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Răzvan Neagu , Violeta Popovici * , Lucia-Elena Ionescu * , Viorel Ordeanu , Andrei Biță , Diana Mihaela Popescu , Emma Adriana Ozon , Cerasela Elena Gird

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Phytochemical Screening and Antibacterial Activity of Commercially Available Essential Oils Combinations with Conventional Antibiotics Against Gram-Positive and Gram-Negative Bacteria

Răzvan Neagu [1,2], Violeta Popovici [3,*], Lucia-Elena Ionescu [4,*], Viorel Ordeanu [5], Andrei Biță [6], Diana-Mihaela Popescu [2], Emma Adriana Ozon [7] and Cerasela Elena Gîrd [1]

[1] Department of Pharmacognosy, Phytochemistry, and Phytotherapy, Faculty of Pharmacy, Carol Davila University of Medicine and Pharmacy, 6 Traian Vuia Street, 020956 Bucharest, Romania; razvan.neagu@drd.umfcd.ro (R.N.); cerasela.gird@umfcd.ro (C.E.G.)

[2] Regenerative Medicine Laboratory, "Cantacuzino" National Military Medical Institute for Research and Development, 103 Spl. Independentei, 050096 Bucharest, Romania; neagu.razvan@cantacuzino.ro (R.N.); popescu.diana@cantacuzino.ro (D.M.P.)

[3] Center for Mountain Economics, "Costin C. Kirițescu" National Institute of Economic Research (INCE-CEMONT), Romanian Academy, 725700 Vatra-Dornei, Romania

[4] Experimental Microbiology Laboratory, "Cantacuzino" National Military Medical Institute for Research and Development, 103 Spl. Independentei, 050096 Bucharest, Romania

[5] Faculty of Pharmacy, "Titu Maiorescu" University, 16 Sincai, 040314, Bucharest, Romania; viorel.ordeanu@prof.utm.ro

[6] Department of Pharmacognosy & Phytotherapy, Faculty of Pharmacy, University of Medicine and Pharmacy of Craiova, Romania; andrei.bita@umfcv.ro

[7] Department of Pharmaceutical Technology and Biopharmacy, Faculty of Pharmacy, Carol Davila University of Medicine and Pharmacy, 6 Traian Vuia Street, 020956 Bucharest, Romania; emma.budura@umfcd.ro

* Correspondence: violeta.popovici@ce-mont.ro (V.P.); ionescu.lucia@cantacuzino.ro (L.E.I.)

Abstract: The present study aims to evaluate the antibacterial activity of five commercially available essential oils (EOs): Lavender (LEO), Clove (CEO), Oregano (OEO), Eucalyptus (EEO), and Peppermint (PEO) against the most-known MDR Gram-positive and Gram-negative bacteria - *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) - alone and in various combinations. Gas Chromatography-Mass Spectrometry (GC-MS) analysis established their complex composition. Then, the antibacterial activity was investigated *in vitro* by diffusimetric antibiogram method, using sterile cellulose discs with Ø 6 mm impregnated with 10 µl of sample and sterile borosilicate glass cylinders loaded with 100 µl of sample; MIC (µg/mL) for each EO was calculated from IZD values (mm) measured after 24 hours. The following EO combinations were evaluated: OEO+CEO, CEO+EEO, CEO+PEO, LEO+EEO, and EEO+PEO. Then, the influence of each dual combination on 3 conventional antibacterial drugs – Neomycin (NEO), Tetracycline (TET), and Bacitracin (BAC) - activity was investigated. The most active EOs against *S. aureus* and *E. coli* were LEO and OEO (IZD = 40 mm). They were followed by the CEO and EEO (IZD = 20-27 mm); PEO exhibited the lowest antibacterial activity (IZD = 15-20 mm). EEO alone showed the highest inhibitory activity on *P. aeruginosa* (IZD = 25-35 mm). It was followed by the CEO, LEO, and EEO (IZD = 7-11 mm), while PEO proved no antibacterial action against it (IZD = 0 mm). Only one synergic action was recorded (OEO+CEO against *P. aeruginosa*); EEO+PEO revealed partial synergism against *S. aureus* and CEO+PEO additive behavior against *E. coli*. Two triple associations with TET showed partial synergism against *E. coli*, and the other 2 ones (with NEO and TET) evidenced the same behavior against *S. aureus*; all contained EEO+PEO or CEO+PEO. Most combinations reported indifference. However, numerous cases were of antagonism between constituents included in double and triple combinations, and EOs with the strongest antibacterial activities belong to the highest antagonistic combinations. A consistent

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statistical analysis supports our results, showing that EOs with moderate antibacterial activities could generate combinations with higher inhibitory effects based on synergistic or additive interactions.

Keywords: Essential Oils; GC-MS; Antibacterial activity; Gram-positive and Gram-negative bacteria; Diffusimetric antibiogram; Antibacterial drugs; Combinations; Interactions

1. Introduction

Currently, treating bacterial diseases is a challenge for modern medicine due to the expansion of antibiotic resistance. Medical research focuses on finding and developing new inhibitory agents for multidrug-resistant (MDR) bacteria. Another potential strategy is to combine classical antibiotic drugs with plant-derived antimicrobials: various plant extracts, phytochemicals, and essential oils (EOs).

The EO chemical composition [1] includes phenolic compounds, terpenes, terpenoids [2], phenylpropanoids, and other aliphatic and aromatic constituents with strong lipophilic properties [3]. Their content varies depending on seasonal variation, climate, subspecies, and even the oil extraction method [4,5], which can lead to consequences for their antibacterial activity [6]. It is essential to decipher the mechanisms of EO antibacterial action and consider the interactions between isolated constituents [7]. EO antibacterial effects could be due to cellular membrane disruption through protein damage, cell content leakage, motive proton force depletion, and cytoplasm coagulation [8–10]. The initial negative impact of EOs on bacterial cell membrane influences the spectrum of bacterial metabolic processes affected by responsible EO phytocompounds (ATPase, lipase, and coagulase activity inhibition, leakage of cell ions, citrate metabolic pathway disruption, efflux pump blockage, fluidization of membrane lipids) [11–14].

Various scientists combined different EOs to identify synergistic associations [15] for therapeutic or food preservative purposes [13]. Moreover, based on differences in antibacterial mechanisms, EOs were coupled with conventional antibiotics [16] because most antibacterial drugs act by a cell wall and protein synthesis inhibition [17]. These combinations were tested against MDR bacterial strains to determine their usefulness. The most studied synergies imply EOs of *Melaleuca alternifolia, Coriandrum sativum, Lippia sidoides, Thymus maroccanus, Cinnamomum zeylanicum, Syzygium aromaticum, Mentha piperita, Origanum vulgare, Rosmarinus officinalis,* combined with antibiotic drugs from various classes (beta-lactams, quinolones, aminoglycozides, chloramphenicol, tetracyclin, polypeptides) against MDR Gram-positive and Gram-negative bacteria [18].

In this context, the present study aims to investigate the antibacterial activity and potential interactions of five commercially available EOs – Lavender (LEO), Clove (CEO), Oregano (OEO), Eucalyptus (EEO), and Peppermint (PEO) - in binary combinations and triple associations with Antibiotic drugs – Tetracycline (TET), Neomycin (NEO) and Bacitracin (BAC) against *S. aureus*, *E coli,* and *P. aeruginosa*. Comparative GC-MS Analysis of autochthonous EOs and standard ones, rarely used antibiotic drugs, and selected EO combinations investigated through 2 different techniques of diffussimetric antibiogram represent the novelty of our research.

2. Results and Discussion

2.1. Gas Chromatography-Mass Spectrometry Analysis

The GC-MS chromatograms of the tested EOs are displayed in Figure 1. The main quantified constituents, expressed as percentages (%), are registered in Table 1. For each EO, both sample and standard compositions are displayed. The GC-MS chromatograms of EOs used as standards are in Supplementary Material, Figure S1. Moreover, all constituents identified and quantified in all EOs are in Table S1 from Supplementary Material.

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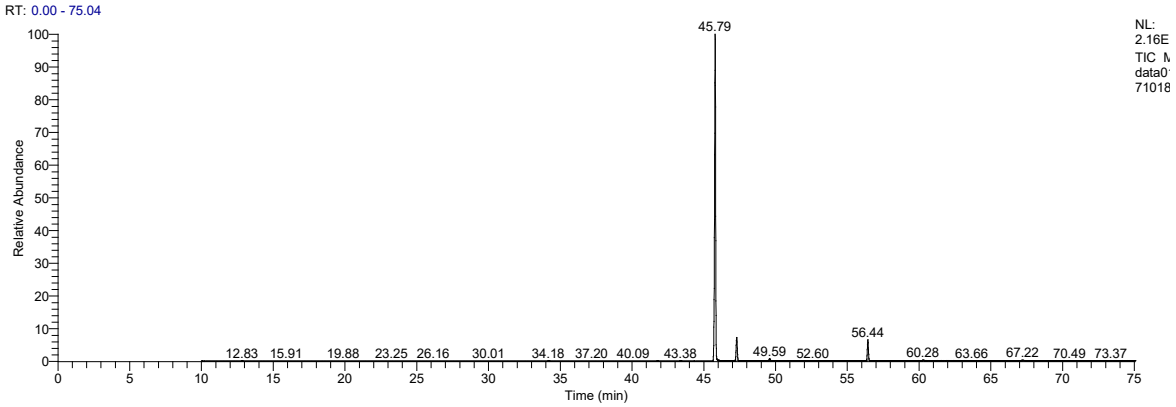

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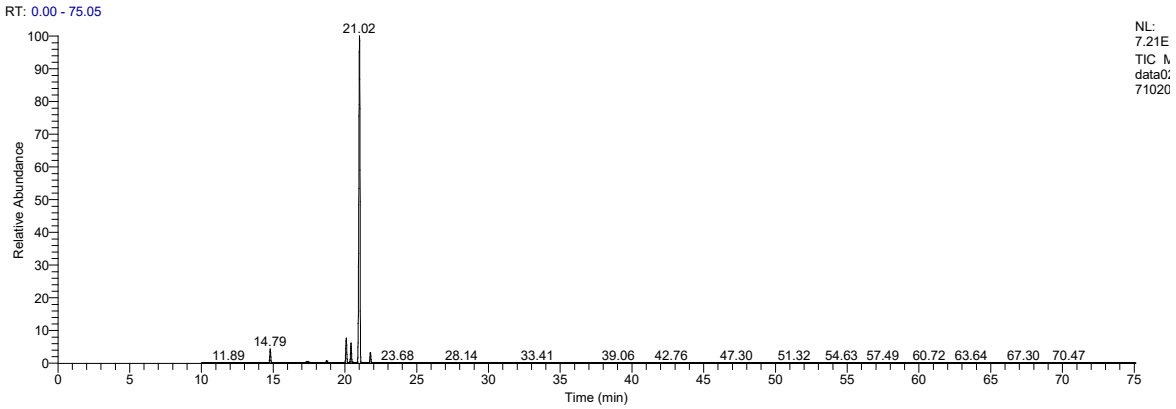

