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Synergistic and Additive Interactions in Essential Oils Obtained from Combined Plant Materials: Enhanced Control of Insect Pests

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Abstract

Essential oils (EOs) obtained from combined plant materials offer a promising alternative to conventional extraction by enhancing chemical diversity and bioactivity through synergistic interactions. Given the growing interest in insecticidal EOs activity and use in agriculture, the chemical composition and insecticidal properties of individual and combined plant EOs from *Cymbopogon citratus*, *Eucalyptus camaldulensis*, *Eucalyptus lehmannii*, *Salvia rosmarinus* and *Thymus vulgaris* were evaluated against aphids. Plant materials were mixed in equal proportions prior to hydrodistillation to produce binary and ternary combinations. GC-MS analysis revealed significant compositional shifts in EOs from combined plant materials. Major compounds in individual oils included citral (53.11%) and neral (29.14%) in *C. citratus*, thymol (70.84%) in *T. vulgaris*, and eucalyptol as the predominant compound in *E. camaldulensis* (66.51%), *E. lehmannii* (56.99%) and *S. rosmarinus* (46.56%), respectively. In the combined oils, the relative abundance of these constituents was altered, and new minor constituents were introduced. Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) revealed that combined plant EOs clustered close to their parental oils, indicating compositional inheritance rather than entirely novel profiles. Insecticidal assays conducted against *Aphis fabae* demonstrated enhanced efficacy of the combined oils, as evidenced by reduced LC_{50} values (1.39 µL mL^{-1} for *E. camaldulensis* + *T. vulgaris*) and pronounced synergistic interactions, indicated by a co-toxicity coefficient (CTC) of 221.58) and elevated synergistic factors. Pearson correlation analysis and Partial Least Squares (PLS) regression jointly identified Acorenone B, thymol and caryophyllene as principal contributors to insecticidal activity, each exhibiting distinct correlation directions. These three compounds ranked highest among the 18 compounds with a Variable Importance in Projection (VIP) scores exceeding 1.0. The integration of these statistical approaches substantiates the insecticidal potential of combined plant-derived EOS and underscores their relevance in advancing sustainable crop protection strategies.

Keywords: essential oils; combined plant material; insecticidal activity; synergism; additivity; multivariate analysis

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1. Introduction

The combination of essential oils (EOs) from different plant species, either through conventional blending of extracted oils, or through the distillation of combined plant materials, introduces a complex chemical matrix that can profoundly influence their biological activities [1,2]. These combinations can lead to various types of interactions among the constituent compounds [3]. Additive effects occur when the combined bioactivity matches the cumulative activity of the individual oils [4]. In contrast, antagonistic interactions happen when one or more components reduce the overall efficacy of the mixture [5]. Synergistic effects are particularly interesting because they produce a combined activity that surpasses the predicted sum of the individual parts [6]. The nature of these interactions depends on several factors, including chemical composition, concentration ratios, and the method used to combine the oils [7]. The distillation of combined plant materials, entailing the simultaneous extraction of multiple botanical sources, can uniquely promote interactions among volatile compounds during the extraction process [8]. This approach has the potential to yield novel chemical profiles exhibiting enhanced antimicrobial activity [9]. Despite its promise, the mechanisms underlying such interactions remain complex and insufficiently elucidated, thereby highlighting the need for further investigation [10].

The global search for environmentally sustainable and effective alternatives to synthetic pesticides has intensified interest in EOs due to their broad-spectrum antimicrobial properties and multifaceted modes of action [11]. Composed mainly of monoterpenes, sesquiterpenes, and oxygenated derivatives, EOs target multiple biological pathways [12]. This complexity reduces the risk of resistance development in pests and pathogens [13]. While extensive research has focused on individual EOs, there is comparatively less information on the chemical and biological impacts of EO combinations, particularly those produced via the distillation of combined plant materials [14,15].

Aromatic and medicinal plants such as *C. citratus*, *S. rosmarinus*, *T. vulgaris* and *Eucalyptus* species are globally distributed and have been traditionally used for their medicinal and pesticidal properties [16,17]. These species have demonstrated significant antimicrobial and insecticidal potential individually [18]. However, the properties of their combined plant material EOs (CPM-EOs) remain underexplored [19]. Leveraging the synergistic potential of these plants species presents promising opportunities for the development of versatile and sustainable biopesticides, tailored to the diverse requirements of contemporary agricultural systems [20]. Agricultural production across many regions faces serious threats from insect pests including *A. fabae*, a major pest of legumes that imposes additional pressures on crop health and productivity [21]. *A. fabae* is also an important vector of viruses on potato, pepper and faba bean in Tunisia [22]. Effective biocontrol agents targeting such pests are crucial components of integrated pest management strategies that could include the use of EOS, representing a more sustainable approach to pest control with minimal environmental impact [23,24].

This study investigates EOs extracted from five aromatic species, *C. citratus*, *E. camaldulensis*, *E. lehmannii*, *S. rosmarinus* and *T. vulgaris*. The research focuses on both individual oils and selected binary and ternary combinations, aiming to evaluate how distillation of combined plant materials influences their chemical profiles and biological activities. Chemical composition was analyzed using gas chromatography–mass spectrometry (GC–MS). Insecticidal efficacy was evaluated against *A. fabae* through contact toxicity testing. Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were performed on the chemical composition data to evaluate the compositional patterns. In addition, Pearson correlation and Partial Least Squares (PLS) regression were performed to identify the main constituents associated with insecticidal activity based on LC_{50} values. These results contribute to a better understanding of the chemical basis of the bioactivity of combined EOs and support their potential role in sustainable crop protection.

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2. Results

2.1. Chemical Composition of Individual and Combined Plant Material Essential Oils

The chemical analysis of individual and CPM-EOs from *C. citratus*, *E. camaldulensis*, *E. lehmannii*, *S. rosmarinus*, and *T. vulgaris*, as well as their binary and ternary mixtures, led to the identification of 41 compounds, representing 98.46% to 99.99% of the total oil composition. The identified constituents were grouped into four major chemical classes: monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes. All EOs, whether individual or combined, were dominated by oxygenated monoterpenes, ranging from 63.12% to 87.28%, followed by monoterpene hydrocarbons (10.01% to 33.6%) (Table 1).

Table 1. Chemical composition (%) of individual and CPM-EOs of *C. citratus* (*CC*), *E. camaldulensis* (*EC*), *E. lehmannii* (*EL*), *S. rosmarinus* (*SR*), and *T. vulgaris* (*TV*) identified by GC-MS analysis.

				Yield (%)						**CPM-EOs**													
				Individual						**Binary**											**Ternary**		
No	Compounds	*RI**	Formula	*CC*	*EC*	*EL*	*SR*	*TV*	*CC + EC*	*CC + EL*	*CC + SR*	*CC + TV*	*EC + EL*	*EC + SR*	*EC + TV*	*EL + SR*	*EL + TV*	*SR + TV*	*CC + TV + EC*	*CC + TV + EL*	*CC + TV + SR*
1	α-Pinene	7.796	$C_{10}H_{16}$	-	24.38	24.18	9.06	1.11	0.8	16.9	3.5	0.66	23.39	9	1.88	13.77	11.97	5.09	0.77	0.71	1.57
2	Camphene	8.241	$C_{10}H_{16}$	-	-	-	4.85	-	-	-	2.28	-	-	2.95	-	4.54	-	3.09	-	-	0.82
3	β-Pinene	9.095	$C_{10}H_{16}$	-	1.21	-	6.64	-	-	-	2.9	-	-	2.78	-	2.98	0.39	3.37	-	-	0.98
4	β-Myrcene	9.582	$C_{10}H_{16}$	9.59	-	-	2.39	1.25	5.91	-	2.93	2.34	-	0.9	1.09	1.51	0.75	2.23	1.03	2.19	1.7
5	3-Carene	10.102	$C_{10}H_{16}$	-	0.45	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	α-Terpinene	10.462	$C_{10}H_{16}$	-	-	-	-	0.97	-	-	-	0.53	-	-	-	-	-	0.79	-	0.54	-
7	*p*-Cymene	10.847	$C_{10}H_{14}$	-	-	-	-	11.01	19.14	-	-	2.92	-	-	20.52	-	-	-	16.81	3.68	-
8	Eucalyptol	10.856	$C_{10}H_{18}O$	-	66.51	56.99	46.56	-	-	73.62	26.13	-	71.27	64.35	-	49.62	35.09	29.02	-	-	10.67
9	Trans-3-Caren-2-ol	11.755	$C_{10}H_{16}O$	-	-	-	-	-	4.07	-	-	-	-	-	-	-	-	-	-	-	-
10	γ-Terpinene	11.825	$C_{10}H_{16}$	-	-	-	-	7.73	-	-	-	3.57	-	-	10.11	-	7.96	6.01	3.38	3.85	4.94

