantially exceeding the efficacy of *Z. armatum* EO (77.22%). Comparable results were observed for *A. baumannii*, with *P. dioica* EO achieving 87.61% inhibition compared to 71.01% for *Z. armatum* EO. *E. coli* was particularly responsive, with *P. dioica* EO leading at 87.87% inhibition and *Z. armatum* EO at 81.22%. These findings are consistent with previous studies on other EOs that have demonstrated strong antibiofilm effects against Gram-negative bacteria, often attributed to disruption of cell membranes and interference with quorum sensing [57]. The EO of *S. terebinthifolia* exhibited behavior very similar to that of *P. dioica* vs. *K. pneumoniae*, achieving 86.80% inhibition vs. 86.36%, respectively. Among Gram-positive bacteria, *S. aureus* biofilms were highly susceptible to *P. dioica* EO (84.36%), which was significantly greater than the inhibition observed with *Z. armatum* EO (69.70%). *L. monocytogenes* exhibited more variable responses, with *P. dioica* EO demonstrating the strongest activity at 81.60%, while *S. terebinthifolia* and *Z. armatum* EOs showed just more moderate inhibitory effects (78.12% and 72.27%, respectively). In contrast, the EO of *S. molle* did not inhibit biofilm formation in *P. aeruginosa* and *S. aureus* but was effective against *E. coli* (54.68% of inhibition), *K. pneumoniae* (34.21% inhibition), and *L. monocytogenes* (14.22% inhibition).

Most EOs exhibited a marked reduction in antibiofilm activity against mature biofilms, likely due to structural and metabolic changes that occur during biofilm maturation (Table 6). As biofilms develop, the extracellular matrix (exosome) becomes more established and cells within the biofilm decrease metabolic activity, increasing resistance to EOs [56,58]. The EO of *S. molle* exhibited a unique and remarkable trend: although it did not inhibit initial biofilm formation in some strains, it showed the highest efficacy against mature biofilms, achieving 71.98% inhibition against *P. aeruginosa* and 52.20% against *S. aureus*. Additionally, this EO showed substantial activity against *E. coli* (47.92%) and *A. baumannii* (40.13%). These data suggest that this EO may be more effective at disrupting mature biofilms or targeting metabolic pathways in sessile cells during later stages of biofilm development [8]. The unique monoterpene blend in *S. molle* EO, particularly α-phellandrene, D-limonene, β-myrcene, and sabinene, may interact more effectively with established biofilm structures, possibly by enhancing membrane permeability in mature sessile cells. The EO of *Z. armatum essential oil* at 20 µg/mL demonstrated notable efficacy against mature biofilms of *S. aureus* (60.09% inhibition) and *P. aeruginosa* (53.70%), representing moderate reductions from initial values but maintaining significant activity. The EO of *S. terebinthifolia* at 20 µL/mL showed 54.38% inhibition against *P. aeruginosa* and 44.21% against *A. baumannii*, representing substantial reductions from initial values (72.22% and 63.96%, respectively). Furthermore, inhibition of the mature *S. aureus* biofilm was 44.43%, only marginally less effective than its effect on the immature biofilm of this strain. In contrast, *P. dioica* EO, which exhibited the highest efficacy during initial biofilm formation, showed drastically reduced activity against mature biofilms. At 20 µL/mL, this EO achieved only 42.88% inhibition against *A. baumannii* and was completely ineffective against *P. aeruginosa*, *E. coli*, *S. aureus*, and *L. monocytogenes*. Only *K. pneumoniae* retained moderate susceptibility (37.17%). This dramatic reduction suggests that the established extracellular matrix of mature biofilms may limit the penetration of this EO, or that its mechanism of action is primarily effective against actively dividing cells during biofilm formation rather than against sessile cells in mature structures. *L. monocytogenes* showed no inhibition by any extract at 24 h, indicating a pronounced ability to form resistant, mature biofilms and highlighting the robustness of *L. monocytogenes* biofilms [58].