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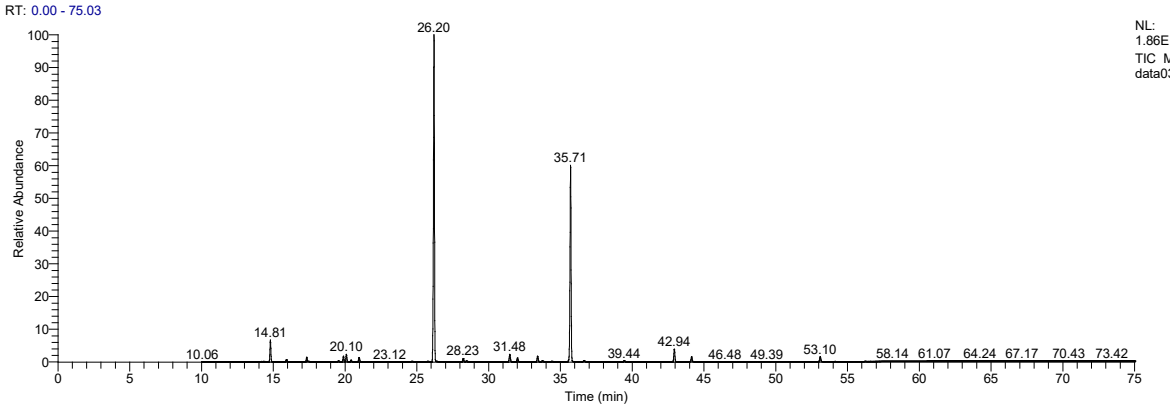

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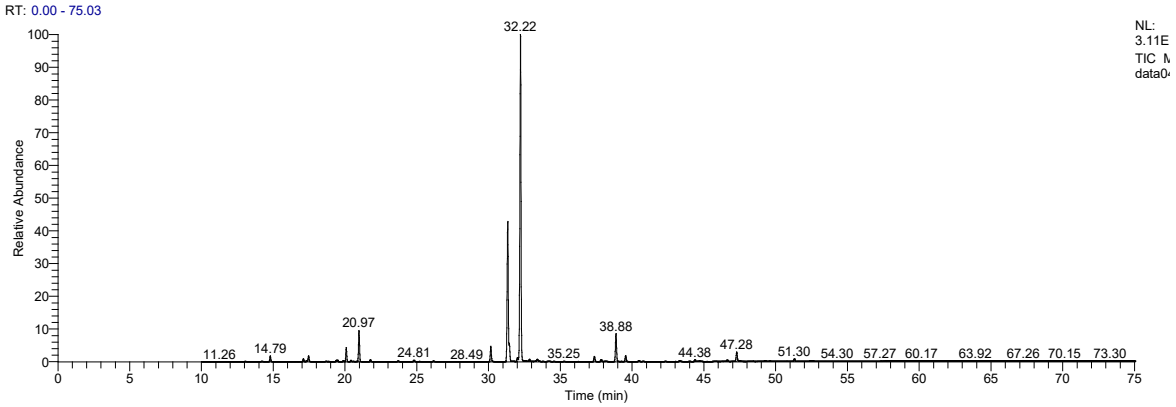

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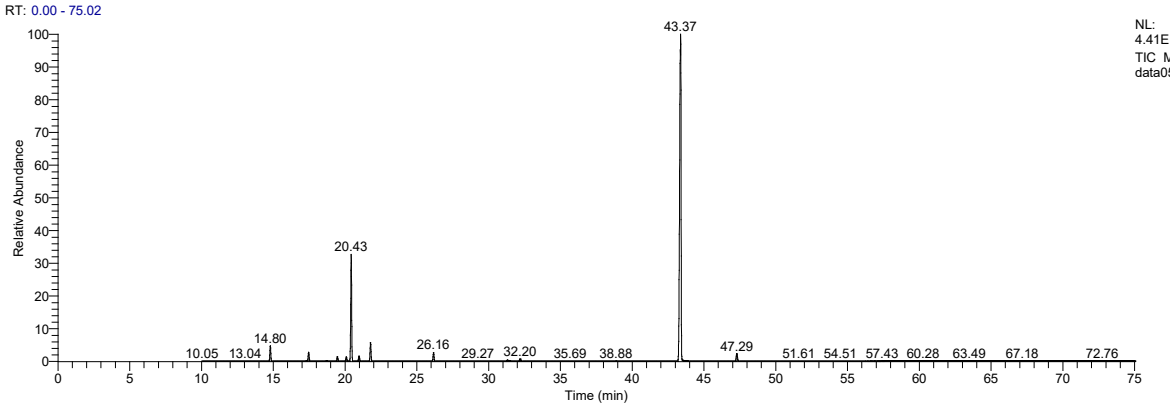

E

Figure 1. GC-MS chromatograms of EOs: Clove Essential Oil (**A**), Eucalyptus Essential Oil (**B**), Lavender Essential Oil (**C**), Peppermint Essential Oil (**D**), Oregano Essential Oil (**E**). The main constituents are evidenced: (**A**) Eugenol (RT=45.79) and Eugenol acetate (RT=56.44); (**B**) Eucalyptol (RT=21.00) and α-Pinene (RT=14.79); (**C**) Linalool (RT=26.20) and Linalyl acetate (RT=36.71); (**D**) Neoisomenthol (RT=32.22) and Limonene (RT=20.97); (**E**) *p*-Thymol (RT=43.37) and *o*-Cymene (RT=20.43).

The main constituents of each EO are as follows: Eugenol (86.22%, Caryophyllene (6.87%) and Eugenol acetate (5.75%) in CEO; Eucalyptol (83.74%), Limonene (5.45%), o-Cymene (4.13%), α-Pinene (2.81%) and γ-Terpinene (2.23%) in EEO; Linalool (52.93%), Linalyl acetate (32.31%), α-Pinene (3.16%), Nerol (2.11%), Camphor (1.34%) and Limonene (1.12%) in LEO; Neoisomenthol (55.09%), Isomenthone (26.52%), Eucalyptol (5.04%), Menthofuran (2.46%), Limonene (2.08%), and Caryophyllene (1.61%) in PEO; *p*-Thymol (72.08%), *o*-Cymene (16.26%), γ-Terpinene (2.98%), α-Pinene (2.39%), Caryophyllene (1.45%), Linalool (1.40%) and β-Pinene (1.39%) in OEO (Figure 1 and Table 1).

Eucalyptol, γ-Terpinene, Linalool, Terpinen 4-ol, Limonene, o-Cymene, α-Pinene, α-Myrcene, β-Pinene, and Camphene are common constituents in 4 EOs: EEO, LEO, PEO, and OEO. Cariophylene is found in CEO, PEO, and OEO. The specific compounds in highest contents are Eugenol and Eugenyl acetate in CEO, Isomenthone, Neoisomenthone, and Menthofuran, and Eucalyptol in PEO, *p*-Thymol, γ-Terpinene, and *o*-Cymene in OEO, Linalool, Linalil-acetate, α-Pinene and Nerol in LEO and Eucalyptol, γ-Terpinene, Limonene, *o*-Cymene and α-Pinene in EEO (Figure 1, Table S1 and Figure S1 from Supplementary Material).

Table 1. The main constituents of EOs quantified by GC-MS Analysis.

RT [min]	Compound name	CEO	EEO	LEO	PEO	OEO
45.79	Eugenol	86.2272	-	-	-	-
47.29	Caryophyllene	6.8733	-	-	1.6170	1.4581
56.44	Eugenol acetate	5.7515	-	-	-	-
21.00	Eucalyptol	-	83.7478	0.7360	5.0452	0.8904
21.77	γ-Terpinene	-	2.2315	0.0000	0.2864	2.9827
31.34	Isomenthone	-	-	-	26.5289	-
26.16	Linalool	-	0.0102	52.9348	0.0670	1.4072
31.48	Camphor	-	0.0138	1.3463	-	-
32.23	Neoisomenthol	-	-	-	55.0951	-
43.29	p-Thymol	-	-	-	0.0896	72.0862
35.69	Linalyl acetate	-	-	32.3146	-	0.0152
42.95	Nerol	-	-	2.1132	-	-
20.10	Limonene	-	5.4506	1.1219	2.0857	0.6877
20.42	o-Cymene	-	4.1323	0.2470	0.1803	16.2692

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14.79	α-Pinene	-	2.8165	3.1612	0.8279	2.3971
17.46	β-Pinene	-	0.2631	0.0478	0.8802	1.3942
30.16	Menthofuran	-	-	-	2.4656	-

Based on literature data and phytochemical profile, the binary combinations between EOs were performed: CEO, EEO, and PEO combined in 3 pairs (CEO+EEO, CEO+PEO, and EEO+PEO). Then, another 2 EOs were added in only 2 combinations: CEO+OEO and EEO+LEO. In total, 5 binary combinations of EOs were analyzed. Each binary combination was combined with 3 Conventional antibiotics: TET, NEO, and BAC, resulting in 15 triple combinations.

2.2. *Antibacterial* Activity

The selected antibiotic drugs belong to 3 different classes. Tetracycline is a broad-spectrum polypeptide antibiotic that exerts a bacteriostatic effect by reversibly binding bacterial 30S ribosomal subunit and blocking protein synthesis [19]. Neomycin is a broad-spectrum aminoglycoside [20] active against Gram-positive and Gram-negative bacteria by linking cellular ribosomes and inhibiting protein synthesis [21]. Bacitracin is a polypeptide antibacterial drug that acts against Gram-positive bacteria by inhibiting cell wall synthesis [21]. Regarding the antibacterial effects of plant-derived products, Vorobets et al. recommend, for increased accuracy, using at least 2 different techniques of diffusimetric antibiogram [22]. Therefore, we used the disk diffusion and cylinder techniques [23]. The data obtained are presented synthetically in Tables 2–4.

Several similarities are observed between the IZD (inhibition zone diameter) measured through both methods. However, the inconsistencies are due to the different diffusion levels of samples' constituents in the culture medium, especially when they are tested in a large volume (100 µl). The scale of measurement was as follows: powerful inhibitory effect at IZD ≥ 35 mm, strong inhibitory effects at 35 > IZD ≥ 25, moderate inhibitory effect at 25 > IZD ≥ 15 mm, mild inhibitory effect when 15 > IZD ≥ 10 mm, and very low inhibitory effect at IZD < 10 mm [24].

Data from Table 2 show that LEO and OEO displayed a powerful inhibitory effect on *S. aureus* (IZD = 40 mm). EEO and PEO had the lowest ones measured by DDM; however, their combination (EEO+PEO) is partially synergistic (FICI = 0.8). The anti-staphylococcal effect of antibiotic drugs increases in order NEO < BAC < TET; NEO and TET form with EEO+PEO partial synergistic triple combinations (Table 2).

Table 2. The antibacterial effects of EOs combinations and classical antibiotics on *S. aureus*, evaluated by diffusimetric method, expressed as IZD (mm), MIC (µg/mL), and FICI (for double and triple combinations).

Technique	***Cellulose disc technique***				***Cylinder technique***			
Sample	***S. aureus***							
	IZD (mm)	**MIC *(µg/mL)***	**FICI**	**Obs**	**IZD (mm)**	**MIC *(µg/mL)***	**FICI**	**Obs**
LEO	40	2.1	-	-	40	21.2	-	-
CEO	27	4.7	-	-	25	54.3	-	-
OEO	40	2.1	-	-	40	21.2	-	-
EEO	20	8.5	-	-	25	54.3	-	-
PEO	25	5.4	-	-	15	150.9	-	-
OEO+CEO	30	3.8	2.6	I	15	150.9	9.9	Ant.
CEO+EEO	15	15.1	5.0	Ant.	27.5	44.9	1.6	I
CEO+PEO	15	15.1	6.0	Ant.	20	84.9	2.2	I
LEO+EEO	40	2.1	1.2	I	30	37.7	2.5	I.
EEO+PEO	35	2.8	0.8	P.S.	25	54.3	1.4	I
NEO	7	86.7	-	-	20	106.2	-	-
OEO+CEO+NEO	25	5.7	3.98	I	20	89.2	6.67	Ant.