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11	Cis-Sabinene hydrate	12.610	$C_{10}H_{18}O$	-	-	12.39	1.25	-	-	-	0.52	-	-	-	-	9.42	0.78	1.18	-	-	-
12	D-Verbenone	13.115	$C_{10}H_{14}O$	-	-	-	-	-	0.73	-	-	-	-	-	-	-	-	-	-	-	-
13	Linalool	13.642	$C_{10}H_{18}O$	-	-	-	-	2.71	0.92	-	1.31	2.01	-	-	1.27	-	-	-	0.57	1.84	1.22
14	Camphor	14.587	$C_{10}H_{16}O$	-	-	-	7.44	-	-	-	9.39	-	-	4.04	-	6.28	-	5.99	-	-	3.99
15	Cis-p-mentha-1(7),8-dien-2-ol	14.636	$C_{10}H_{16}O$	-	-	-	-	-	-	-	-	-	-	-	0.83	-	-	-	-	-	-
16	Isopinocarveol	15.142	$C_{10}H_{16}O$	-	0.72	-	-	-	-	-	-	-	-	-	-	-	1.06	-	-	-	-
17	β-Citronellene	15.245	$C_{10}H_{18}$	0.91	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	1,6-Octadiene, 3,7-dimethyl-	15.693	$C_{10}H_{18}$	2.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	Isoneral	15.859	$C_{10}H_{16}O$	-	-	-	-	-	2.68	-	-	2.45	-	-	-	-	-	-	-	2.45	-
20	endo-Borneol	15.972	$C_{10}H_{18}O$	-	-	-	11.35	-	-	-	-	-	-	-	-	2.41	-	4.93	-	-	10.13
21	Terpinen-4-ol	16.045	$C_{10}H_{18}O$	-	-	-	-	-	-	-	-	-	-	-	1.52	-	-	-	0.72	-	-
22	Isoborneol	16.084	$C_{10}H_{18}O$	-	-	-	-	3.01	-	-	5.02	-	-	2.79	-	-	1.46	2.82	-	-	-
23	Isobornyl formate	16.621	$C_{11}H_{18}O_2$	-	-	-	-	-	-	-	3.23	-	-	-	-	-	-	-	-	-	-
24	α-Terpineol	16.853	$C_{10}H_{18}O$	-	-	2.66	-	-	2.57	0.59	-	-	1.9	3.9	3.81	3.59	2.29	-	1.49	-	-
25	Neral	17.741	$C_{10}H_{16}O$	29.14	-	-	-	-	17.23	2.69	9.8	12.34	-	-	-	-	-	-	6.43	10.7	7.68

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26	Bornyl acetate	18.429	$C_{12}H_{20}O_2$	-	-	-	2.12	-	-	-	-	-	-	0.88	-	0.68	-	1.9	-					
27	Citral	18.699	$C_{10}H_{16}O$	53.11	-	-	-	-	33.2	5.11	21.45	24.12	-	-	-	-	-	-	16.97	20.9	15.95			
28	α-Terpinyl acetate	20.403	$C_{12}H_{20}O_2$	-	3.56	2.41	-	-	-	1	-	-	1.65	1.63	-	1.36	1.36	-	-	-	-			
29	Ylangene	20.838	$C_{15}H_{24}$	-	-	-	-	-	-	-	0.33	-	-	-	-	-	-	-	-	-	-			
30	Nerolidyl acetate	21.165	$C_{17}H_{28}O_2$	-	-	-	-	-	-	-	1.99	-	-	-	-	-	-	-	-	-	-			
31	Geranyl acetate	21.213	$C_{12}H_{20}O_2$	4.43	-	-	-	-	6.84	-	-	-	-	-	-	-	-	-	-	-	-			
32	Thymol	21.69	$C_{10}H_{14}O$	-	-	-	-	70.84	-	-	-	45.78	-	-	55.69	-	35.77	33.56	50.46	49.67	37.64			
33	Caryophyllene	22.122	$C_{15}H_{24}$	-	-	-	8.34	-	-	-	4.35	-	-	2.88	-	3.17	-	-	-	-	-			
34	Aromandendrene	22.665	$C_{15}H_{24}$	-	-	-	-	-	1.4	-	-	-	-	0.85	-	-	-	-	-	-	-			
35	cis-α-Bisabolene	23.041	$C_{15}H_{24}$	-	-	-	-	-	-	-	1.75	-	-	-	-	-	-	-	-	-	-			
36	β-Longipinene	23.121	$C_{15}H_{24}$	-	-	-	-	-	-	-		-	-	-	-	0.63	-	-	-	-	-			
37	7-epi-cis-sesquisabinene hydrate	23.631	$C_{15}H_{26}O$	-	-	-	-	-	-	-	0.69	-	-	-	-	-	-	-	-	-	-			
38	Acorenone B	24.174	$C_{15}H_{24}O$	-	-	-	-	-	-	-	-	1.76	-	-	-	-	-	-	-	1.9	1.24			
39	Caryophyllene oxide	26.789	$C_{15}H_{24}O$	-	-	-	-	1.36	-	-	2.42	1.5	-	-	-	-	1.1	-	-	1.5	1.46			
40	Globulol	26.989	$C_{15}H_{26}O$	-	-	-	-	-	2.97	-	-	-	-	-	3.2	-	-	-	1.3	-	-			

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41	(-)-Spathulenol	27.064	$C_{15}H_{24}O$	-	3.09	0.37	-	-	-	-	-	-	1.77	3.02	-	-	-	-	-	-	-
Monoterpene hydrocarbons %				13.3	26.04	24.78	22.92	22.07	25.85	16.9	11.6	10.02	23.39	15.63	33.6	22.8	21.07	20.58	21.99	10.97	10.01
Oxygenated monoterpenes %				86.68	70.79	74.45	68.72	76.56	68.24	83.01	76.85	86.7	74.82	77.59	63.12	73.36	77.81	79.4	76.64	85.56	87.28
Sesquiterpene hydrocarbons %				0	0	0	8.34	0	1.4	0	6.43	0	0	3.73	0	3.8	0	0	0	0	0
Oxygenated sesquiterpenes %				0	3.09	0.37	0	1.36	2.97	0	5.1	3.26	1.77	3.02	3.2	0	1.1	0	1.3	3.4	2.7
Total identified %				99.98	99.92	99.6	99.98	99.99	98.46	99.91	99.98	99.98	99.98	99.97	99.92	99.96	99.98	99.98	99.93	99.93	99.99

*: Retention index on the HP-5MS capillary column.

In *C. citratus* EO, six chemical components, accounting for 99.98% of the total composition were identified. The oil was primarily composed of oxygenated monoterpenes (86.68%) and monoterpene hydrocarbons (13.3%), with citral (53.11%), neral (29.14%) and β-myrcene (9.59%) as the most abundant compounds. *E. camaldulensis* EO contained seven constituents (99.92%), dominated by oxygenated monoterpenes (70.79%) and monoterpene hydrocarbons (26.04%), particularly eucalyptol (66.51%) and α-pinene (24.38%). Similarly, in *E. lehmannii* EO oxygenated monoterpenes (74.45%) and monoterpene hydrocarbons (24.78%) were the most abundant compounds with six major chemicals representing the 99.6% of the total composition, with eucalyptol (56.99%) and α-pinene (24.18%) as key components. In *S. rosmarinus* EO, ten chemical constituents were identified (99.98%), comprising oxygenated monoterpenes (68.72%) and monoterpene hydrocarbons (22.92%), where eucalyptol (46.56%), endo-borneol (11.35%), and α-pinene (9.06%) were predominant. Finally, *T. vulgaris* EO contained nine components (99.99%), rich in oxygenated monoterpenes (76.56%) and monoterpene hydrocarbons (22.07%), with thymol (70.84%), *p*-cymene (11.01%), and γ-terpinene (7.73%) as the major compounds.

Binary combinations involving *C. citratus* resulted in notable shifts in chemical profiles, reflecting both synergistic interactions and selective transformations depending on the companion species. In the *C. citratus* + *E. camaldulensis* oil, citral (33.2%) and neral (17.23%) were the main constituents, indicating partial retention of the citrus profile, whereas eucalyptol, characteristic of *E. camaldulensis*, was completely absent. Remarkably, *p*-cymene (19.14%) appeared as a newly formed compound, indicating possible chemical transformation during the distillation process. In the blend with *E. lehmannii*, eucalyptol became the dominant compound (73.62%), a level exceeding that of *E. lehmannii* alone (56.99%), while citral and neral dropped sharply to 5.11% and 2.69%, respectively. Additionally, α-pinene declined from 24.18% to 16.9%, reflecting rebalancing of the volatile fraction. The *C. citratus* + *S. rosmarinus* mixture showed a moderate retention of citral (21.45%) and neral (9.8%), while camphor increased from 7.44% to 9.39%. In contrast, other major *S. rosmarinus* volatiles such as eucalyptol, α-pinene, camphene, and β-pinene were reduced to 26.13%, 3.5%, 2.28%, and 2.9%, respectively. In the *C. citratus* + *T. vulgaris* blend, thymol became the dominant constituent (45.78%), underscoring the strong phenolic imprint of *T. vulgaris*, while citral (24.12%) and neral (12.34%) were well retained. The appearance of isoneral (2.45%) alongside reduced levels of *p*-cymene (2.92%) and γ-terpinene (3.57%), further highlights the emergence of new volatiles and reorganization of the monoterpene fraction during distillation.