The comparison of crystal violet and MTT assay results revealed a critical distinction between biomass reduction and bacterial metabolic inhibition, particularly evident for the

EO of *P. dioica* oil against mature biofilms. While this EO demonstrated high metabolic inhibition in mature biofilms (83.64% for *A. baumannii* and 69.34% for *P. aeruginosa*), it showed markedly reduced biomass removal (42.88% and 0.00%, respectively) in the crystal violet assay. This discrepancy indicates that the *P. dioica* EO, rich in eugenol, effectively kills or inhibits the metabolic activity of sessile cells within mature biofilms but does not efficiently disrupt or remove the established extracellular matrix. This finding suggests that essential oils exert a strong, direct cytotoxic effect on sessile cells before the biofilm matrix is detached or disrupted. This mechanism is widely associated with essential oil components disrupting membrane integrity and critical cellular processes [59,60]. Conversely, the EO of *S. molle* exhibited a reversed pattern: moderate biomass reduction in mature biofilms (71.98% against *P. aeruginosa* and 52.20% against *S. aureus* in the crystal violet assay) with lower metabolic inhibition values, suggesting that this EO may more effectively penetrate and disrupt the extracellular matrix. This differential activity profile highlights the importance of evaluating both biomass and metabolic parameters when assessing antibiofilm efficacy, as different essential oils may act through distinct mechanisms. Remarkably, the EO of *P. nigrum* achieved 88.49% metabolic inhibition against *A. baumannii* in immature biofilm, and a significant effect against *E. coli* (68.22%). Both the crystal violet and MTT assays demonstrated that the EO of *P. dioica* could be a promising candidate for antibiofilm activity during initial biofilm formation, exhibiting high and consistent efficacy against all tested bacterial strains. However, its efficacy against mature biofilms is complex: while it maintains strong metabolic inhibition of sessile cells, it shows limited capacity to remove established biofilm biomass. This suggests that this EO is most effective as a preventive agent against biofilm formation or in combination strategies targeting both cellular metabolism and matrix disruption. The distinctive activity profile of *S. molle* EO against mature biofilm biomass, despite lower metabolic inhibition, merits further investigation as a complementary approach for disrupting established biofilm structures. Table 9 show a comparative overview of the five EOs' biological activity following the study herein performed.

2.5. Chemical Composition and Biological Activities

Essential oils, containing complex mixtures of monoterpenes and sesquiterpenes, exhibit broad spectra of biological activity. These findings underscore the importance of chemical profiling for predicting potential therapeutic applications, as both individual components and their combinations may influence pharmacological properties. While the distinct biological activities of the five studied essential oils can be associated with their unique chemical compositions, it is important to note that the presence and concentration of specific compounds or compound classes are only part of the explanation for the observed effects. The roles of synergistic or antagonistic interactions among constituents, as well as the contribution of minor components, remain largely unexplored.

For example, the exceptional antibiofilm efficacy and potent antioxidant and neuroprotective activities of *P. dioica* EO may be partly influenced by its high eugenol content, a phenylpropanoid well-documented for broad-spectrum antimicrobial, antioxidant, and anti-inflammatory properties [61]. Eugenol's ability to disrupt bacterial cell membranes and interfere with cellular processes is hypothesized to contribute to its antibiofilm effects, while its neuroprotective properties include neutralizing free radicals and suppressing reactive oxygen species [62,63]. However, our data do not directly validate the contribution of eugenol alone, as the observed activities likely result from the combined action of multiple constituents within the EO.

Table 9. Comparative overview of the biological activities of the Eos.