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CEO+EEO+NEO	25	5.7	1.94	I	37.5	25.4	1.15	I
CEO+PEO+NEO	12.5	22.8	9.33	Ant.	22	73.7	2.52	I
LEO+EEO+NEO	23.5	6.5	3.83	I	25	57.1	4.27	Ant.
EEO+PEO+NEO	26	5.3	1.66	I	15	15.9	0.53	P.S.
TET	25	6.8	-	-	29	50.5	-	-
OEO+CEO+TET	25	5.7	4.87	Ant.	40	21.2	1.8	I
CEO+EEO+TET	22	7.4	3.52	I	15	150.9	8.52	Ant.
CEO+PEO+TET	25	5.7	3.09	I	45	16.8	0.74	P.S.
LEO+EEO+TET	26	5.3	4.83	Ant.	30	37.7	3.20	I
EEO+PEO+TET	26	5.3	2.37	I	10	339.7	15.22	Ant.
BAC	17	14.7	-	-	25	67.9	-	-
OEO+CEO+BAC	30	4.0	3.02	I	30	47.2	3.77	I
CEO+EEO+BAC	32.5	3.4	1.35	I	0	-	-	-
CEO+PEO+BAC	17.5	11.6	5.38	Ant.	0	-	-	-
LEO+EEO+BAC	25	5.7	3.76	I	27	48.9	5.32	Ant.
EEO+PEO+BAC	32.5	3.4	1.25	I	20	89.2	3.54	I

LEO—Lavender Essential Oil; CEO—Clove Essential Oil; OEO—Oregano Essential Oil; EEO—Eucalyptus Essential Oil; PEO—Peppermint Essential Oil; NEO—Neomicin; TET—Tetracyclin; BAC—Bacitracin (https://microbiologie-clinique.com/antibiotic-family-abbreviation.html). IZD—Inhibition zone diameter (mm); the scale of measurement was as follows: very strong inhibitory effect at IZD ≥ 35 mm, strong inhibitory effects at 35 > IZD ≥ 25, moderate inhibitory effect at 25 > IZD ≥ 15 mm, mild inhibitory effect when 15 > IZD ≥ 10 mm, and very low inhibitory effect at IZD < 10 mm. MIC—Minimum inhibitory concentration (µg/mL), FICI—Fractional inhibitory concentration index. If IZD = 0 mm. the substance has no effect; there is no MIC. FICI ≤ 0.5 indicates synergism (S). 0.5 < FICI < 1 means partial synergism (PS); FICI = 1 indicates additive effects (Add.); 1 < FICI ≤ 4 —indifference (I); FICI > 4 is antagonism (Ant.) [25]; 4 < FICI < 10 — low antagonism; 10 < FICI < 15 — moderate antagonism; 15 < FICI < 20 —strong antagonism; FICI > 20 — very strong antagonism; FICI1 — determined by DDM (disc diffusion method); FICI2 — determined by cylinder diffusion technique. Sa — *S. aureus*, Ec — *E. coli*, Pa — *P. aeruginosa.*

Data from Table 3 shows that OEO and LEO exhibited the highest antibacterial effect on *E. coli*. Conversely, PEO and BAC had no inhibitory activity against it, and CEO, EEO, TET, and NEO displayed moderate ones. In this context, CEO+PEO reveals additive antibacterial activity on *E coli,* and triple combinations with TET (CEO+PEO+TET and EEO+PEO+TET) act partially synergistically.

Table 3. The antibacterial effect of essential oils (EOs) combinations and classical antibiotics on *E. coli* evaluated by diffusimetric method, expressed as IZD (mm), MIC (µg/mL), and FICI (for double and triple combinations).

Technique	***Cellulose disc technique***				***Cylinder technique***			
Sample	***E. coli***							
	IZD (mm)	**MIC *(µg/mL)***	**FICI**	**Obs**	**IZD (mm)**	**MIC *(µg/mL)***	**FICI**	**Obs**
LEO	40	2.1	-	-	40	21.2	-	-
CEO	22	7.0	-	-	15	150.9	-	-
OEO	45	1.7	-	-	45	16.8	-	-
EEO	25	5.4	-	-	30	37.7	-	-
PEO	0	-	-	-	10	339.7	-	-
OEO+CEO	25	5.4	4.0	I	15	150.9	10.0	Ant
CEO+EEO	15	15.1	4.9	Ant	10	339.7	11.3	Ant
CEO+PEO	22	7.0	1.0	Add.	0	-	-	-

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LEO+EEO	12	23.6	15.6	Ant	15	150.9	11.1	Ant
EEO+PEO	13	20.1	3.7	I	12	235.9	7.0	Ant
NEO	15	18.9	-		25	67.9	-	-
OEO+CEO+NEO	30	4.0	3.7	I	0	-	-	-
CEO+EEO+NEO	15	15.9	6.05	Ant	27	48.9	2.33	I
CEO+PEO+NEO	25	5.7	1.11	I	20	89.2	2.16	I
LEO+EEO+NEO	15	15.9	11.35	Ant	25	57.1	5.04	Ant
EEO+PEO+NEO	15	15.9	3.78	I	20	89.2	3.93	I
TET	14	21.7	-	-	30	47.2	-	-
OEO+CEO+TET	25	5.7	4.42	Ant	25	57.1	4.96	Ant
CEO+EEO+TET	26	5.3	1.97	I	28	45.5	2.46	I
CEO+PEO+TET	27	4.9	0.92	P.S.	26	52.8	1.59	I
LEO+EEO+TET	26	5.3	3.74	I	27.5	47.2	4.47	Ant
EEO+PEO+TET	30	4.0	0.92	P.S.	30	39.6	1.99	I
BAC	0	-	-	-	0	-	-	-
OEO+CEO+BAC	25	5.7	4.16	Ant	15	158.5	10.48	Ant
CEO+EEO+BAC	13	21.1	6.91	Ant	8	557.4	18.47	Ant
CEO+PEO+BAC	20	8.9	1.27	I	0	-	-	-
LEO+EEO+BAC	10	35.7	23.61	Ant	10	356.7	26.28	Ant
EEO+PEO+BAC	9	44.0	8.14	Ant	10	356.7	10.51	Ant

LEO—Lavender Essential Oil; CEO—Clove Essential Oil; OEO—Oregano Essential Oil; EEO—Eucalyptus Essential Oil; PEO—Peppermint Essential Oil; NEO—Neomycin; TET—Tetracycline; BAC—Bacitracin; IZD—Inhibition zone diameter (mm); the scale of measurement was as follows: very strong inhibitory effect at IZD ≥ 35 mm, strong inhibitory effects at 35 > IZD ≥ 25, moderate inhibitory effect at 25 > IZD ≥ 15 mm, mild inhibitory effect when 15 > IZD ≥ 10 mm, and very low inhibitory effect at IZD < 10 mm. MIC—Minimum inhibitory concentration (µg/mL), FICI—Fractional inhibitory concentration; FICI ≤ 0.5 indicates synergism (S). 0.5 < FICI < 1 means partial synergism (PS); FICI = 1 indicates additive effects (Add.); 1 < FICI < 4 —indifference (I); FICI ≥ 4 is antagonism (Ant.) [25]; 4 < FICI < 10 — low antagonism; 10 < FICI < 15 — moderate antagonism; 15 < FICI < 20 —strong antagonism; FICI > 20 — very strong antagonism; FICI1 — determined by DDM (disc diffusion method); FICI2 — determined by cylinder diffusion technique. Sa — *S. aureus*, Ec — *E. coli*, Pa — *P. aeruginosa.*

Table 4 also shows that PEO and BAC had no inhibitory effects on *P. aeruginosa;* most EO triple combinations with BAC act similarly through DDM. Moreover, all the other EOs evidenced very low/low/moderate antibacterial activity on *P. aeruginosa.* One binary combination is synergistic – OEO+CEO, evaluated by cylinder technique.

Table 4. The antibacterial activity of EO combinations and classical antibiotics against *P. aeruginosa,* evaluated by diffusimetric method, expressed as IZD (mm), MIC (µg/mL), and FICI (for double and triple combinations).

Technique	***Cellulose disc technique***				***Cylinder technique***			
Sample	***P. aeruginosa***							
	IZD (mm)	**MIC *(µg/mL)***	**FICI**	**Obs**	**IZD (mm)**	**MIC *(µg/mL)***	**FICI**	**Obs**
LEO	11	28.1	-	-	7	693.2	-	-
CEO	10	33.9	-	-	8	530.9	-	-
OEO	11	28.1	-	-	10	339.7	-	-
EEO	25	5.4	-	-	35	27.7	-	-
PEO	0	-	-	-	0	-	-	-
OEO+CEO	7.5	60.6	3.9	I	30	37.7	0.2	S
CEO+EEO	7.5	60.6	13.0	Ant	35	27.7	1.1	I

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CEO+PEO	7.5	60.6	1.8	I	0	-	-	-
LEO+EEO	8	53.0	11.7	Ant	16	132.7	5.0	Ant
EEO+PEO	8	53.0	9.8	Ant	17	117.5	4.2	Ant
NEO	10	42.4	-	-	20	106.2	-	-
OEO+CEO+NEO	0	-	-	-	22	73.7	1.16	I
CEO+EEO+NEO	12.5	22.8	5.56	Ant	15	158.5	7.5	Ant
CEO+PEO+NEO	15	15.9	1.29	I	0	-	-	-
LEO+EEO+NEO	9	44.0	10.73	Ant	14	182.0	2.05	I
EEO+PEO+NEO	17	12.3	2.56	I	21	80.9	3.68	I
TET	15	18.9	-	-	25	67.9	-	-
OEO+CEO+TET	0	-	-	-	7	727.9	14.23	Ant
CEO+EEO+TET	18	11.0	2.93	I	23	67.4	3.54	I
CEO+PEO+TET	14	18.2	1.49	I	15	158.5	2.62	I
LEO+EEO+TET	14	18.2	4.97	Ant	15	158.5	8.27	Ant
EEO+PEO+TET	15	15.9	3.78	I	22	73.7	3.74	I
BAC	0	-	-	-	0	-	-	-
OEO+CEO+BAC	0	-	-	-	0	-	-	-
CEO+EEO+BAC	0	-	-	-	22	73.7	2.79	I
CEO+PEO+BAC	0	-	-	-	0	-	-	-
LEO+EEO+BAC	0	-	-	-	21	80.9	3.03	I
EEO+PEO+BAC	6	99.1	18.35	Ant	20	89.2	3.22	I

LEO—Lavender Essential Oil; CEO—Clove Essential Oil; OEO—Oregano Essential Oil; EEO—Eucalyptus Essential Oil; PEO—Peppermint Essential Oil; NEO—Neomycin; TET—Tetracyclin; BAC—Bacitracin. IZD—Inhibition zone diameter (mm); the scale of measurement was as follows: very strong inhibitory effect at IZD ≥ 35 mm, strong inhibitory effects at 35 > IZD ≥ 25, moderate inhibitory effect at 25 > IZD ≥ 15 mm, mild inhibitory effect when 15 > IZD ≥ 10 mm, and very low inhibitory effect at IZD < 10 mm. MIC—Minimum inhibitory concentration (μg/mL), FICI—Fractional inhibitory concentration. If IZD = 0 mm, the substance has no effect and no MIC; FICI ≤ 0.5 —synergism (S). 0.5 < FICI < 1 —partial synergism (PS); FICI = 1 —additive effects (Add.); 1 < FICI ≤ 4 —indifference (I); FICI ≥ 4 —antagonism (Ant.) [25]; 4 < FICI < 10 — low antagonism; 10 < FICI < 15 — moderate antagonism; 15 < FICI < 20 —strong antagonism; FICI > 20 — very strong antagonism; FICI1 — determined by DDM (disc diffusion method); FICI2 — determined by cylinder diffusion technique. Sa — *S. aureus*, Ec — *E. coli*, Pa — *P. aeruginosa.*

Data from Tables 3 and 4 show that IZD sizes recorded on Gram-negative bacteria (especially on *P. aeruginosa*) are higher through the cylinder technique than those measured by DDM. Moreover, Vorobets et al. noted that, in their study, the cylinder technique was the most sensitive, recording the highest IZD values [22].