Binary blends containing *E. camaldulensis* exhibited distinct compositional behaviors, ranging from preservation to transformation of key constituents depending on the combined species. Thus, in the *E. camaldulensis* + *E. lehmannii* oil, the major components, eucalyptol (71.27%) and α-pinene

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(23.39%) were largely retained, indicating compositional stability and chemical compatibility between the two species. Conversely, combination with *S. rosmarinus* led to a profile still dominated by eucalyptol (64.35%) and α-pinene (9.0%), alongside camphor (4.04%), while camphene (2.95%) and β-pinene (2.78%) were preserved at low levels, reflecting moderate rebalancing without major loss of original markers. In contrast, the blend with *T. vulgaris* revealed marked compositional shifts: thymol emerged as the dominant compound (55.69%), slightly reduced from its original concentration in *T. vulgaris* (70.84%). Notably, eucalyptol, originally the primary component of *E. camaldulensis* (66.51%) was no longer detectable, indicating possible chemical transformation. Monoterpene hydrocarbons, including α-pinene and β-pinene were also drastically reduced from 24.38% and 1.21% to 1.88% and undetectable levels, respectively. Meanwhile, *p*-cymene and γ-terpinene present in *T. vulgaris* at 11.01% and 7.73% were enriched to 20.52% and 10.11% respectively. These compositional modifications reflect a clear shift toward the phenolic and aromatic profile of *T. vulgaris* accompanied by a complete attenuation of the eucalyptol-rich signature characteristic of *E. camaldulensis*.

Combinations with *E. lehmannii* resulted in compositional changes influenced by the accompanying species. In the *E. lehmannii* + *S. rosmarinus* blend, eucalyptol remained the predominant compound at 49.62%, reflecting substantial retention from both original oils (56.99% and 46.56% respectively). Camphor, a key constituent of *S. rosmarinus* (7.44%), was slightly reduced to 6.28%, while α-pinene initially abundant in both oils (24.18% and 9.06% respectively) appeared at an intermediate level of 13.77%, indicating a compositional balance between the two sources. In contrast, the combination of *E. lehmannii* with *T. vulgaris* yielded an oil dominated by thymol (35.77%) and eucalyptol (35.09%), both substantially reduced from their original concentrations. *p*-Cymene was completely eliminated, α-pinene declined to 11.97%, while γ-terpinene increased slightly from 7.73 to 7.96%, demonstrating a partial merging of the two oils.

Similar compositional patterns were observed in the combination of *S. rosmarinus* with *T. vulgaris*, where thymol (33.56%) and eucalyptol (29.02%) emerged as the predominant compounds shaping the blend's chemical profile. *p*-Cymene was completely eliminated, and α-pinene decreased to 5.09%, reflecting selective changes that enhance the blend's chemical diversity.

Regarding ternary CPM-EOs, eucalyptol was detected exclusively in *C. citratus* + *T. vulgaris* + *S. rosmarinus*, where it accounted for 10.67%. Thymol, the principal phenolic compound of *T. vulgaris*, consistently dominated all ternary blends, with concentrations varying from 37.64% to 50.46%. *p*-Cymene, another major compound from *T. vulgaris*, was present at 16.81% and 3.68% in blends containing *E. camaldulensis* and *E. lehmannii*, respectively, but was entirely absent in *S. rosmarinus* blend. Citral (15.95–20.9%) and neral (6.43–10.7%) characteristic of *C. citratus* were well preserved across all blends, contributing to their distinctive citrus aroma. Camphor, a marker compound of *S. rosmarinus*, was detected only in its corresponding ternary blend at 3.99%. These compositional variations illustrate the complex interactions occurring in ternary CPM-EOs, where the phenolic dominance of thymol is balanced by the citrus freshness of citral and neral, while other compounds such as eucalyptol, *p*-cymene, and camphor modulate the aroma profile and potentially the biological activity of the oils.

2.2. Multivariate Analysis of Essential Oil Compositions

2.2.1. Principal Component Analysis (PCA)

PCA was performed to explore patterns in the chemical composition of individual and CPM-EOs. The scree plot indicated that the first three principal components account for approximately 95.9% of the total variance, with PC1 explaining 47.53%, PC2 explaining 34.87%, and PC3 contributing an additional 13.65%. Given the sharp decline in eigenvalues after the third component, only PC1 and PC2, together explaining 82.4% of the variance, were considered for graphical representation.

The PCA score plot (Figure 1) revealed distinct groupings driven by the distribution of key chemical constituents in individual and CPM-EOs. Detailed PCA loadings (PC1, PC2 and PC3) for all individual, binary, and ternary CPM-EOs are provided in Supplementary Table S1**.** *E. camaldulensis*

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and *E. lehmannii* exhibited strong negative loadings on PC1 (–0.90 and –0.89, respectively), primarily influenced by eucalyptol, a dominant compound strongly correlated with this axis. Similarly, *S. rosmarinus* also loads strongly negatively on PC1 (–0.87) but displays a moderate positive loading on PC2 (0.40), suggesting some chemical similarity with the *Eucalyptus* species while being differentiated by compounds such as camphor, which contributes to its unique profile. *T. vulgaris* showed a strong negative loading on PC2 (–0.88), primarily associated with thymol, underscoring its distinct chemical signature. In contrast, *C. citratus* exhibited moderate loadings near the origin for both PC1 (–0.06) and PC2 (–0.28), indicating a balanced distribution across these components. However, it was primarily characterized by its high loading on PC3 (0.94).

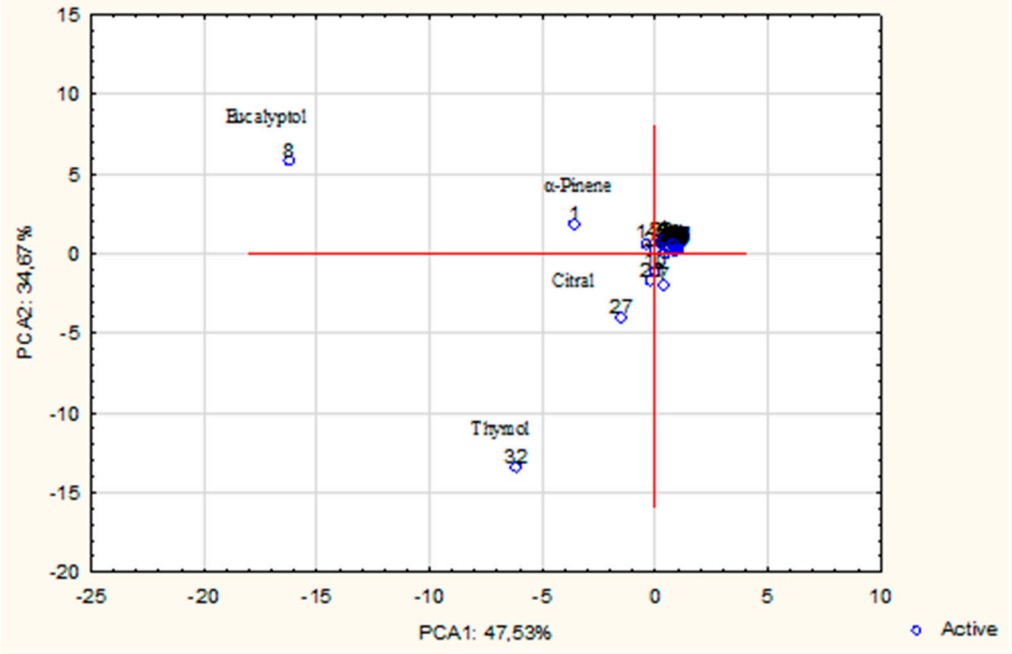

Figure 1. Principal component analysis (PCA) of EOs composition: Projection on PC1 and PC2.

The binary and ternary CPM-EOs exhibited clear spatial distributions in the PCA space, shaped by the dominant constituents of their parent oils. Binary combinations involving *Eucalyptus* species, including pairings within the genus and with other plants such as *C. citratus* + *E. lehmannii* and *E. camaldulensis* + *S. rosmarinus*, showed strong negative loadings on PC1, reflecting high levels of eucalyptol and α-pinene. Mixtures involving *T. vulgaris*, such as *C. citratus* + *T. vulgaris* and *E. camaldulensis* + *T.* vulgaris were positioned along PC2 due to thymol content. Combinations with *C. citratus*, especially *C. citratus* + *E. camaldulensis* and *C. citratus* + *S. rosmarinus*, exhibited strong positive loadings on PC3, indicating a distinct citral-driven profile. Ternary mixtures, such as *C. citratus* + *T. vulgaris* + *E. camaldulensis* and *C. citratus* + *T. vulgaris* + *E. lehmannii*, loaded strongly on PC2 (thymol-driven), with moderate PC3 contributions reflecting citral influence. Similarly, *C. citratus* + *T. vulgaris* + *S. rosmarinus* showed strong negative loadings on both PC1 and PC2, indicating a combined influence of eucalyptol, α-pinene, and thymol, with minor citral input. These spatial patterns illustrate the compositional interplay and synergistic effects emerging from plant material combinations.