Activity	*P. dioica*	*Z. armatum*	*S. terebinthifolia*	*S. molle*	*P. nigrum*	Overall Best Performers
Antioxidant (DPPH test)	Superior (IC_{50} = 0.13 µg/mL)	Moderate (required larger quantity)	Moderate (lower DPPH activity)	Strong (IC_{50} = 0.31 µg/mL)	Excellent (IC_{50} = 0.42 µg/mL)	*P. dioica*, *P. nigrum* *S. molle*
Antioxidant (TEAC)	Superior and balanced	High (7.77 µMTE/g)	Moderate (3.87 µMTE/g)	Lowest (2.79 µMTE/g)	Intermediate (5.07 µMTE/g)	*P. dioica* *Z. armatum*
Anti-Arthritic	Moderate (IC_{50} = 6.59 µg/mL)	Strongest (IC_{50} = 3.82 µg/mL)	Second strongest (IC_{50} = 4.78 µg/mL)	Weakest (IC_{50} = 12.27 µg/mL)	Moderate (IC_{50} = 8.67 µg/mL)	*Z. armatum*
AChE Inhibition	Highest (86.9%)	Limited (33.4%)	Substantial (69.4%)	Substantial (68.9%)	Substantial (65.2%)	*P. dioica*
BChE Inhibition	Measurable (68.3%)	Ineffective (0%)	Strongest (99.2%)	Ineffective (0%)	Measurable (57.8%)	*S. terebinthifolia*
Tyrosinase Inhibition	Strong (IC_{50} = 2.56 µg/mL)	No detectable	Moderate (IC_{50} = 6.37 µg/mL)	Strong (IC_{50} = 2.6 µg/mL)	Strong (IC_{50} = 2.65 µg/mL)	*P. dioica*, *S. molle*, *P. nigrum*
Antibiofilm (immature biofilm)	Highest efficacy across all strains (e.g., 92.43% against *P. aeruginosa*)	Moderate, dose-dependent	Good, especially against *K. pneumoniae* (86.80%)	Failed against several strains	Remarkable against *A. baumannii* (88.49%) and *E. coli* (68.22%)	*P. dioica*
Antibiofilm (mature biofilm)	High and consistent efficacy across all strains (e.g., 83.64% against *A. baumannii*)	Drastically reduced activity (0% against several)	Significantly reduced efficacy	Maintained significant efficacy against *P. aeruginosa* (71.98%) & *S. aureus* (52.20%)	Drastically reduced activity	*P. dioica*

Similarly, *Z. armatum* EO demonstrated the strongest anti-arthritic potential and moderate antioxidant activity but exhibited limited AChE inhibition and no detectable inhibition of BChE or tyrosinase. Linalool, the main component of this EO, is recognized for anti-inflammatory, antioxidant, and neuroprotective properties [64]. Its ability to decrease AChE expression and mediate anti-inflammatory mechanisms may underline the anti-arthritic effects observed [65,66]. However, as with eugenol, the specific role of linalool in the overall activity of the EO remains to be experimentally confirmed. Furthermore, the presence of methyl cinnamate in *Z. armatum* EO did not translate into tyrosinase inhibition, highlighting the complexity of predicting bioactivity based solely on composition.

P. nigrum EO exhibited strong antioxidant and antibiofilm activities, as well as moderate anti-arthritic and neuroprotective effects. Among its main constituents, sabinene and caryophyllene are known for their respective antifungal, antibacterial, antioxidant, and anti-inflammatory activities [67–69]. The combined—or potentially synergistic—effects of these and other compounds likely contribute to the broad spectrum of biological activities observed, but further studies are needed to delineate their individual contributions.

The high content of monoterpene hydrocarbons within *S. molle* and *S. terebinthifolia* EOs is thought to contribute to their antimicrobial, antioxidant, and anti-inflammatory activities [70]. Compounds such as α-pinene, α-phellandrene, D-limonene, β-myrcene, and p-cymene have all been associated with such properties [71–73]. The potent BChE inhibition observed for *S. terebinthifolia* EO and the unique efficacy of S. molle against mature biofilms may result from specific combinations of their predominant constituents, yet these attributions remain hypothetical in the absence of studies on isolated components and reconstituted mixtures.