All comparative data regarding antibacterial activity expressed as IZD (mm) for EOs, Conventional Antibiotics, double and triple combinations, and p-values are displayed in Figure 2. Thus, we can see that double EO combinations lead to an increasing antibacterial activity against *P aeruginosa* in the cylinder technique (Figure 2A,C). Generally, using the cylinder technique, NEO reduces the EO inhibitory activity on bacteria teste (Figure 2D), except on *P. aeruginosa.*

On the other hand, triple combinations with TET have a higher antibacterial activity on all tested strains than double EO combinations. Contrariwise, BAC mixed with double EO combinations diminished IZD values (Figure 2F).

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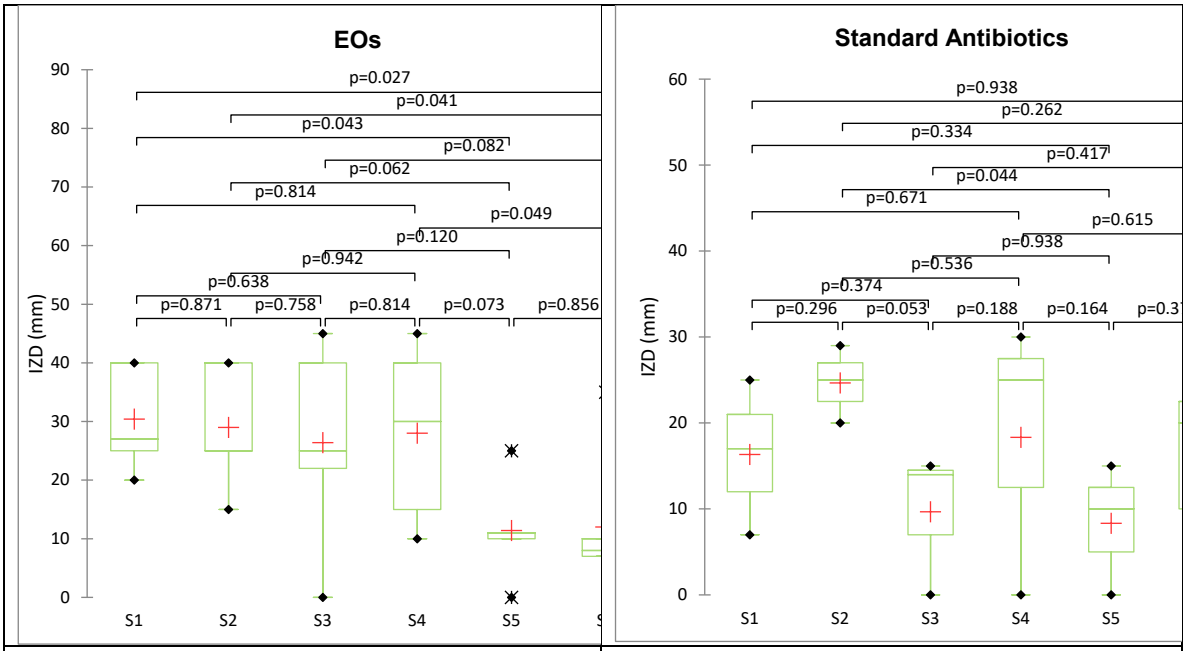

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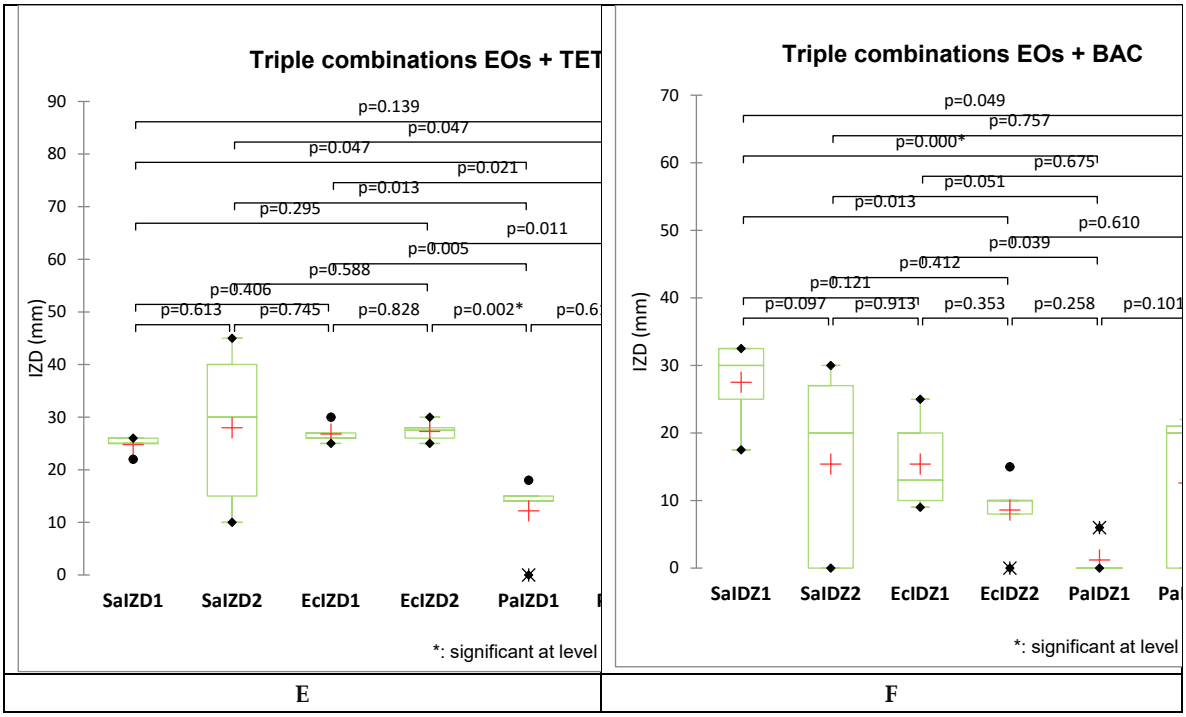

Figure 2. Comparative presentation of antibacterial activities evaluated by both diffusimetric methods (disc diffusion – 1 and cylinder technique -2) for all EOs (**A**), conventional antibiotics (**B**), Double EOs combinations (**C**), Triple combinations EOs + NEO (**D**), EOs + TET (**E**), EOs + BAC (**F**). * Statistically significant differences. LEO—Lavender Essential Oil; CEO—Clove Essential Oil; OEO—Oregano Essential Oil; EEO—Eucalyptus Essential Oil; PEO—Peppermint Essential Oil; NEO—Neomycin; TET—Tetracycline; BAC—Bacitracin; S1-S6 from A-D: S1 — SaIZD1; S2 — SaIZD2; S3 — EcIZD1; S4 — EcIZD2; S5 — PaIZD1; S6 — PaIZD2; IZD—Inhibition zone diameter (mm), the scale of measurement was as follows: powerful inhibitory effect at IZD ≥ 35 mm, strong inhibitory effects at 35 > IZD ≥ 25 moderate inhibitory effect at 25 > IZD ≥ 15 mm, mild inhibitory effect when 15 > IZD ≥ 10 mm, and no inhibitory effect at IZD < 10 mm. Sa — *S. aureus,* Ec — *E. coli,* Pa — *P. aeruginosa.*

MIC-values calculation from the IZD ones led to evaluating the interaction between EOs in binary combinations and EOs and conventional Antibiotics in triple combinations, possibly by analyzing each FIC index (Figure 3).

Bacteria	***S. aureus***		***E. coli***		***P. aeruginosa***	
Combination	SaFICI1	SaFICI2	EcFICI1	EcFICI2	PaFICI1	PaFICI2
CEO+EEO	5.00	1.60	4.90	11.30	13.00	1.10
CEO+EEO+BAC	1.35		6.91	18.47		2.79
CEO+EEO+NEO	1.94	1.15	6.05	2.33	5.56	7.50
CEO+EEO+TET	3.52	8.52	1.97	2.46	2.93	3.54
CEO+PEO	6.00	2.20	1.00		1.80	
CEO+PEO+BAC	5.38		1.27			
CEO+PEO+NEO	9.33	2.52	1.11	2.16	1.29	
CEO+PEO+TET	3.09	0.74	0.92	1.59	1.49	2.62
EEO+PEO	0.80	1.40	3.70	7.00	9.80	4.20
EEO+PEO+BAC	1.25	3.54	8.14	10.51	18.35	3.22
EEO+PEO+NEO	1.66	0.53	3.78	3.93	2.56	3.68
EEO+PEO+TET	2.37	15.22	0.92	1.99	3.78	3.74

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LEO+EEO	1.20	2.50	15.60	11.10	11.70	5.00
OEO+CEO	2.60	9.90	4.00	10.00	3.90	0.20
LEO+EEO+NEO	3.83	4.27	11.35	5.04	10.73	2.05
OEO+CEO+NEO	3.98	6.67	3.70			1.16
LEO+EEO+BAC	3.76	5.32	23.61	26.28		3.03
OEO+CEO+BAC	3.02	3.77	4.16	10.48		
LEO+EEO+TET	4.83	3.20	3.74	4.47	4.97	8.27
OEO+CEO+TET	4.87	1.80	4.42	4.96		14.23

Figure 3. Heat Map of FICI values for double and triple combinations. LEO—Lavender Essential Oil; CEO—Clove Essential Oil; OEO—Oregano Essential Oil; EEO—Eucalyptus Essential Oil; PEO—Peppermint Essential Oil; NEO—Neomycin; TET—Tetracycline; BAC—Bacitracin. IZD—Inhibition zone diameter (mm), MIC—Minimum inhibitory concentration (µg/mL), FICI—Fractional inhibitory concentration index. If IZD = 0 mm, it has no effect; no MIC exists. FICI ≤ 0.5 indicates synergism (S). 0.5 < FICI < 1— partial synergism (PS); FICI = 1 —additive effects (Add.); 1 < FICI ≤ 4 —indifference (I); FICI > 4 —antagonism (Ant.) [25] ; 4 < FICI < 10 — low antagonism; 10 < FICI < 15 — moderate antagonism; 15 < FICI < 20 —strong antagonism; FICI > 20 —very strong antagonism; FICI1 — determined by DDM (disc diffusion method); FICI2 —determined by cylinder diffusion technique. Sa — *S. aureus*, Ec — *E. coli*, Pa — *P. aeruginosa*.