2.2.2. Hierarchical Cluster Analysis (HCA)

The hierarchical cluster analysis (HCA) dendrogram, constructed using Euclidean distances and the UPGMA method, revealed clear groupings among the EOs and their combinations, in agreement with the patterns observed in the PCA. Three primary clusters emerged based on compositional similarity (Figure 2).

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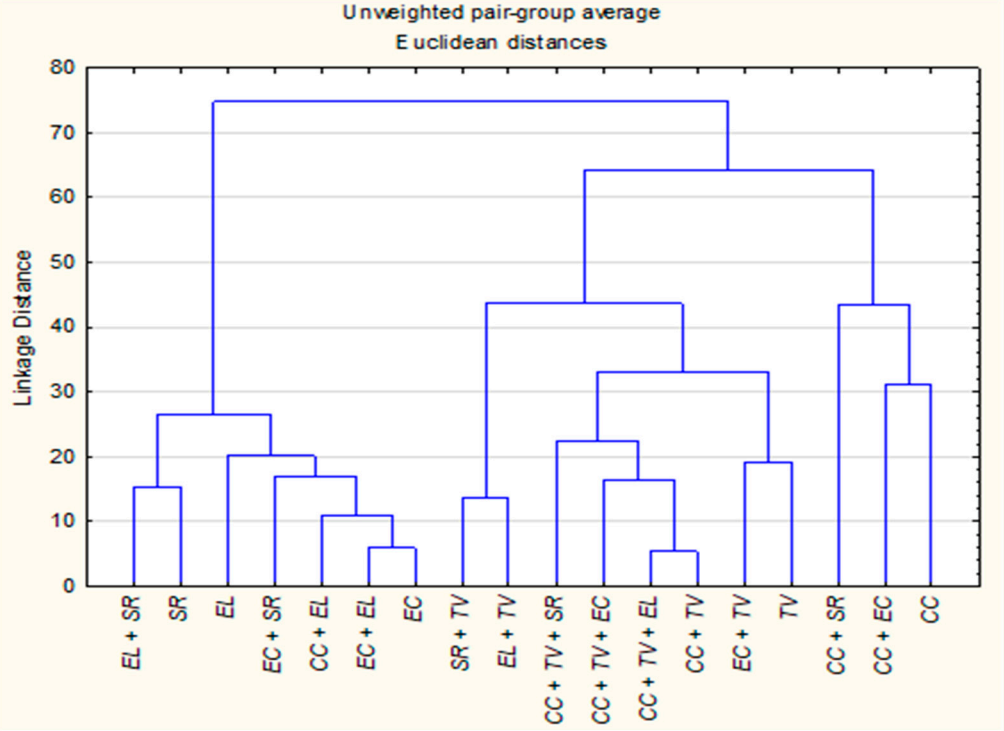

Figure 2. Hierarchical clustering dendrogram of individual and CPM-EOs of *C. citratus* (*CC*), *E. camaldulensis* (*EC*), *E. lehmannii* (*EL*), *S. rosmarinus* (*SR*), and *T. vulgaris* (*TV*) based on their chemical composition. Clustering was performed using Euclidean distances and the UPGMA method.

Cluster A consisted of *Eucalyptus* species, *S. rosmarinus* and their mixtures. The close proximity between *E. camaldulensis* and *E. lehmannii* (distance = 16.2) reflects a high degree of shared constituents, further emphasized by the even closer distance between *E. camaldulensis* and its blend with *E. lehmannii* (distance = 5.9). Additionally, *E. camaldulensis* + *S. rosmarinus* (distance = 17.5 from *E. camaldulensis* and 22.3 from *E. lehmannii*) supports a trend of compositional alignment within this cluster, likely driven by common oxygenated monoterpenes such as eucalyptol and α-pinene. Cluster B was dominated by *T. vulgaris* and its mixtures, characterized by high phenolic content. While *T. vulgaris* alone was chemically distinct from *E. camaldulensis* (distance = 101.1), their combination showed a greatly reduced distance of 19.18, indicating a closer chemical relationship. Mixtures involving *C. citratus* and *T. vulgaris* also exhibited strong chemical affinity. For instance, *C. citratus* + *T. vulgaris* and *C. citratus* + *T. vulgaris* + *E. lehmannii* displayed a very low inter-sample distance of 5.4, while *C. citratus* + *T. vulgaris* + *E. camaldulensis* showed a slightly higher but still close distance of 17.9. These shifts suggest enhanced chemical integration, likely driven by synergistic interactions between thymol and citral components. The mixture *E. lehmannii* + *T. vulgaris* (distance = 45.9) also supports the potential for moderate compositional blending between phenolic-rich and monoterpene-based oils. Cluster C encompassed *C. citratus* and its combined derivatives. Although *C. citratus* alone was chemically distant from most other oils (distance > 90 from *E. camaldulensis* and *T. vulgaris*), its mixtures consistently reduced inter-sample distances and frequently occupied intermediate positions between Clusters A and B. Notable examples include *C. citratus* + *E. camaldulensis* (distance = 31.2 from *C. citratus*), *C. citratus* + *S. rosmarinus* (48.1) all suggesting partial compositional blending. These results indicate that combining plant material modifies the volatile profile in a manner that reduces chemical distances between initially dissimilar oils.

2.3. Insecticidal activity

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2.3.1. Probit Analysis of Individual and Combined Plant Material Essential Oils

Probit regression analysis revealed notable variation in the insecticidal efficacy of both individual and CPM-EOs against *A. fabae* after 24 h of exposure. Among the individual oils tested, *E. camaldulensis* demonstrated the highest insecticidal potency, with the lowest LC_{50} value of 2.45 µL mL^{-1}, indicating its efficiency was obtained with the smallest concentration to kill 50% of the aphid population. This was followed by *E. lehmannii* with LC_{50} of 2.90 µL mL^{-1}, *C. citratus* at 3.24 µL mL^{-1}, and *T. vulgaris* at 3.71 µL mL^{-1}. The least effective individual oil was *S. a rosmarinus*, which exhibited the highest LC_{50} value of 4.41 µL mL^{-1}, reflecting lower toxicity (Table 3).

Table 3. Probit regression results of individual and CPM-EOs against *A. fabae* (24 h post-treatment).

EO Species/Combinations	LC_{50} (µL mL^{-1})	Intercept ± SE	Slope ± SE	χ^2	*p*-value
C. citratus	3.24	2.044 ± 0.463	–0.631 ± 0.126	25.24	0.000001
E. camaldulensis	2.45	0.333 ± 0.229	–0.136 ± 0.035	14.90	0.000114
E. lehmannii	2.90	0.580 ± 0.240	–0.200 ± 0.041	24.32	0.000001
S. rosmarinus	4.41	1.122 ± 0.257	–0.133 ± 0.034	15.24	0.000095
T. vulgaris	3.71	0.837 ± 0.250	–0.226 ± 0.042	29.19	<0.000001
C. citratus + *E. camaldulensis*	1.75	1.608 ± 0.468	–0.917 ± 0.220	17.32	0.000032
C. citratus + *E. lehmannii*	2.35	2.307 ± 0.627	–0.981 ± 0.240	16.64	0.000045
C. citratus + *S. rosmarinus*	2.38	1.494 ± 0.389	–0.628 ± 0.131	23.01	0.000002
C. citratus + *T. vulgaris*	1.75	1.608 ± 0.468	–0.917 ± 0.22	17.32	0.000032
E. camaldulensis + *E. lehmannii*	3.28	0.905 ± 0.263	–0.276 ± 0.050	30.88	<0.000001
E. camaldulensis + *S. rosmarinus*	2.87	1.243 ± 0.320	–0.433 ± 0.082	28.09	<0.000001
E. camaldulensis + *T. vulgaris*	1.39	1.352 ± 0.435	–0.972 ± 0.242	16.12	0.000060
E. lehmannii + *S. rosmarinus*	2.82	0.777 ± 0.260	–0.276 ± 0.052	28.61	<0.000001
E. lehmannii + *T. vulgaris*	1.51	1.430 ± 0.443	–0.945 ± 0.232	16.54	0.000048
S. rosmarinus + *T. vulgaris*	1.63	1.515 ± 0.454	–0.927 ± 0.225	16.97	0.000038
C. citratus + *T. vulgaris* + *E. camaldulensis*	-	-	-	-	-
C. citratus + *T. vulgaris* + *E. lehmannii*	-	-	-	-	-
C. citratus + *T. vulgaris* + *S. rosmarinus*	-	-	-	-	-

- :Ternary combinations were not subjected to Probit regression analysis, as they caused 100% mortality at the lowest concentration tested (2 µL mL-1), making it impossible to fit a dose–response model.