Overall, while the unique chemical compositions of the five essential oils provide a plausible rationale for their distinct biological activities—including the high eugenol content in P. dioica EO and elevated linalool in *Z. armatum* EO—these relationships should be interpreted with caution until experimentally validated. Our findings highlight the need for additional studies to clarify the roles of individual compounds and their interactions, through testing of pure components and defined blends.

Based on a comprehensive evaluation of all tested biological activities, *P. dioica* EO emerges as a promising candidate among the five essential oils studied. It consistently demonstrated superior or highest efficacy in antibiofilm activity, both against forming and mature biofilms, and maintained a balanced and strong antioxidant profile [56]. Furthermore, it exhibited strong inhibition against both acetylcholinesterase and tyrosinase [74,75]. On the other hand, *Z. armatum* EO exhibited the strongest anti-arthritic potential, and *S. terebinthifolia* EO was particularly effective at inhibiting butyrylcholinesterase [3]. However, these observations warrant further investigation to confirm the underlying mechanisms and the specific contribution of individual constituents.

The observed biological activities of the tested essential oils are closely related to their chemical compositions. For example, the notable multi-target efficacy of *P. dioica* EO may be influenced by its high eugenol content (85.26%), a phenylpropanoid widely reported in the literature for its broad-spectrum antimicrobial, antioxidant, and anti-inflammatory properties [62]. Eugenol's potential ability to compromise bacterial cell membranes and interfere with cellular processes has been linked to antibiofilm effects, while its neuroprotective actions include neutralizing free radicals and suppressing reactive oxygen species [63]. Similarly, the strong anti-arthritic activity of *Z. armatum* could be associated with its high linalool content, known for its anti-inflammatory properties [76]. The antioxidant and antibiofilm activities of *P. nigrum* could be partly attributable to sabinene and caryophyllene, and the prominent monoterpene hydrocarbons in *S. molle* and *S. terebinthifolia* (such as α-pinene, β-myrcene, α-phellandrene, and *p*-cymene) have also been linked to diverse bio-

logical effects [73,77,78]. However, it is important to note that these attributions are based on compositional analysis and supporting literature, and the actual activities observed in essential oil mixtures may result from complex interactions (synergistic or antagonistic) among multiple components. Further studies—such as testing isolated constituents and reconstituted mixtures—are needed to experimentally validate the contribution of individual compounds to the overall biological profile. These findings highlight the critical role of detailed chemical characterization as a foundation for understanding the therapeutic potential of essential oils, while also underscoring the necessity for mechanistic and validation studies [43].

3. Materials and Methods

The experiments were performed on five essential oils, present on the market as "pink" or "black" pepper: *Pimenta dioica* (vapour-distillation lot number146790, origin Jamaica, produced and distributed by "L'Alalambicco", Rivergaro, Italy), *Piper nigrum* (vapour-distillation, lot 2023, origin Sri Lanka, distributed by "Santa Bianca SSA, Pomarance, Italy.), *Schinus molle* (vapour-distillation, lot 240,718, origin Perù, produced and distributed by "Terra Amazonas"), *Schinus terebinthifolia* (vapour-distillation, lot number B092F, origin Madagascar, produced and distributed by "L' Aromoteca", Assago, Italy), *Zanthoxylum armatum* (lot number 1125, vapour-distillation, produced in Nepal and distributed by "Santa Bianca SSA, Pomarance, Italy). Samples were stored in the dark and at room temperature until the analysis procedures.