Thus, a binary combination (OEO+CEO) reported synergism against *P. aeruginosa* (PaFICI2 = 0.2) assessed by cylinder technique.

Partial synergism was revealed by another EO pair (EEO+PEO) against *S. aureus* (SaFICI1 = 0.8) using DDM. EEO+PEO in triple combinations (with NEO and TET) evidenced partial synergism against *S. aureus* (SaFICI2 = 0.53) and *E. coli* (EcFICI1 = 0.92).

PEO was previously tested on *S. aureus* in association with Ciprofloxacin and against *E. coli* was found synergistic combinations with Ampicilin, Erytromicin, Oxytetracycline, and Gentamycin [26,27]. In our study, a binary EO combination (CEO+PEO) recorded additive effects against *E. coli* (EcFICI1 = 1), and another triple combination (CEO+PEO+TET) has shown partial synergism against *S. aureus* and *E. coli* (SaFICI2 = 0.74 and EcFICI1 = 0.92). Previous studies revealed synergistic effects of CEO combined with Ampicillin and Gentamycin against another *Staphylococcus* sp, *S. epidermidis.* [28].

Moreover, CEO+PEO+TET and EEO+PEO+NEO did not show antagonism (FICI < 4). Most combinations displayed indifference and/or low antagonism. There were no recorded binary or triple combinations with an exclusive antagonism against all bacteria tested (Figure 3). However, LEO+EEO+BAC revealed powerful antagonism against E. coli (FICI > 20) and LEO+EEO+NEO moderate/low ones; the binary EOs combination (LEO+EEO) has shown antagonism on both Gram-negative bacteria (strong/moderate against *E. coli* and moderate/low against *P. aeruginosa*). In contrast, the third triple combination (LEO+EEO+TET) recorded low antagonism only against *P. aeruginosa.* Another triple combinations with BAC, CEO+EEO+BAC, EEO+PEO+BAC, and OEO+CEO+BAC show antagonistic interactions against *E coli* (low/strong or low/moderate, Figure 3), while CEO+EEO and OEO+CEO act similarly, and EEO+PEO and CEO+EEO+NEO, registered low agonism against *P. aeruginosa*. CEO+EEO+TET and EEO+PEO+TET did not record an agonist behavior against both Gram-negative bacteria, while CEO+PEO+TET and EEO+PEO+NEO showed only partial synergism and indifference against all bacteria by both methods (Figure 3).

2.3. *Data Analysis*

The correlations between chemical composition and antibacterial activity (expressed as IZD) were analyzed through Principal Component Analysis (Figure 4A–C).

The Correlation biplot from Figure 4A has 2 principal components which explain total data variances (PC1 = 62.52% and PC2 = 37.48%). All antibacterial activities and constituents are linked to

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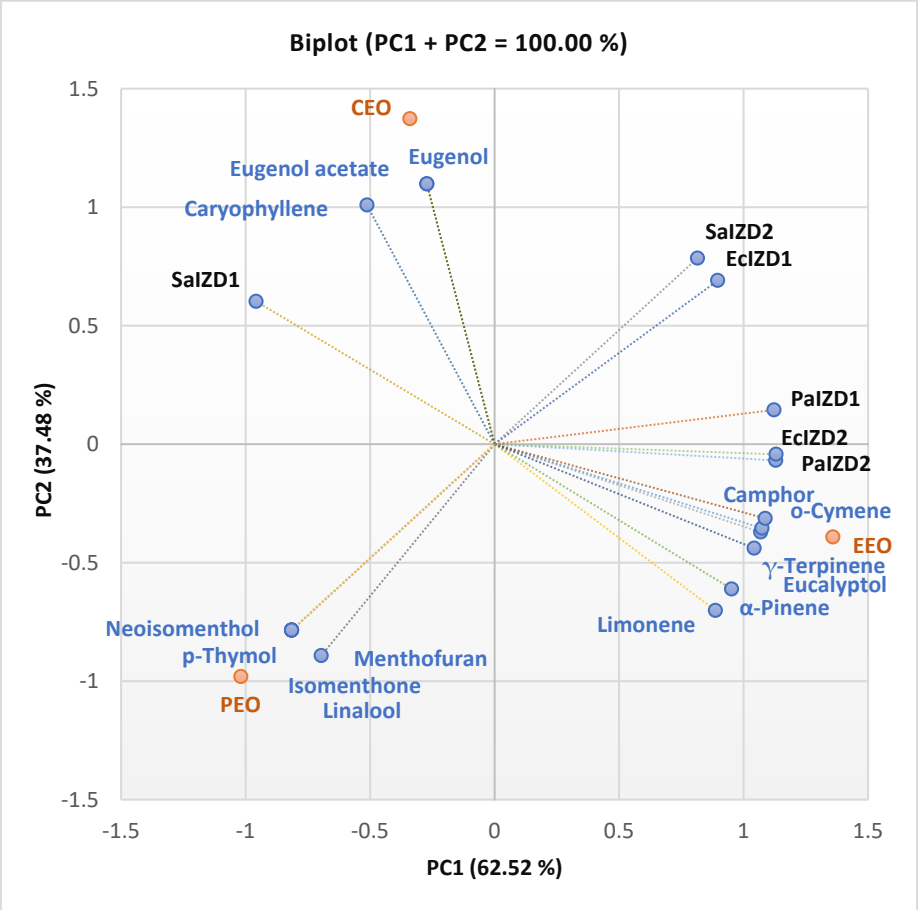
PC1, while only 4 compounds are associated with PC2 (Linalool, Eugenol acetate, Eugenol, and Caryophyllene). The Correlation matrix from Supplementary Material and Figure 3A indicates that Eucalyptol, Limonene, *o*-Cymene, γ-Terpinene, and Camphor are highly correlated with PaIZD and EcIZD evaluated by cylinder technique ($r = 0.936 – 0.976$, $p > 0.05$) and strongly correlated with PaIZD1 ($r = 0.864 – 0.918$, $p > 0.05$). Limonene and α-Pinen display a good correlation with PaIZD2 and EcIZD2 ($r = 0.807 – 0.873$, $p > 0.05$) and a moderate one with PaIZD1 ($r = 0.698$, $r = 0.766$, $p > 0.05$). α-Pinen shows a significant negative correlation with SaIZD1 ($r = -0.999$, $p < 0.05$), and all the others, previously mentioned, display a high one ($r = - [0.961\text{-}0.994]$, $p < 0.05$).

Contrariwise, Caryophyllene, Eugenol, and Eugenol acetate are highly and moderately correlated with SaIZD1 ($r = 0.858$, $r = 0.721$, $p < 0.05$). Neoisomenthol, Isomenthol, Menthofuran, and p-Thymol substantially negatively correlate with SaIZD2 ($r = -0.999$, $p < 0.05$), while Linalool shows a high negative one ($r = 0.990$, $p > 0.05$). All exhibit a considerable negative correlation with EcIZD1 ($r = - [0.968, 0.994]$, $p > 0.05$) and a good to moderate one with EcIZD1 ($r = - [0.803, 0.711]$, $p > 0.05$).



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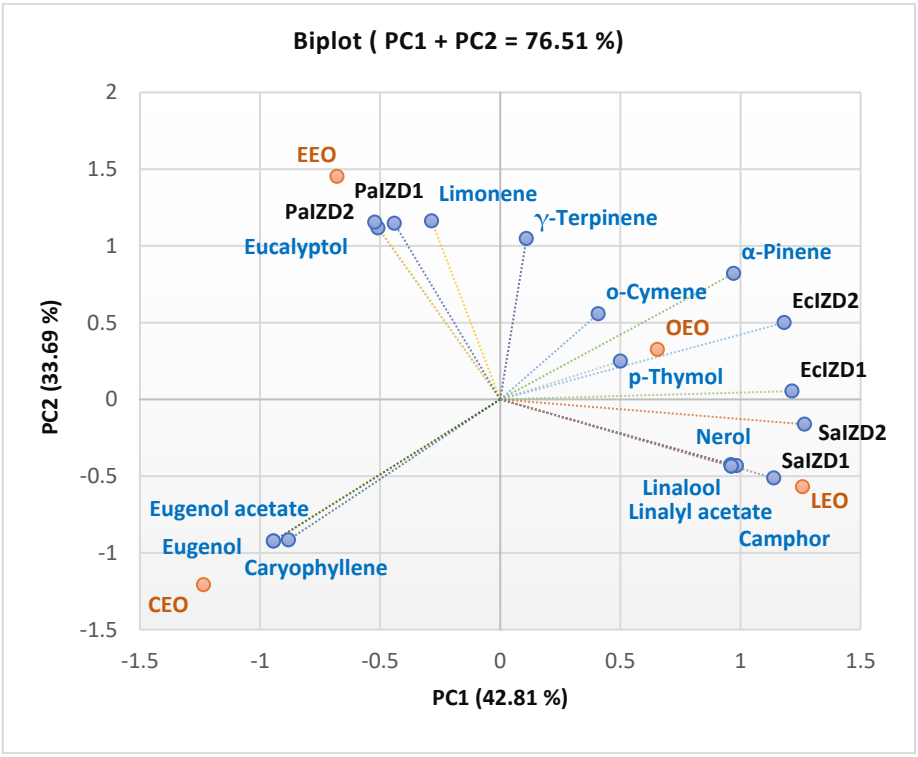

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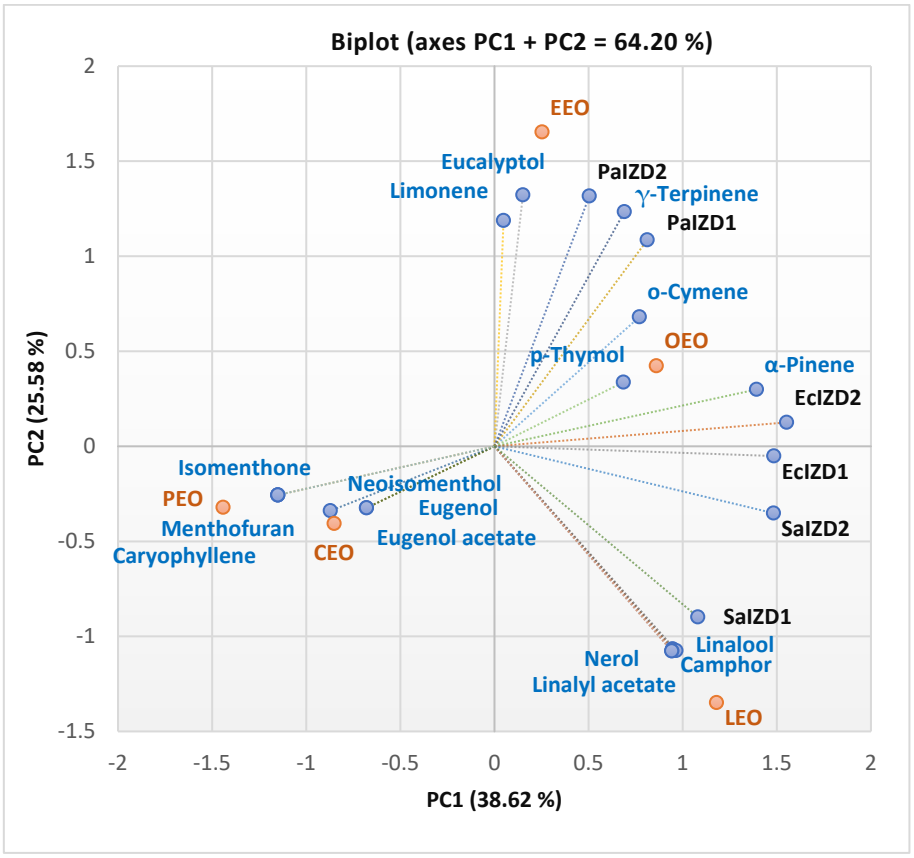

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Figure 4. Correlations between EOs antibacterial activity (expressed as IZD) and the main phytoconstituents: (**A**) EEO-PEO-CEO group; (**B**) CEO-OEO-EEO-LEO group; (**C**) All EOs. IZD—Inhibition zone diameter (mm), Sa — *S. aureus*, Ec — *E. coli*, Pa — *P. aeruginosa*, LEO—Lavender Essential Oil; CEO—Clove Essential Oil; OEO—Oregano Essential Oil; EEO—Eucalyptus Essential Oil; PEO—Peppermint Essential Oil; IZD1 —determined by DDM (disc diffusion method); IZD2 — determined by cylinder technique.