When evaluating CPM-EOs, binary mixtures generally lead to enhanced insecticidal activity compared to individual ones. The binary combination of *E. camaldulensis* + *T. vulgaris* was the most potent, achieving an LC_{50} of 1.39 µL mL^{-1}, indicating good synergy between components inducing a strong increase in toxicity. Other binary mixtures involving *C. citratus* also showed substantial improvements, with LC_{50} values ranging from 1.75 to 2.38 µL mL^{-1}, demonstrating a relative synergistic or additive effect. Ternary combinations resulted in complete mortality (100%) at the lowest tested concentration of 2 µL mL^{-1}, which precluded fitting dose–response models but strongly suggests powerful synergistic effects among the three oils.

2.3.2. Assessment of Synergistic Interactions Using Co-Toxicity Coefficient and Synergistic Factors

The evaluation of CPM-EOs against *A. fabae* revealed a spectrum of interaction types, ranging from strong synergism to additive or slight antagonism. *C. citratus* stood out as a key synergistic component. Its binary combinations with *E. camaldulensis*, *E. lehmannii*, *S. rosmarinus*, and *T. vulgaris* consistently exhibited strong synergistic effects, with co-toxicity coefficients (CTC) ranging from 130.64 to 198.57 and synergistic factors (SF) exceeding 1.2 in all cases (Table 4).

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Table 4. Lethal concentration value (LC_{50}), co-toxicity coefficient (CTC) and synergistic factors (SF) of CPM-EOs against *A. fabae* (24 h post-treatment).

EO Species/Combination	LC_{50} (µL mL^{-1})	Expected LC_{50} (µL mL^{-1})	CTC	SF vs. A	SF vs. B	Effect Interpretation
C. citratus + *E. camaldulensis*	1.75	2.85	162.57	1.85	1.40	Strong synergism
C. citratus + *E. lehmannii*	2.35	3.07	130.64	1.38	1.23	Strong synergism
C. citratus + *S. rosmarinus*	2.38	3.83	160.71	1.36	1.85	Strong synergism
C. citratus + *T. vulgaris*	1.75	3.48	198.57	1.85	2.12	Strong synergism
E. camaldulensis + *E. lehmannii*	3.28	2.68	81.58	0.75	0.88	Additive/Slight antagonism
E. camaldulensis + *S. rosmarinus*	2.87	3.43	119.52	0.85	1.54	Additive to moderate synergism
E. camaldulensis + *T. vulgaris*	1.39	3.08	221.58	1.76	2.67	Strong synergism
E. lehmannii + *S. rosmarinus*	2.82	3.66	129.47	1.03	1.56	Strong synergism
E. lehmannii + *T. vulgaris*	1.51	3.31	218.81	1.92	2.46	Strong synergism
S. rosmarinus + *T. vulgaris*	1.63	4.06	249.08	2.71	2.27	Strong synergism
C. citratus + *T. vulgaris* + *E. camaldulensis*	-	-	-	-	-	Strong synergism*
C. citratus + *T. vulgaris* + *E. lehmannii*	-	-	-	-	-	Strong synergism*
C. citratus + *T. vulgaris* + *S. rosmarinus*	-	-	-	-	-	Strong synergism*

Interaction types were interpreted using both the co-toxicity coefficient (CTC) and the synergistic factor (SF). A CTC value > 120 indicates synergism, 80–120 indicates an additive effect, and < 80 indicates antagonism. Similarly, an SF > 1 suggests synergism, SF = 1 indicates additivity, and SF < 1 suggests antagonism. *: The inability to estimate LC_{50} values due to 100% mortality at minimal doses (2 µL mL^{-1}), indicating strong synergistic effects.

T. vulgaris also contributed significantly to enhanced efficacy when paired with other species. Combinations with *E. camaldulensis*, *E. lehmannii*, and *S. rosmarinus* demonstrated particularly strong synergy, reflected by CTC values greater than 218 and SF values above 2, indicating substantial improvement in insecticidal activity. *S. rosmarinus*, despite being the least toxic as a single oil, displayed notable synergistic effects in combinations. When paired with *C. citratus* or *T. vulgaris*, the mixtures achieved high CTC values (up to 249.08) and strong SF values (>2.2), underscoring its supportive role in enhancing toxicity. However, in combination with *E. camaldulensis*, the interaction was weaker, classified as additive to moderate synergism (CTC = 119.52), and showed uneven SF values (0.85 and 1.54), suggesting variable contribution depending on the pairing. In contrast, the mixture of *E. camaldulensis* and *E. lehmannii* showed the weakest interaction, classified as additive to slight antagonism. This combination yielded a CTC of 81.58 and SF values of 0.75 and 0.88, indicating little to no enhancement and potential interference in insecticidal action.

Ternary mixtures comprising *C. citratus*, *T. vulgaris*, and either *S. rosmarinus* or, an *Eucalyptus* species caused complete mortality at the lowest tested dose (2 µL mL^{-1}). This prevented LC_{50} estimation but clearly indicated potent synergistic interactions. In summary, *C. citratus* and *T. vulgaris* were the primary drivers of synergy across combinations, while *S. rosmarinus* enhanced their effects significantly. Conversely, pairings involving only *Eucalyptus* species provided limited or even slightly antagonistic results, emphasizing the importance of strategic plant material selection for optimal insecticidal efficacy.

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2.3.3. Correlation and Regression Analysis Based on LC_{50}

To identify the chemical constituents most strongly associated with insecticidal activity, correlation and regression analyses were performed using LC_{50} values as indicators of toxicity. Pearson correlation analysis provided insight into the strength and direction of linear relationships between key constituents and bioactivity. To further explore these associations in a multivariate context and account for potential interactions between compounds, partial least squares (PLS) regression was applied. This approach allowed the development of a predictive model and highlighted the key constituents contributing to the overall insecticidal effect of EOs.

Pearson correlation between major constituents and LC_{50} values

Among the 41 EOs constituents analyzed, three exhibited statistically significant correlations with LC_{50} values. Thymol ($r = –0.5018$, $p = 0.034$) and Acorenone B ($r = –0.5119$, $p = 0.030$) were negatively correlated, indicating that higher concentrations of these compounds corresponded to lower LC_{50} values and therefore greater insecticidal activity. Caryophyllene was positively correlated with LC_{50} ($r = 0.5267$, $p = 0.025$), suggesting that higher concentrations of this compound were associated with higher LC_{50} values and, therefore, reduced insecticidal potency. No other constituent of the EOs showed a statistically significant correlation with LC_{50} values. The complete list of correlation coefficients and p values for all analyzed compounds is provided in Table 5.

Table 5. Pearson correlation coefficients (r) and p-values between EOs compounds and LC_{50} values.

Chemical Compound	r	*p*-value	Signifiance
α-Pinene	0.3942	0.105	NS
Camphene	0.3829	0.117	NS
β-Pinene	0.4248	0.079	NS
β-Myrcene	0.0837	0.741	NS
3-Carene	0.0514	0.839	NS
α-Terpinene	-0.0325	0.898	NS
p-Cymene	-0.3312	0.179	NS
Eucalyptol	0.4664	0.051	NS
Trans-3-Caren-2-ol	-0.1043	0.680	NS
γ-Terpinene	-0.4387	0.069	NS
Cis-Sabinene hydrate	0.2340	0.350	NS
D-Verbenone	-0.1043	0.680	NS
Linalool	-0.2652	0.287	NS
Camphor	0.2265	0.366	NS
Cis-p-mentha-1(7),8-dien-2-ol	-0.1845	0.464	NS
Isopinocarveol	-0.1045	0.680	NS
β-Citronellene	0.2272	0.364	NS
1,6-Octadiene, 3,7-dimethyl-	0.2272	0.364	NS
Isoneral	-0.3578	0.145	NS
endo-Borneol	0.0884	0.727	NS
Terpinen-4-ol	-0.3383	0.170	NS
Isoborneol	0.1521	0.547	NS
Isobornyl formate	0.0359	0.888	NS
α-Terpineol	0.0019	0.994	NS
Neral	-0.1616	0.522	NS
Bornyl acetate	0.3534	0.150	NS
Citral	-0.2101	0.403	NS
α-Terpinyl acetate	0.2596	0.298	NS
Ylangene	0.0359	0.888	NS
Nerolidyl acetate	0.0359	0.888	NS
Geranyl acetate	0.0370	0.884	NS
Thymol	**-0.5018**	**0.034**	*
Caryophyllene	**0.5267**	**0.025**	*
Aromandendrene	-0.0144	0.955	NS
cis-α-Bisabolene	0.0359	0.888	NS
β-Longipinene	0.1338	0.597	NS

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7-epi-cis-Sesquisabinene hydrate	0.0359	0.888	NS
Acorenone B	**-0.5119**	**0.030**	*
Caryophyllene oxide	-0.2312	0.356	NS
Globulol	-0.3233	0.191	NS
(-)-Spathulenol	0.2442	0.329	NS

* Indicates statistically significant correlation ($p < 0.05$); NS = not significant.