3.1. Analysis of the Essential Oils

GC/MS analyses were performed on an Agilent 6850 Ser. II apparatus (Agilent, Roma, Italy), fitted with a fused silica DB-5 capillary column (30 m, 0.25 mm i.d., 0.33 µm film thickness, Agilent, Roma, Italy), coupled to an Agilent Mass Selective Detector MSD 5973; ionization energy voltage 70 eV; electron multiplier voltage energy 2000 V. Mass spectra were scanned in the range 40–500 amu, scan time 5 scans/s. Column temperature: 40 °C, with 5 min initial hold, and then to 270 °C at 2 °C/min, 270 °C (20 min); injection mode, splitless (1 µL of a 1:1000 *n*-hexane solution); injector and detector temperatures 250 °C and 290 °C, respectively. Helium was used as the carrier gas (1.0 mL/min). The majority of constituents were identified by comparing their Kovats retention indices (Ri), which were determined relative to the retention times (Rt) of *n*-alkanes (C10-C35), to either those in the literature [79,80] and mass spectra on the column, or those of authentic compounds available in our laboratories via the NIST 02 and Wiley 275 libraries [81]. Peak area normalization was used to get component relative concentrations. No response factors were calculated.

3.2. Evaluation of the Antioxidant Activity

The antioxidant activity was evaluated following the protocol described by Fratianni et al. [82]. Briefly, EOs were mixed with acetone (Sigma-Aldrich, Milan, Italy) at a 1:1 (*v*/*v*) ratio. After 1 h of storage at room temperature, 1% methanol–HCl was added to each mixture at a ratio of 1:2 (*v*/*v*). Samples were then kept at room temperature for an additional hour and centrifuged at 13,000 rpm for 5 min. The supernatant was collected, while the residue was subjected to a second extraction under the same conditions. The two supernatants were combined and used for subsequent analyses.

3.2.1. DPPH Radical Scavenging Assay

The free radical scavenging activity of the oils was assessed using the diphenyl-1-picrylhydrazyl (DPPH) assay, adapted to a microplate format [83]. Samples were appropriately diluted in methanol and, to avoid evaporation, they were kept in microtubes,

then mixed with 303 µL of a methanolic DPPH solution (153 mM). The reading of results by the spectrophotometer took place by filling a multiplate with the appropriate volume. Absorbance was measured at 517 nm using a UV–Vis spectrophotometer (Cary Varian, Milan, Italy, equipped with a Cary 50MPR multiplate reader (Varian, Milan, Italy)). The absorbance of the DPPH solution without a sample was used as the control. All measurements were performed in triplicate, and results were expressed as mean ± standard deviation (SD). The concentration of sample (µg) required to inhibit 50% of the DPPH radical activity in 1 mL of solution was defined as the IC_{50} value. The IC50 value was calculated through GraphPad PRISM 10.5.0 software.

3.2.2. ABTS Radical Cation Decolorization Assay

Antioxidant capacity was also evaluated using the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay [84]. ABTS diammonium salt, potassium persulfate, Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and ethanol 96° were obtained from Sigma-Aldrich (Milan, Italy). A freshly prepared 2.5 mM methanolic Trolox® solution was used as the antioxidant standard. ABTS and potassium persulfate were dissolved in distilled water at final concentrations of 7 mM and 2.45 mM, respectively, mixed, and incubated in the dark at room temperature to generate the ABTS radical cation ($ABTS^+$). The resulting solution was diluted with ethanol to obtain an absorbance of 1.00 at 734 nm (UV–Vis spectrophotometer, Cary Varian, Milan, Italy, equipped with a Cary 50MPR multiplate reader). Subsequently, 100 µL of samples (final concentrations ranging from 0.0001 to 0.0100 mg/mL) or 100 µL Trolox® standard solutions (0–20 mM) were mixed in 1.5 microtubes with 1 mL of the $ABTS^+$ solution, and absorbance was recorded after 4 min. Results were expressed as µmol Trolox® equivalent antioxidant capacity (TEAC) ± SD, based on triplicate measurements.