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Various authors revealed synergy between Eugenol and Chloramphenicol, Norfloxacin and Oxacillin against *E. coli* and *P. aeruginosa,* Menthol with Oxytetracycline against *E coli*, Thymol with Norfloxacin and Bacitracin against *S. aureus*, and Novobiocin and Penicillin against *E. coli* [18].

Figure 4B displays the correlations between antibacterial activity and phytochemicals quantified in CEO, OEO, LEO, and EEO. In the correlation biplot, the 2 principal components explain 76.58% of the total data variance (PC1 = 42.81% and PC2 = 33.69%). Antibacterial activities against *S. aureus* and *E. coli* and most phytochemicals are linked with PC1. In contrast, inhibitory activity on *P. aeruginosa* and Eucalyptol, Limonene, Caryophyllene, and γ-Terpinene are associated with PC2. Eucalyptol and Limonene are significantly correlated with PaIZD (1 and 2). α-Pinen, o-Cymene, and p-Thymol show a good to moderate correlation with EcIZD (1 and 2), r = 0.821 – 0.589, $p < 0.05$. Finally, Antibacterial activity against S. aureus is moderately correlated with *p*-Thymol, Camphor, Linalool, Linalyl acetate, and Nerol.

In the Correlation biplot from Figure 4C, the two principal components explained 64.20% of the total data variance, with 38.62% attributed to the first (PC1) and 25.58% to the second (PC2). The PC1 was associated with SaIZD, EcIZD, and some EOs constituents (α-Pinene, Neoisomenthol, Isomenthol, and Menthofuran). At the same time, PC2 was linked with PaIZD and other phytochemicals (Eucalyptol, Lymonene, γ-Terpinene, Camphor, Linalool, Linalyl-acetate, and Nerol). It shows the place of all EOs reported to phytochemical profile and antibacterial activity on Gram-positive and Gram-negative bacteria evaluated through both methods.

Using PCA, we analyzed the influence of each component's antibacterial activity (expressed as MIC (μg/mL)) on those of double and triple combinations and then on interactions (expressed as FICI value). All results are illustrated in Figure 5.

Figure 5A shows that SaMIC (1 and 2) have a significant moderate to good correlation with SaFICI (1 and 2): r = 0.759, r = 0.892, $p < 0.05$, while PaMIC1 moderately correlates with PAFICI1 (r = 0.670, $p < 0.05$). EcMIC (1 and 2) show a low to moderate correlation with EcFICI (1 and 2), r = 0.469 – 0.589, $p > 0.05$ (Figure 5A).

When TET is selected antibiotic (Figure 5B), EcMIC (1 and 2) is moderately correlated with EcFICIs (r = 0.628, r = 0.567, $p > 0.05$). The same observation is available for PaMIC1 – PaFICI1 (r = 0.585, $p < 0.05$), while PaMIC2 shows a very low correlation with PaFICI2 (r = 0.075, $p > 0.05$). SaMICs display a minimal negative correlation with SaFICI (r = −0.034, r = −0.192, $p < 0.05$).

Figure 5C, with triple combinations containing BAC, indicates a significant powerful correlation between EcMICs and EcFICIs (r = 0.913, r = 0.850, $p < 0.05$), and PaMIC1 and PaFICI1 (r = 0.865, $p < 0.05$). Moreover, EcFICI1 significantly intercorrelates with EcFICI2, and PaFICI1 reports a moderate intercorrelation with PaFICI2 (r = 0.915, r = 0.654, $p < 0.05$).

In the case of CEO+OEO+EEO+LEO combined with all 3 antibiotics, Figure 5D shows that EcMIC2 is significantly correlated with EcFICI2 (r = 0.905, $p < 0.05$), while EcMIC1 has an appreciable correlation with EcFICI1 (r = 0.754, $p < 0.05$). PaMIC1 and SaMIC2 are moderately correlated with PaFICI1 and SaFICI2 (r = 0.607, r = 0.579, $p < 0.05$). Moreover, EcFICI1 is highly correlated with EcFICI2 (r = 0.890, $p < 0.05$) and SaFICI1 moderately correlates with SaFICI2 (r = 0.669, $p < 0.05$).

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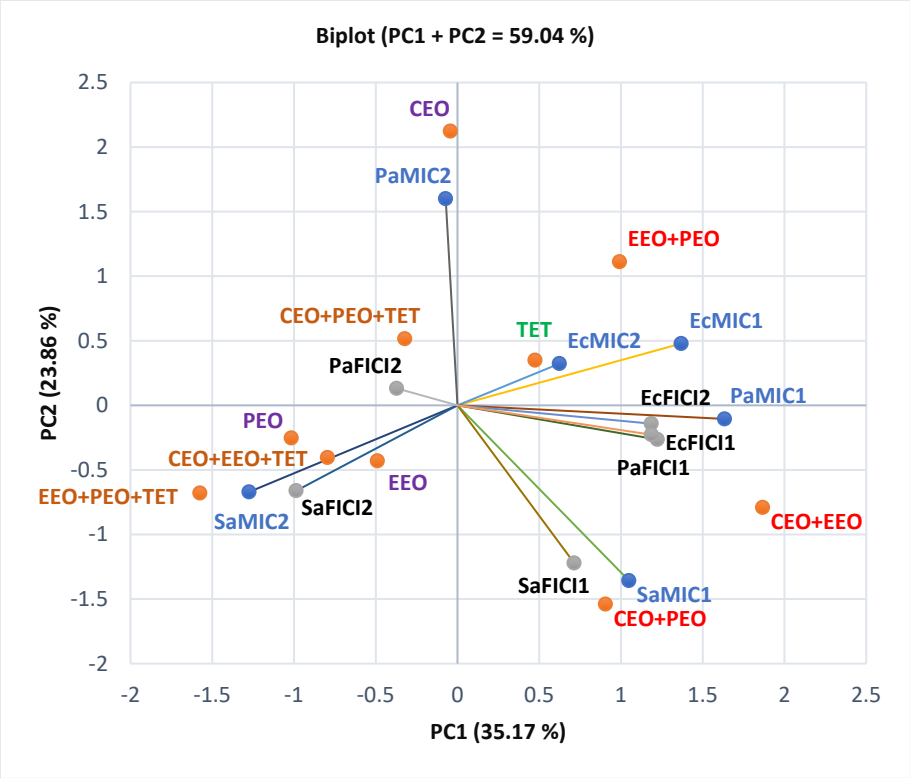

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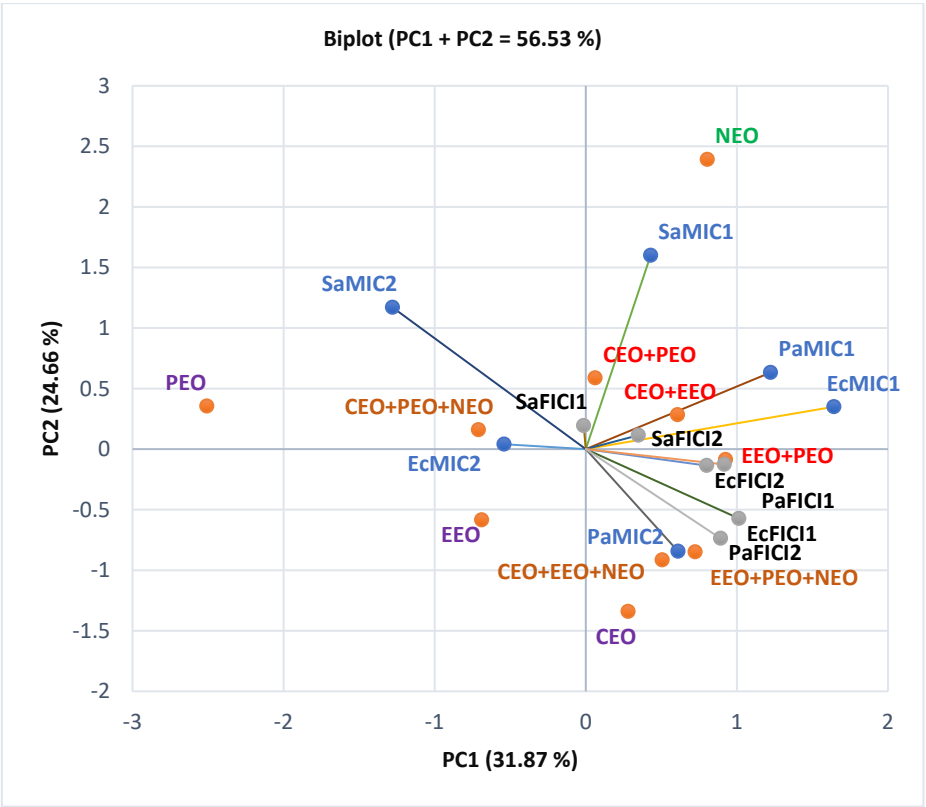