Partial least square regression modeling

The PLS regression model extracted two components, which together explained 59.39% of the variance in insecticidal activity (LC_{50}). Detailed statistics for each component, including R^2X, R^2Y, eigenvalues, Q^2, and the number of iterations, are presented in Supplementary Table S2. The first component explained 19.44% of the variance in the predictor variables (R^2X) and 42.09% of the variance in the response variable (R^2Y), with an eigenvalue of 6.96. The associated Q^2 value was 0.15, indicating moderate predictive accuracy. This component was statistically significant. The second component contributed 11.77% to R^2X, bringing the cumulative R^2X to 31.21% and R^2Y to 59.39%. Its eigenvalue was 2.45, but the Q^2 value was negative (-0.63) and the component was not statistically significant. Although the cumulative R^2Y increased with the addition of the second component, the corresponding decrease in Q^2 suggests limited predictive contribution beyond the first component. The overall performance of the model is summarized in Figure 3.

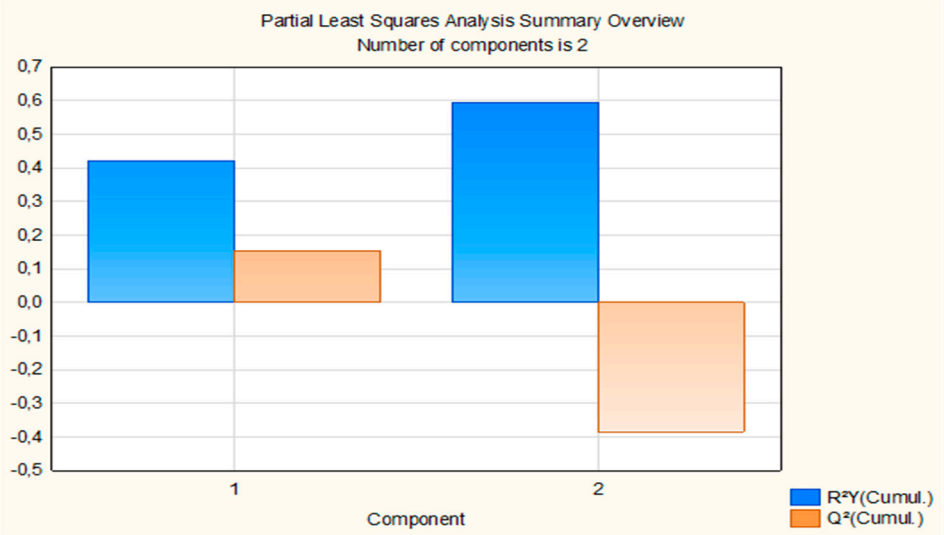

Figure 3. Cumulative R^2Y and Q^2 statistics of the two PLS components explaining variance in LC_{50}.

The VIP scores from the PLS regression model were used to assess the relative importance of the 41 identified compounds in predicting insecticidal activity. VIP scores quantify the contribution of each chemical constituent used as a predictor variable to explain variation in the response variable, and compounds with VIP scores greater than 1 are considered significant contributors to the model. As shown in Figure 4, 18 compounds exceeded the commonly used threshold of VIP > 1, indicating a significant contribution to the model. These 18 compounds are summarized in Table 6, ranked according to their VIP scores. The complete list of compounds and their associated VIP scores are presented in Supplementary Table S3.

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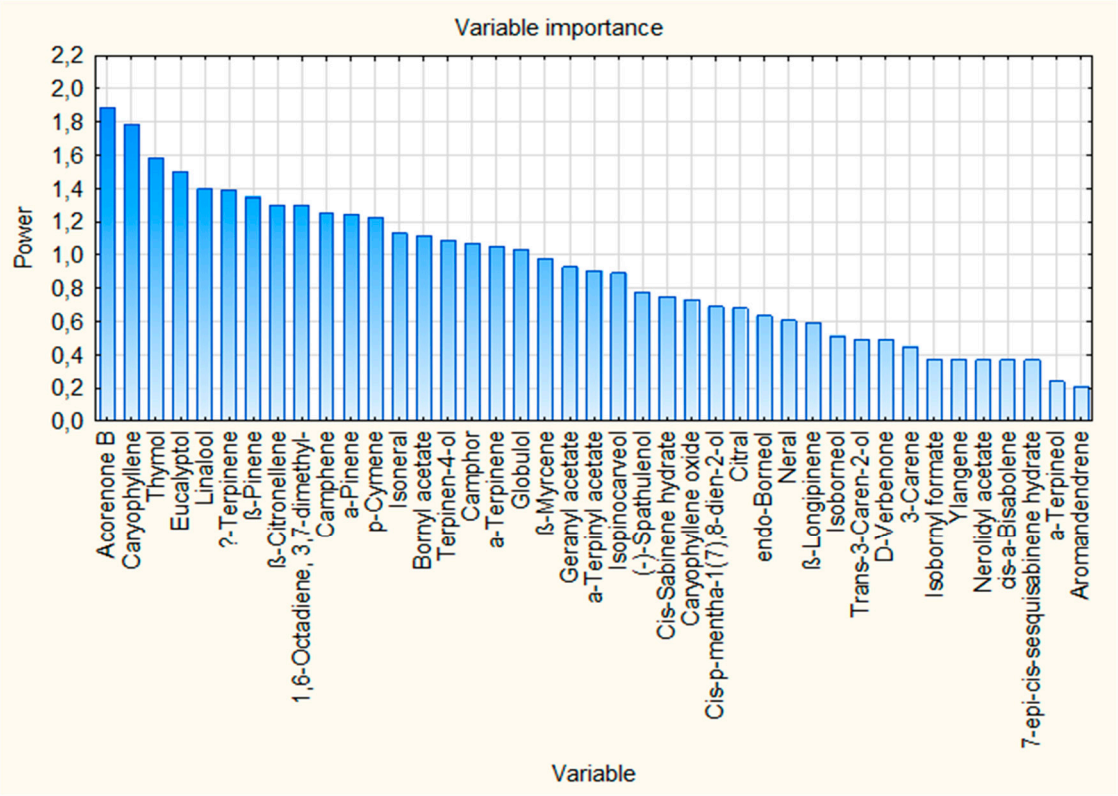

Figure 4. VIP scores of all identified chemical compounds from PLS regression analysis.

Table 6. Compounds with VIP scores greater than 1 in the PLS regression analysis.

Rank	Variable number	Chemical compound	VIP score
1	38	Acorenone B	1.886
2	33	Caryophyllene	1.785
3	32	Thymol	1.580
4	8	Eucalyptol	1.498
5	13	Linalool	1.394
6	10	γ-Terpinene	1.388
7	3	ß-Pinene	1.345
9	17	ß-Citronellene	1.297
9	18	1,6-Octadiene, 3,7-dimethyl-	1.297
10	2	Camphene	1.249
11	1	α-Pinene	1.243
12	7	*p*-Cymene	1.224
13	19	Isoneral	1.127
14	26	Bornyl acetate	1.114
15	21	Terpinen-4-ol	1.085
16	14	Camphor	1.069
17	6	α-Terpinene	1.045
18	40	Globulol	1.028

The top 18 compounds ranked by their variable importance in projection (VIP). Variable numbers correspond to those used in the chemical composition table (Table 2). Compounds with identical VIP scores share the same rank.

Acorenone B (VIP =1.886), caryophyllene (VIP =1.785) and thymol (VIP =1.580) were identified as the compounds with the highest VIP scores, indicating their dominant influence on the PLS

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regression model. It is noteworthy that these same compounds also showed statistically significant correlations with insecticidal activity in the Pearson correlation analysis presented previously. The convergence of the results of these two independent statistical approaches reinforces their biological relevance and underlines their role as main contributors to the observed insecticidal effect. Besides these compounds, several other compounds with VIP greater than 1, such as eucalyptol, linalool, and γ-terpinene, also showed notable importance in the model and could contribute through additive or synergistic interactions. This observation supports the notion that insecticidal activity arises from the collective contribution of multiple constituents, rather than being solely driven by a single dominant compound.