3.3. Anti-Arthritic Activity

The in vitro anti-arthritic activity was determined by assessing the inhibition of bovine serum albumin (BSA) denaturation, according to the method described by Fratianni and coworkers [85]. The reaction mixture consisted of 2 mL of a 0.5% (*w*/*v*) BSA solution (96% purity; Sigma-Aldrich, Milan, Italy) prepared in 0.05 M tris–phosphate-buffered saline (pH 6.5), along with 2 mL of the sample. A solution containing BSA and methanol served as the control. After heating at 72 °C followed by cooling, absorbance was measured at 660 nm (UV–Vis spectrophotometer, Cary Varian, Milan, Italy, equipped with a Cary 50 MPR multiplate reader). The concentration of sample required to inhibit 50% of BSA denaturation (IC_{50}) was calculated and compared with that of diclofenac sodium, used as the positive control. All assays were performed in triplicate, and data were expressed as mean ± SD. The IC50 value was calculated through GraphPad PRISM 10.5.0 software.

3.4. Cholinesterase Inhibitory Activities

The in vitro inhibitory activities against acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were evaluated using the spectrophotometric method of Ellman et al. [86]. All assays were conducted in triplicate using 96-well microtiter plates. For AChE inhibition, acetylcholine was used as the substrate. The reaction mixture contained 550 µL of 0.1 M sodium phosphate buffer (pH 8.0), 50 µL of sample, and 5–20 ng of AChE (from *Electrophorus electricus*, 1000 U/mg). Samples and the positive control (galantamine; Sigma-Aldrich, Milan, Italy) were dissolved in 10% DMSO. After incubation for 15 min at room temperature, acetylcholine (10 µL) and 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB; 10 µL) were added. The formation of the yellow 5-thio-2-nitrobenzoate anion was monitored at 412 nm for 10 min. For BChE inhibition, butyrylthiocholine was used as the substrate. The reaction mixture consisted of 550 µL of 0.1 M potassium phosphate buffer (pH 7.0), 50 µL of

sample, and 10–50 ng of equine serum BChE (≥10 U/mg protein). After incubation at room temperature for 15 min, butyrylthiocholine and DTNB were added, and absorbance was monitored at 412 nm (Cary Varian, Milan, Italy, equipped with a Cary 50MPR multiplate reader). Enzyme inhibitory activity was expressed as a percentage of inhibition; galatamin was used as a positive control.

The following equation allows us to calculate the percentage of inhibition:

$$1 - (B - b) \times 100/(A - a) \%\text{inhibition} = 1 - (B - b) \times 100/(A - a),$$

where B = the initial enzyme reaction with the sample; b = the initial reaction with the sample but without the enzyme; A = the initial reaction with the enzyme; a = the initial reaction without the enzyme.

3.5. Tyrosinase Inhibition Assay

Tyrosinase inhibitory activity was evaluated according to the method described by Khatib et al. [87], with minor modifications. Samples were initially dissolved in DMSO. The reaction mixture, consisting of 70 µL of phosphate buffer (pH 6.8), tyrosinase solution (10 U/mL), and the sample, was added directly to 96-well microtiter plates and incubated at 37 °C for 5 min. Subsequently, 0.5 mM L-dihydroxyphenylalanine (L-DOPA) was added as the substrate, and absorbance was measured at 475 nm after 10 min of incubation at 37 °C (UV–Vis spectrophotometer, Cary Varian, Milan, Italy, equipped with a Cary 50MPR multiplate reader). Kojic acid was used as a positive control, while phosphate buffer served as the blank. Tyrosinase inhibitory activity was expressed as IC_{50} values, calculated from concentration–response curves. All experiments were conducted in triplicate, and results were expressed as mean ± SD.

3.6. Microbial Properties

The bacterial strains used in this study included *Acinetobacter baumannii* (ATCC 19606), *Escherichia coli* (DSM 8579), a freshly cultured hospital isolate of *Klebsiella pneumonia*, *Listeria monocytogenes* (ATCC 7644), *Pseudomonas aeruginosa* (DSM 50071), and *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC 25923). All strains were grown in Luria–Bertani broth for 18 h under aerobic conditions at 37 °C or 35 °C, depending on the strain, with agitation at 80 rpm (Corning LSE, Pisa, Italy) prior to experimental assays. Resazurin, crystal violet, MTT, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Milan, Italy).