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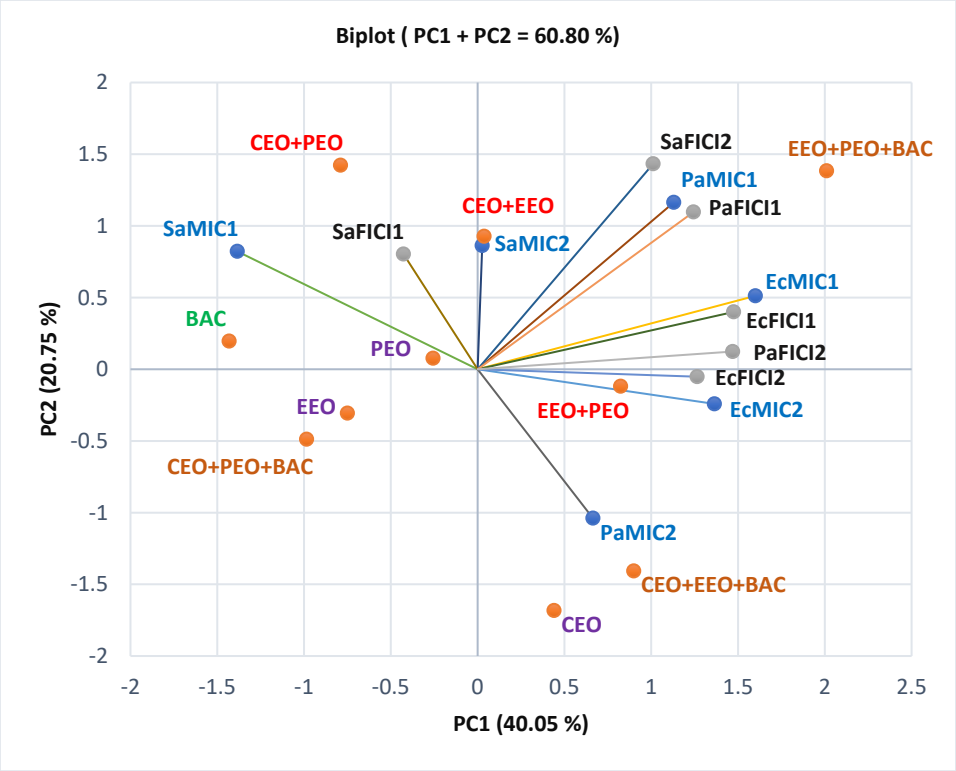

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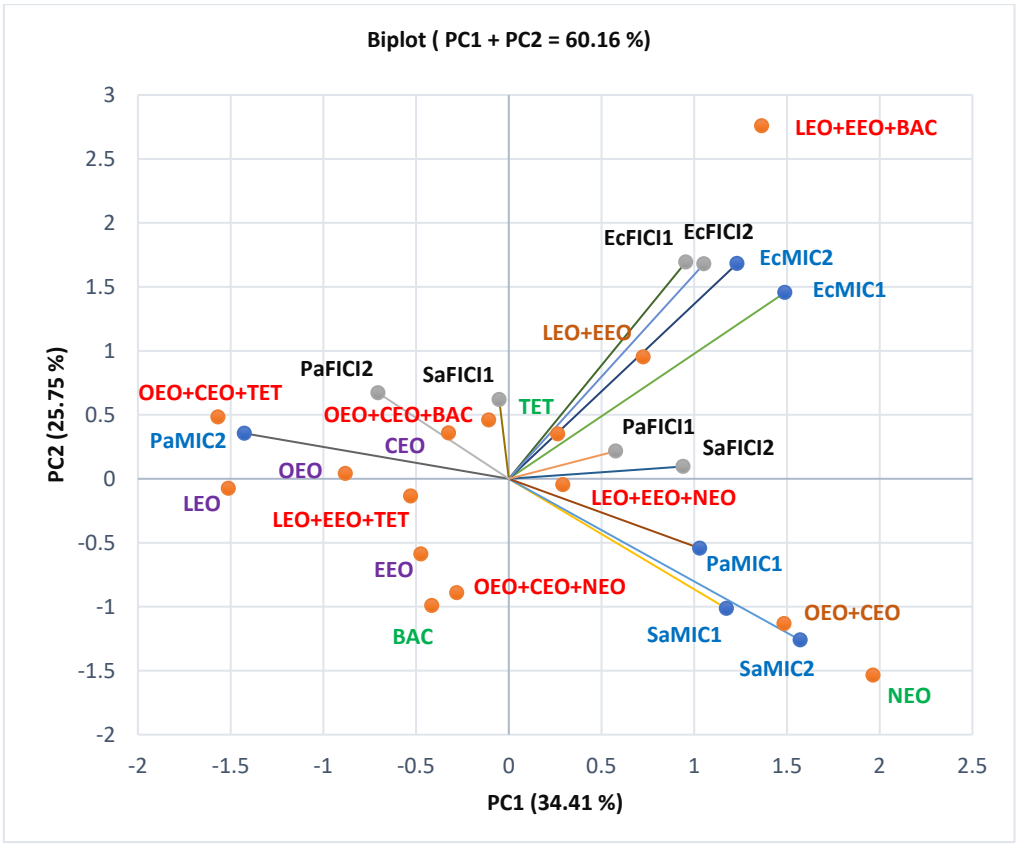

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Figure 5. Correlation of MIC values of EOs and those of Conventional Antibiotics in double and triple combinations and FICI value. (**A**–**C**). CEO + PEO + EEO Group in triple combinations with various antibiotics: (**A**) TET, (**B**) NEO, and (**C**) BAC; (**D**) CEO + OEO + EEO + LEO In double and triple combinations, with 3 antibiotics. LEO—Lavender Essential Oil; CEO—Clove Essential Oil; OEO—Oregano Essential Oil; EEO—Eucalyptus Essential Oil; PEO—Peppermint Essential Oil; NEO—

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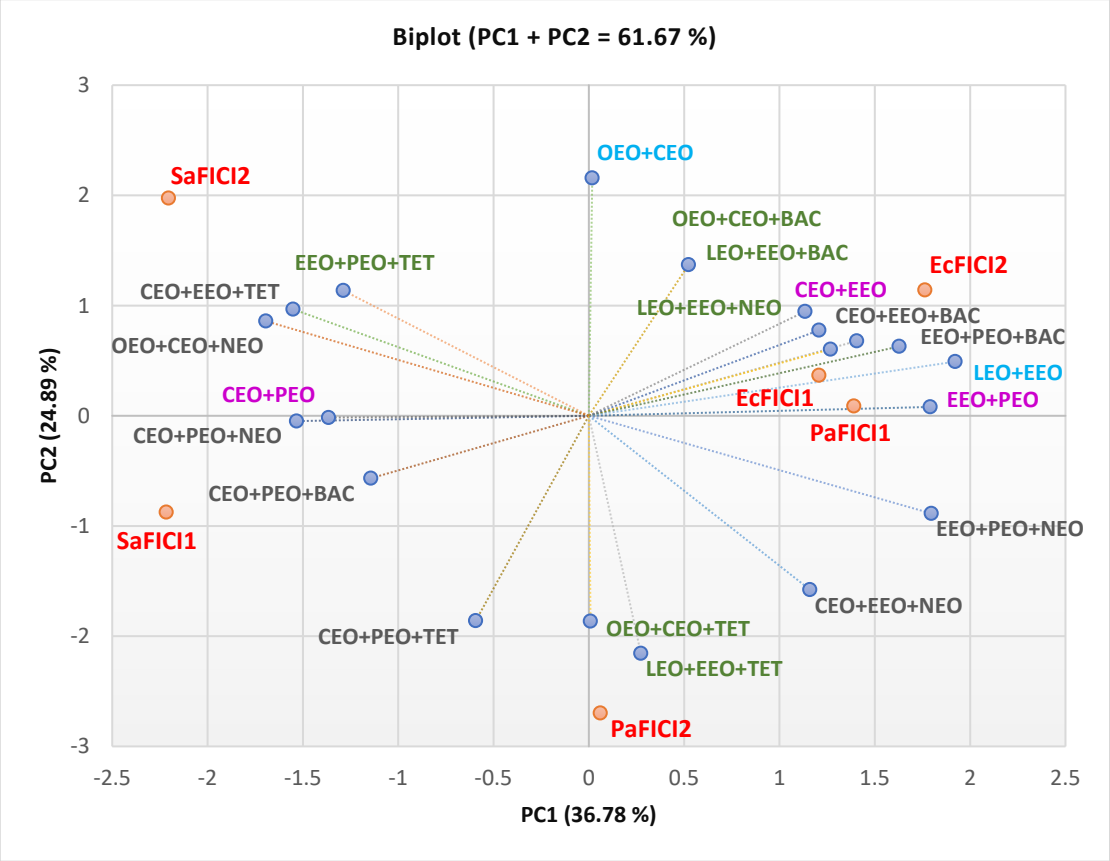
Neomycin; TET—Tetracycline; BAC—Bacitracin (https://microbiologie-clinique.com/antibiotic-family-abbreviation.html). IZD—Inhibition zone diameter (mm), MIC—Minimum inhibitory concentration (μg/mL), FICI—Fractional inhibitory concentration index. FICI1 was determined by DDM (disc diffusion method), and the cylinder diffusion technique provided FICI2. Sa — *S. aureus*, Ec — *E. coli*, Pa — *P. aeruginosa*.

All combinations and FICI values places are illustrated in the Correlation Biplot (Figure 6A) and the Dendrogram (Figure 6B).



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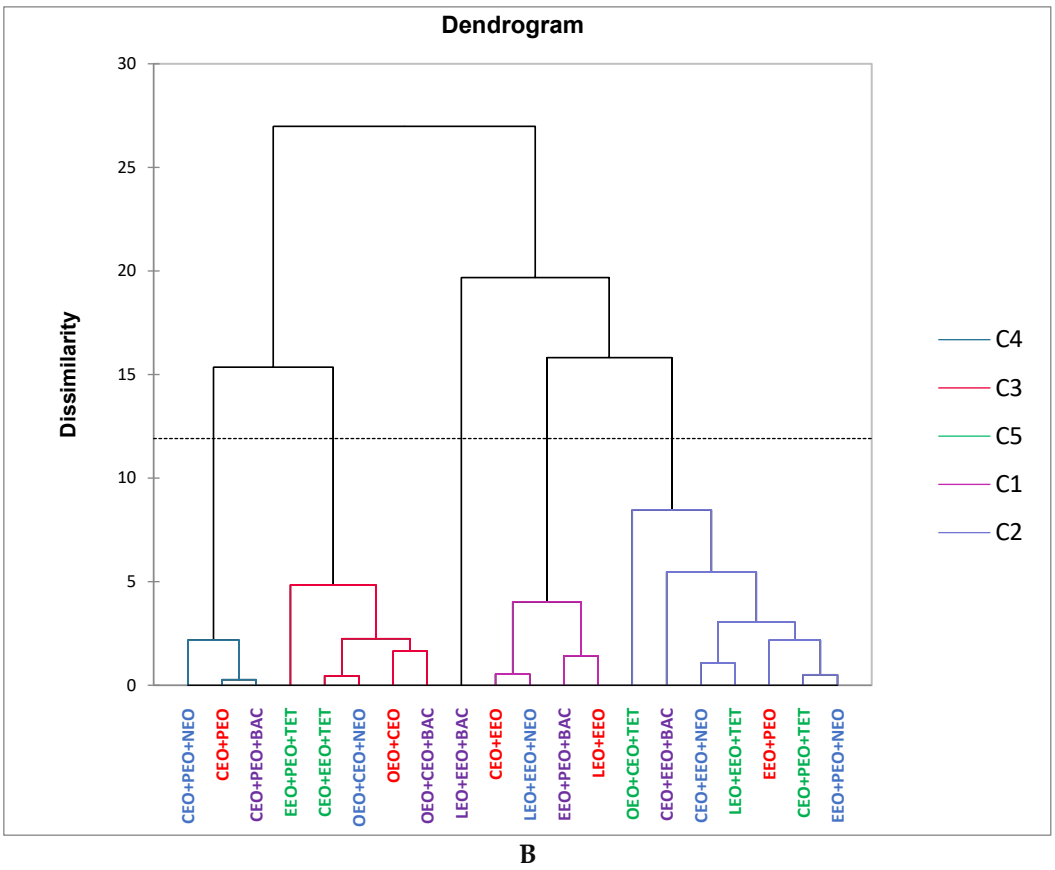