3. Discussion

The chemical composition of EOs is a primary determinant of their biological activity, and in this study, the profiles of the tested EOs largely conformed to established chemotypes. Citral is the primary component of EOs derived from *Cymbopogon* species. In the present study, *C. citratus* was rich in citral (53.11%) and neral (29.14%), closely aligning with values observed in the Brasilian (citral 55.48%, neral 35.40%) and Egyptian (citral 31.57%, neral 13.42%) chemotypes [25,26]. Similar citral-dominant profiles have also been reported in Indonesian [27], Indian [28], and Vietnamese [29] *C. citratus* oils, further confirming the consistency of this chemotype across diverse geographical regions. *E. camaldulensis* and *E. lehmannii* were both dominated by eucalyptol, which is consistent with previous findings, where eucalyptol typically ranges from 20 to 40% [30,31]. However, our results showed a higher relative abundance of eucalyptol, ranging from 50 to 70%. Previous studies have demonstrated significant variability in the chemical composition of *Eucalpytus* EOs depending on factors such as geographic origin, climate, soil conditions, harvesting time, and extraction method [32,33]. *S. rosmarinus* EO was mainly composed of eucalyptol (46.56%) followed by endo-borneol, and α-pinene. This composition indicates a predominance of eucalyptol chemotype, consistent with typical profiles previously reported for this species [34–36]. Other chemotypes have been also described including camphor [37], verbenone [38], α-pinene [39] and myrcene [40]. The *T. vulgaris* EO was characterized by a predominance of thymol, indicating that the studied oil belongs to the thymol chemotype, in agreement with the literature [41,42]. However, the relative proportion of thymol in our samples was notably higher (reaching 70%) than that reported in previous studies, where values generally remained close to 40% [43–45]. In addition to the thymol chemotype observed in the present study, other chemotypes of *T. vulgaris* EO were reported including carvacrol [46], linalool [47] and α-terpineol [48].

Upon combining and distilling plant materials, the resulting EOs showed strong compositional inheritance from their respective parent profiles, as indicated by the clustering of major components. However, consistent and notable differences were observed, including suppression of certain constituents, emergence of new minor compounds, and significant shifts in relative abundance. In particular, combinations involving *C. citratus* with *Eucalyptus species* showed distinct profiles, likely due to the interaction of citral and eucalyptol during the distillation process, which may enhance or suppress certain volatiles. In contrast, blends with *T. vulgaris* revealed that thymol emerged as the dominant compound, underscoring the strong phenolic imprint of *T. vulgaris*. On the other hand, combinations of chemically similar oils, such as *E. camaldulensis, E. lehmannii* and *S. rosmarinus*, showed less differentiation, indicating limited synergy in their volatile profiles. These observations highlight the importance of strategic EO selection in combined distillation to achieve both compositional and functional diversity. Similar transformations were previously observed during the combined plant material distillation of *C. citratus* and *Hyptis suaveolens*, where the resulting oil showed new major constituents, piperitone (40.8%) and p-menth-4(8)-ene (13.2%) being absent in the individual oils. In contrast, key compounds such as β-pinene and sabinene (from *Hyptis suaveolens*), and neral and geranial (from *C. citratus)* disappeared, while limonene and α-pinene, present only in *Hyptis suaveolens*, increased markedly in the combined oil. These changes were attributed to thermal and acid-catalyzed reactions, including cyclization, isomerization, and dehydration, leading to the

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conversion of citral isomers (neral and geranial) to piperitone, rearrangement of sabinene to p-menth-4(8)-ene and the transformation of myrcene and β-pinene into limonene [2]. Additionally, Sánchez-Velandia et *al*. [49] reported that in situ chemical reactions can occur during distillation, for example, *α*-pinene undergoes acid-catalyzed rearrangement to camphene, which is further oxidized to camphor, while myrcene, formed via β-pinene pyrolysis, can be converted to d-citronellal and ultimately cyclized to produce l-menthol. These transformations demonstrate how naturally occurring compounds can undergo significant structural changes during extraction and processing, influencing both their physical characteristics and biological activity [50]. These reactions may also produce artefacts, compounds not present in the original biomass, which can contribute to the complexity and sometimes unpredictability of the resulting EO [51]. Moreover, such transformations may influence not only aroma but also the oil's biological activity, with potential implications for therapeutic, antimicrobial, or preservative efficacy [52].

These compositional changes are attributable to a range of interacting factors, both chemical and physical, inherent to the distillation process [53,54]. Hydrodistillation exposes EO constituents to elevated temperatures, prolonged contact with water, and oxygen, creating conditions favorable for a variety of chemical transformations [55,56]. Thermal degradation is a primary factor, especially affecting heat-sensitive compounds like monoterpenes and sesquiterpenes, which can volatilize, decompose, or rearrange into different structures under high temperatures [57,58]. Simultaneously, hydrolysis reactions in the aqueous environment can cleave esters and glycosides, generating corresponding alcohols and acids, which have different volatilities and polarities, affecting both extraction efficiency and oil composition [59,60]. Oxidative reactions, driven by the presence of dissolved oxygen or trace catalytic metals, may further transform reactive aldehydes, phenolics, or unsaturated compounds into oxidized derivatives, including peroxides or epoxides, that are not present in the raw plant material [61,63]. Physical factors such as differences in vapor pressure cause more volatile compounds to distill preferentially, potentially suppressing less volatile components or causing uneven release [64]. Moreover, the extraction procedure can influence physical properties such as viscosity, coloring, and odor, which may affect EOs quality [65]. When different plant matrices are combined, their anatomical and microstructural differences, such as trichome density, oil gland structure, and moisture content, can influence the release kinetics of EO constituents, leading to suppression or enhancement of certain compounds [66].

The insecticidal assay against *A. fabae* revealed variable toxicity levels among the tested oils. Among the individual ones, *E. camaldulensis* exhibited the highest toxicity, followed by *E. lehmannii* consistent with literature attributing strong insecticidal activity to eucalyptol-rich oils [67]. For instance, Khedhri et *al*. [68] demonstrated potent toxicity of four *Eucalyptus* species against *A. fabae* with LC_{50} ranging from 0.264 to 0.39 mg mL^{-1}. *C. citratus* EO also showed considerable activity, likely due to its high content in citral and neral, which have been reported to be strong insecticidal and repellent compounds against *A. fabae* [69]. Comparable efficacy was observed against related species including *A. gossypii*, *A. citricola* and *M. persicae*, further supporting the broad-spectrum activity of these oxygenated monoterpenes against the Aphididae family [69–72]. Similarly, *T. vulgaris* EO exhibited notable insecticidal activity which is attributed to its high thymol content, a monoterpene phenol with well-documented neurotoxic effects on insects [73]. In aphid species, several mechanisms have been described for thymol, including modulation of GABA-gated chloride channels and interference with the tyramine–octopamine signaling pathway, both of which are crucial for neural transmission and behavioral regulation in insects [74]. In the present study, *S. rosmarinus* EO exhibited the lowest activity, with LC_{50} value of 4.41 µL mL^{-1}. These results are consistent with those obtained by Casas et *al*. [75] who reported a similar LC_{50} value (4.61 µL mL^{-1}) against *Myzus persicae*.

When EOs were combined, they generally exhibited enhanced toxicity when compared to individual oils. For instance, *E. camaldulensis* + *T. vulgaris* mixture demonstrated the lowest LC_{50} value of 1.39 µL mL^{-1}. The co-toxicity coefficient (CTC) and synergistic factor (SF) analyses further confirmed strong synergy in combinations involving *C. citratus*, *T. vulgaris*, and *S. rosmarinus*. Statistical analyses identified acorenone B, thymol and caryophyllene as key contributors to

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insecticidal activity observed in this study. In agreement, acorenone B has been reported to inhibit both acetylcholinesterase and butyrylcholinesterase, with IC_{50} values of 40.8 μg mL^{-1} and 10.9 μg mL^{-1} respectively, indicating a neuroinhibitory mode of action that disrupts cholinergic neurotransmission and leads to paralysis and death in insects. Consistently, acorenone-rich EOs exhibited high ecotoxicity, further supporting the contribution of acorenone-type sesquiterpenes to EO-mediated toxicity [76]. These results are in line with the findings of Bora et *al*. [77], who reported that using multi-component EO blends with different mechanism of action, can significantly improve pest control efficiency and delay resistance development. In line with these observations, a study on *Sitophilus zeamais* demonstrated that optimized mixtures of plant volatile compounds, including thymol, carvacrol, phellandrene, γ-terpinene, pulegone, and δ-3-carene, exhibit synergistic insecticidal activity through multitarget effects, impacting neurotransmission and reducing detoxification enzyme activities [78].These findings are consistent with previous study showing strong synergism among the major constituents (Eucalyptol, carvacrol, pulegone, and eugenol) of *Rosmarinus officinalis*, *Origanum compactum*, *Mentha pulegium*, *Thymus satureioides*, *Myrtus communis* and *Eugenia aromatica* against *Callosobruchus maculatus*, which can enhance insecticidal activity through improved stability, absorption, cuticular penetration and multi-target neurophysiological effects [79]. Similarly, binary mixtures of thymol and eucalyptol demonstrated synergistic toxicity against *Helicoverpa armigera*, inhibiting detoxification and neurophysiological enzymes more effectively than single compounds [80]. Recent modeling-based research on *Musca domestica* demonstrated that the insecticidal activity of thyme EOs is primarily attributed to thymol, *p*-cymene and γ-terpinene, identified as the main active constituents through component effect analysis. Mixture modeling confirmed synergistic interactions among all binary combinations of these compounds, with the optimal ternary ratio (*p*-cymene: γ-terpinene: thymol = 32:23:45) achieving the highest mortality rate of 87.5%. This optimized blend significantly outperformed individual components, underscoring the crucial role of compositional optimization and synergistic interactions among terpenoid constituents in enhancing the efficacy and consistency of EO-based insecticides [81]. Several reports have highlighted the insecticidal potential of caryophyllene [82–84]. For example, Sun et *al*. [85] demonstrated that caryophyllene, α-pinene and β-myrcene act as major bioactive components responsible for the insecticidal and repellent activity of *Peucedanum terebinthinaceum* EO. Additionally, Chohan et *al*. [86] reported that β-caryophyllene and α-pinene exhibit strong fumigant toxicity against *Myzus persicae*, significantly affecting genes responsible for reproduction, dispersion, and insect growth. Moreover, β-caryophyllene-rich leaf EO of *Psidium guajava* showed strong contact toxicity against *Sitophilus zeamais*, highlighting the eco-friendly potential of this compound as natural insecticide and control agent [87].