3.6.1. Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) was determined using the resazurin-based microtiter plate assay [85] performed in flat-bottom 96-well plates.

Resazurin functions as an oxidation–reduction indicator commonly employed to assess cell viability and proliferation. Initially, it is blue, non-fluorescent, and non-toxic dye. When reduced by oxidoreductase enzymes within living cells, resazurin is converted into resorufin, which is pink and fluorescent. Resorufin can be further reduced to hydroresorufin, an uncolored and non-fluorescent compound. To prepare the resazurin solution, 270 mg of dye was dissolved in 40 mL of sterilized deionized water. For the assay, serial dilutions of the samples (previously diluted 1:10 *v*/*v* with DMSO) were performed directly in the plate. To each well, 10 µL of the resazurin solution was added, followed by 30 µL of 3.3× concentrated isosensitized broth, 10 µL of a bacterial suspension (final concentration 5×10^6 cfu/mL), and Luria broth or sterile solution, to have a final volume of 250 µL. The plates were sealed with parafilm to minimize evaporation.

As a positive control, tetracycline—a broad-spectrum antibiotic dissolved in DMSO—was added to one column of the plate. Negative controls consisted of wells containing

Luria–Bertani broth, resazurin, and bacteria without any test samples. Following incubation at 37 °C for 24 h (35 °C for *A. baumannii* under identical conditions), bacterial viability was assessed based on the resazurin colorimetric change. Sterile dimethyl sulfoxide (DMSO) and tetracycline (1 mg/mL in DMSO; Sigma-Aldrich) were used as the negative and positive controls, respectively. All measurements were carried out in triplicate, and data were reported as mean values ± standard deviation.

3.6.2. Effects on Biofilm Formation

The ability of the EOs to interfere with bacterial biofilm formation was evaluated using the crystal violet (CV) staining assay (Sigma-Aldrich, Italy) in flat-bottom 96-well microtiter plates (Falcon, VWR International, Milan, Italy) [85]. Briefly, 10 µL of overnight bacterial culture, adjusted to 0.5 McFarland in fresh Luria–Bertani (LB) broth, was dispensed into each well, together with EOs at final concentrations of 10 and 20 µL/mL and sterile LB medium, to a final volume of 250 µL. Plates were sealed with parafilm to prevent evaporation and incubated for 48 h at 37 °C or 35 °C, depending on the bacterial strain. After incubation, planktonic cells were carefully removed, and adherent cells were gently washed twice with sterile phosphate-buffered saline (PBS). Sessile cells were fixed by adding 200 µL of methanol for 15 min, after which the fixing solution was discarded. Once the plates were air-dried, each well was stained with 200 µL of 2% (*w*/*v*) CV solution for 20 min. Excess dye was removed, and the wells were gently rinsed with sterile PBS and left to dry. The bound CV was subsequently solubilized by adding 200 µL of 20% (*w*/*v*) glacial acetic acid, and absorbance was measured at 540 nm (Cary Varian, Milan, Italy, equipped with a Cary 50MPR multiplate reader). Biofilm adhesion was expressed as a percentage relative to the untreated control (bacteria grown without EOs, assumed as 0% inhibition), following the formula:

$$\% \text{ of inhibition} = 100 \times [(\text{mean O.D. of biofilm of the untreated control} - \text{mean of biofilm treated with essential oils})/(\text{mean O.D. of biofilm of the control})]$$

All experiments were carried out in triplicate, and the results were reported as mean ± standard deviation.

3.6.3. Effects on Mature Biofilms

For the evaluation of mature biofilms, 10 µL of overnight bacterial cultures standardized to 0.5 McFarland in LB broth were inoculated into flat-bottom 96-well microtiter plates with 240 µL of LB broth, resulting in a final volume of 250 µL per well. Plates were sealed with parafilm and incubated for 24 h at 37 °C or 35 °C (for *A. baumannii*). After removal of planktonic cells, EOs were added at final concentrations of 10 and 20 µL/mL, and fresh LB broth (240 and 230 µL, respectively) was added to restore the final volume to 250 µL. Plates were then incubated for an additional 24 h under the same conditions. Biofilm inhibition was calculated relative to untreated mature biofilms following the same procedure described above.

3.6.4. Effects on the Metabolic Activity of Sessile Sells

The effect of the EOs on the metabolic activity of sessile bacterial cells was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay (Sigma-Aldrich, Italy) [88]. The EOs were tested at concentrations of 10 and 20 µL/mL and were added either at the beginning of bacterial growth or after 24 h of incubation to evaluate their impact on developing and mature biofilms, respectively. After a total incubation period of 48 h, planktonic cells were removed, and each well was supplemented with 150 µL of PBS and 30 µL of 0.3% (*w*/*v*) MTT solution. Plates were incubated for

2 h at 37 °C or 35 °C, depending on the bacterial strain. Subsequently, the MTT solution was discarded, and wells were washed twice with 200 µL of sterile PBS. The resulting formazan crystals were dissolved by adding 200 µL of dimethyl sulfoxide (DMSO), and absorbance was measured at 570 nm (Cary Varian, Milan, Italy, equipped with a Cary 50MPR multiplate reader). The inhibitory effect was calculated assuming for the untreated control an inhibition = 0%, and following the formula:

$$\% \text{ of inhibition} = 100 \times [(\text{mean O.D. of biofilm of the control} - \text{mean of biofilm treated with essential oils})/(\text{mean O.D. of biofilm of the control})]$$

3.7. Statistical Analysis

Principal Components Analysis (PCA) and Hierarchical Cluster Heatmap (HCH) were provided by using Matlab 2021a software and were used to assess the similarity between different species analyzed on the following original variables: linalool, methyl cinnamate, *p*-mentha-1(7),8-diene, α-pinene, terpinen-4-ol, α-terpinene, *p*-cymene, γ-cadinene, β-myrcene, α-phellandrene, bicyclogermacrene, camphene, camphor, cadina-1(10),4-diene, D-limonene, sabinene, caryophyllene oxide, eugenol, caryophyllene, cyclofenchene, β-elemene, δ-elemene, α-thujene, and copaene.

4. Conclusions

This comprehensive investigation of five pepper-related essential oils revealed significant and diverse biological activities in vitro, suggesting potential for further exploration as natural agents in various sectors. Among the tested EOs, *P. dioica* demonstrated the broadest and most potent activity profile, while *Z. armatum* and *S. terebinthifolia* exhibited specialized effects—most notably anti-arthritic and butyrylcholinesterase inhibition, respectively. Structure–activity relationships highlighted the importance of specific bioactive components, such as eugenol in *P. dioica* and linalool in *Z. armatum*, in shaping these profiles. The strong antibiofilm activities observed, particularly against ESKAPE and foodborne pathogens, are promising from a microbiological perspective. However, it is important to underscore that these findings are limited to in vitro conditions and do not address critical factors such as cytotoxicity, therapeutic window, bioavailability, realistic exposure levels, or formulation stability. Further studies are necessary to evaluate the safety, toxicity, and efficacy of these essential oils in relevant biological systems, as well as to develop optimized delivery systems suitable for practical use. Additionally, the complexity of essential oil mixtures and the likelihood of synergistic or antagonistic interactions among their constituents warrant detailed mechanistic studies and in vivo validation.

In summary, while our results contribute to the growing body of evidence supporting the bioactivity of pepper-derived essential oils, their translation to food, pharmaceutical, or biomedical applications requires extensive further research, particularly regarding safety and feasibility.

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