4. Materials and Methods

4.1. Plant Material

During the spring of 2025 (March-April), five plant species (*C. citratus*, *E. camaldulensis*, *E. lehmannii*, *S. rosmarinus* and *T. vulgaris*) were collected from various regions across Tunisia. For *C. citratus*, *E. camaldulensis* and *E. lehmannii*, leaves were harvested, while the aerial parts were gathered for *S. rosmarinus* and *T. vulgaris*. To ensure representative sampling, plant materials were randomly harvested from several individual plants or trees within each species. The samples were then combined to form homogenized samples, as summarized in Table 7 After collection, the plant materials were placed in a glass greenhouse and air-dried under ambient conditions for five days. Once dried, they were stored in paper bags at room temperature until further analysis. All plant species were taxonomically identified by Professor Lamia Hamrouni following standard procedures. Corresponding voucher specimens have been deposited in the herbarium division of the National Institute of Researches on Rural Engineering, Water and Forests (INRGREF).

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Table 7. Plant species, used part, period and harvesting sites.

Species	Used part	Harvesting period	Location
C. citratus	Leaves	March-April/2025	Kairouan
E. camaldulensis	Leaves		Zarniza arboreta
E. lehmannii	Leaves		Babbouche, Ain drahem
S. rosmarinus	Aerial parts		Tborsok, Beja
T. vulgaris	Aerial parts		

4.2. Essential oil Extraction and Combinations

EOs were extracted by hydro-distillation of dried plant material using a clevenger-type apparatus (SAF Wärmetechnik LabHEAT® KM-ME, 1000 mL, SAF GmbH, Hamm, Germany) for 3 h. Extractions were performed on individual species (*C. citratus*, *E. camaldulensis*, *E. lehmannii*, *S. rosmarinus*, and *T. vulgaris*) as well as on binary (1:1) and ternary (1:1:1) mixtures, prepared by mixing the dried materials in equal weight ratios prior to distillation. The extracted oils were dried over anhydrous sodium sulfate and stored at 4 °C in amber glass vials until further use.

4.3. Gas Chromatography Mass Spectrometric (GC-MS) Analysis

The GC-MS analysis was conducted using an HP 8890 gas chromatograph coupled to an HP 5977B mass spectrometer equipped with an HP-5MS UI column (30 m×0.25 mm; 0.25 µm) (Agilent Technologies, Santa Clara, CA, USA). The injector and the detector temperatures were set at 250 °C and 280 °C, respectively. The oven temperature was programmed from 45 °C (held 1 min) to 250 °C (held 6 min) at a rate of 5 °C/min, for a total runtime of 48 min. Helium was used as the carrier gas at a constant flow rate of 1 mL min^{-1}. Samples (1 µL) were injected in split mode with a 1:100 ratio. Mass spectrometry data were acquired in scan mode over a mass range of m/z 30 to 600. Components were identified by comparing their retention times with those of pure reference samples and by comparing their linear retention indices (LRI) relative to the n-alkane series. Mass spectra were compared to those present in commercial libraries (Wiley 7, NIST 05) and/or reported in the literature [88].

4.4. Insecticidal Activity

4.4.1. Aphid Sampling and Rearing

Pathogen-free apterous parthenogenetic populations of *A. fabae* were collected from faba bean (*Vicia faba*) crops in the Cap Bon region (36.69° N, 10.49° E). Colonies were maintained on potted faba bean plants in a controlled-environment growth chamber at 23 °C ± 1, a relative humidity 60 ± 5%, and a 16:8 h light:dark photoperiod. Aphids and host plants were kept in insect-proof cages equipped with fine mesh vents to ensure proper ventilation and prevent contamination.

4.4.2. Contact Toxicity Bioassay

The insecticidal activity of individual and CPM-EOs was evaluated against *A. fabae* using a direct contact toxicity test. The assays were conducted using concentrations of 2, 4, 6, 8, 10, and 12 µL mL^{-1}. For each treatment, an emulsion was prepared by mixing the appropriate volume of EO with 1% (v/v) Tween 20 solution. Ten wingless adult aphids were gently transferred into a fresh faba bean leaf placed on Whatman filter paper discs inside a sterile 90 mm petri dish. Each group was then sprayed with 1 mL of the prepared EO emulsion, ensuring even coverage of both aphids and the leaf surface. Control groups were treated with 1 mL of 1% Tween 20 solution without EOs. All treatments, including the control, were replicated five times. Mortality was assessed 24 h post-application. Aphids were considered dead if they showed no movement of legs or antennae upon gentle probing with a fine brush. Lethal concentration value (LC_{50}) with 95% confidence interval was estimated using PROBIT analysis.

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4.4.3. Interaction Assessment

The co-toxicity coefficient (CTC) was calculated to evaluate the interaction effects of CPM-EOs on *A. fabae* toxicity. For each mixture, the expected LC_{50} was determined by averaging the LC_{50} values of the individual EOs. The CTC was then calculated by dividing the expected LC_{50} by the observed LC_{50} of the mixture and multiplying by 100. A CTC value greater than 120 was considered indicative of strong synergism, values between 80 and 120 indicated additive (cumulative) effects, and values less than 80 suggested antagonism [89]. Additionally, the synergistic factor (SF) was calculated to assess further the interaction between EOs mixtures [90]. The SF was determined separately for each component of the mixture by dividing the LC_{50} of the individual EO by the LC_{50} of the corresponding mixture. Specifically, SF vs. A = LC_{50} of EO A/LC_{50} of the mixture, and SF vs. B = LC_{50} of EO B/LC_{50} of the mixture. An SF value greater than 1 indicated synergism, a value equal to 1 indicated an additive effect, and a value less than 1 suggested antagonism. Both CTC and SF were used together to comprehensively assess the nature of interactions between EOs in the toxicity bioassays against *A. fabae*.

4.5. Statistical and Chemometric Analysis

All experiments were conducted in triplicate, and the results are presented as mean ± standard deviation. Statistical analyses were performed using STATISTICA software version 10. Probit analysis was performed to estimate lethal concentration (LC_{50}) with 95% confidence interval. Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were conducted to examine patterns in the chemical composition of individual and CPM-EOs. HCA was based on Euclidean distance and clustering was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). To study the relationship between EO composition and insecticidal activity, chemometric approaches were used. Pearson correlation analysis identified significant linear associations between the abundance of individual compounds and their insecticidal effects based on LC_{50} values. In addition, partial least squares (PLS) regression was used to determine the main bioactive constituents contributing to insecticidal activity. The VIP (Variable Importance in Projection) scores of the PLS model highlighted the most influential compounds. These multivariate chemometric analyses provided a better understanding of the associations between the chemical profiles of EOs and their insecticidal efficacy.

5. Conclusions

The combination of plant material facilitates effective compositional blending, as evidenced by multivariate analyses that revealed distinct chemical profiles predominantly influenced by key constituents such as eucalyptol, thymol, and citral. These compositional alterations were mirrored in the observed biological activities. In insecticidal bioassay against *A. fabae*, *E. camaldulensis* exhibited the highest toxicity among the individual essential oils tested. Notably, combinations involving *C. citratus, Eucalyptus* species and *T. vulgaris* demonstrated pronounced synergistic effects, significantly increasing toxicity and reducing LC_{50} values. The mixture of *E. camaldulensis* + *T. vulgaris* proved to be the most potent. Althought, *S. rosmarinus* displayed the lowest individual toxicity, its inclusion in blends contributed positively to overall insecticidal efficacy. In contrast, combinations composed solely of *Eucalyptus* species exhibited limited or mildly antagonistic interactions, underscoring the importance of selecting chemically complementary oils. Multivariate analysis further identified acorenone B, thymol and caryophyllene as principal contributors to the insecticidal activity observed. These compounds exhibited potent toxicity against *A. fabae*, likely through mechanisms involving neurophysiological disruption and interference with reproductive, developmental, and behavioral processes. Their effects appear to be enhanced through synergistic interactions, thereby amplifying overall efficacy. These findings underscore the potential of essential oils enriched in these bioactive constituents as environmentally sustainable alternatives to conventional synthetic insecticides.

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Further research is warranted to optimize formulation strategies, assess field-level applications, and elucidate the molecular mechanisms underpinning their bioactivity.

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