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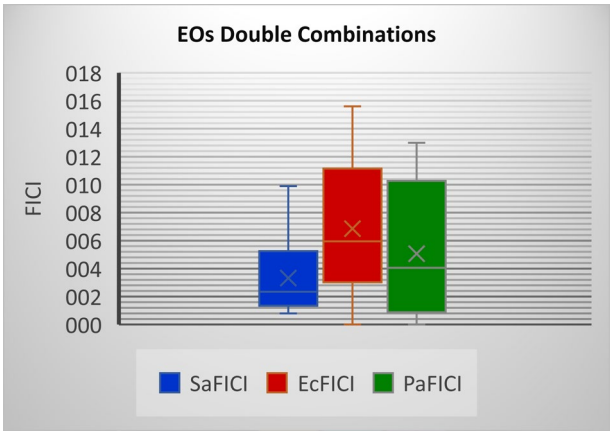
Figure 6. (**A**) Correlations between all tested double and triple combinations and FICI values. (**B**) AHC Dendrogram, with 5 Clusters: C1 = LEO+EEO+NEO; C2 = CEO+EEO+NEO; C3 = CEO+EEO+TET; C4 = CEO+PEO; C5 = LEO+EEO+BAC. LEO—Lavender Essential Oil; CEO—Clove Essential Oil; OEO—Oregano Essential Oil; EEO—Eucalyptus Essential Oil; PEO—Peppermint Essential Oil; NEO—Neomycin; TET—Tetracycline; BAC—Bacitracin. FICI—Fractional inhibitory concentration index. FICI1 is determined by DDM (disc diffusion method), and the cylinder diffusion technique determines FICI2. Sa — *S. aureus*, Ec — *E. coli*, Pa — *P. aeruginosa*.

An overview of the influence of each conventional antibiotic added on binary EO combinations on the interactions related to antibacterial activity determined through both diffusimetric techniques is displayed in Figure 7.



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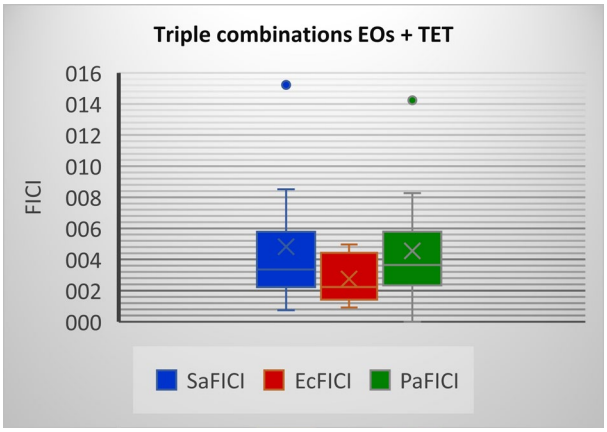

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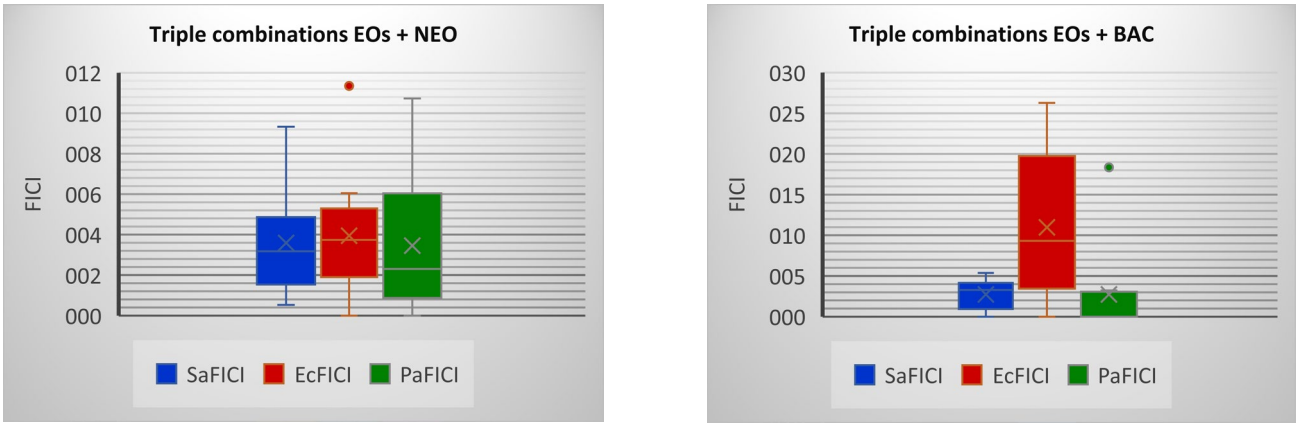

C D

Figure 7. Box&Whisker Plots display the overall FICI changes when binary EO combinations are associated with Conventional Antibiotics. (**A**) Binary EO combinations**;** (**B**) Triple combinations with TET; (**C**) Triple combinations with NEO; (**D**) Triple combinations with BAC. LEO—Lavender Essential Oil; CEO—Clove Essential Oil; OEO—Oregano Essential Oil; EEO—Eucalyptus Essential Oil; PEO—Peppermint Essential Oil; NEO—Neomycin; TET—Tetracycline; BAC—Bacitracin. FICI—Fractional inhibitory concentration index. Sa — *S. aureus*, Ec — *E. coli*, Pa — *P. aeruginosa.*

Compared to EO binary combinations, TET and NEO diminished EcFICI and PaFICI (Figure 7A–C). Contrariwise, BAC substantially decreased PaFICI and increased EcFICI (Figure 7A,D).

4. Materials and Methods

4.1. *Materials and Equipment*

Commercially available EOs (OEO, CEO, EEO, PEO, and LEO) were provided by "Laboratoarele Fares Biovital SRL" (Orastie, Romania) [29].

EOs used as GC-MS Standards were purchased from established manufacturers: Oregano Essential oil was provided by Carl Roth GmbH & Co. KG (Karlsruhe, Germany), Clove and Peppermint Essential oils were supplied by Sigma Aldrich Corporation (St. Louis, MO, USA), and Lavender and Eucalyptus Essential oils were provided by HWI Group (Ruelzheim, Germany). Certificates of Analysis for proving their purity are available in Supplementary Material.

All chemicals and reagents were of analytical grade. Conventional antibiotics (Neomycin sulfate, Bacitracin, and Tetracycline hydrochloride) and Poly (ethylene glycol)-block-poly (propylene glycol)-block-poly (ethylene glycol) (Poloxamer 407) were supplied from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). Bacterial strains were purchased from authorized suppliers for the sale of ATCC products: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). The bacterial strains were cultured in Mueller Hinton agar (Thermo Fisher, Dreieich, Germany).

The laboratory equipment consisted of EnSight multimodal plate reader (PerkinElmer Inc., Waltham, MA, USA), adjustable incubator (Memmert GmbH + Co.KG. Büchenbach. Germany), microplate shaking incubator (Heidolph Instruments GmbH & Co. KG. Schwabach, Germany), laminar flow class II microbiological hood (Jouan SA, Saint-Herblain, Pays de la Loire, France), Evoqua double water still (Evoqua Water Technologies GmbH. Barsbüttel, Germany), an electronic balance (Ohaus Corporation, Parsippany, NJ, USA), and NUNC™ MaxiSorp™ 96-well plates (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA).

4.2. *GC-MS Analysis*

GC/MS analysis was performed to identify and quantify the constituents of essential oils of Cloves, Eucalyptus, Lavender, Peppermint, and oregano by perceptible elucidation comparing their mass spectra with reference spectra (NIST Library 2020). We used a Thermo Scientific Focus GC with

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an AI/AS 3000 autosampler coupled with a DSQ II mass detector and equipped with a TraceGOLD TG-624 column 60 × 0.25 × 1.4 (mm). The injection volume was 1 µL, at 1.3 mL/min flow and a split ratio of 1:50 using helium as carrier gas. The initial oven temperature was set to 110 °C and maintained for 5 min. After, it was increased to 220 °C at the rate of 2 °C/min and maintained for 15 min. MS transfer line temperature was kept at 240 °C. The ion source temperature was 230 °C, and the electron impact ionization (EI) was set at 70 eV. Spectra were scrutinized in the full scan mode over the 50 to 450 mass range, and all constituents' retention times (RT) were recorded [30].

4.3. *In Vitro Evaluation of Antibacterial Activity*

The in vitro evaluation of the antibacterial activity of the tested solutions was done using a semi-quantitative method (diffusimetric antibiogram adapted from Kirby-Bauer [31]). The results - expressed as IZD (mm) - were measured after 24 h of contact between tested solutions and pathogenic bacteria.

4.3.1. Bacterial Inoculum

The bacteria inoculum was obtained using the direct colony suspension method (CLSI) [32]. Hence, a saline suspension (0.9%) of bacterial colonies from a 24-hour agar plate was prepared, adjusting it to the 0.5 McFarland standard, with around 10^8 CFU/mL (CFU = colony-forming unit) [32].

4.3.2. Sample Solutions

The O/W emulsions were prepared with an EO concentration of 30% *w/w*; the emulsifier was Poloxamer 407 5% in water, as previously mentioned [29,33].

Each O/W emulsion was diluted with double distilled water to assess each EO stock solution's final concentration (25 mg/mL)[29].

The binary EO combinations were prepared in a 1:1 ratio.

The antibiotic drugs (Neomycin, Bacitracin, and Tetracycline) were dissolved in double distilled water with a stock solution's final concentration of 5120 µg/mL.

The triple combinations contain equal parts of each constituent (1:1:1).

4.3.3. Technique

The minimum and maximum diameters of the inhibition zones (IZD) were measured in mm, and the arithmetic mean was calculated using one decimal place. Sterile cellulose discs with Ø 6 mm were impregnated with 10 µL of the sample solutions. Sterile cylinders (made of borosilicate glass) with Ø 6 mm were loaded with 100 µL of previously prepared solutions.

The Petri plates with the test samples were incubated at 37 °C and read after 24 hours. The Minimum Inhibitory Concentration (MIC) is calculated from the arithmetic mean of IZD.

The negative control was a non-impregnated sterile cellulose disk.

The indicative method to evaluate the Quantity/Effect correlation (Q/Ef index) specific for antimicrobial agents screening consists of MIC-value calculation:

$$Effect\ (MIC) = \frac{\text{Q (µg)}}{\text{V (µL)}}$$

Q — Quantity (µg) of sample solution applied.

V —the volume of the environment in which the tested sample diffused and inhibited microbial multiplication.

V is calculated from the average of the IZD, to which the classic cylinder volume formula is applied:

$$\text{V (µL)} = \pi R^2 \times G$$

π = 3.14 (the circle constant);

R^2 — the square of the radius from the mean IZD (mm);

G — the cylinder generator, respectively the thickness of the culture medium layer (mm).

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$$V\ (\mu L) = (\phi\ (mm) \times 2^{-1})^2 \times 3.14 \times 3\ (mm)$$

The fractional inhibitory concentration index (FICI) was calculated for double and triple combinations as follows:

$FICI_{AB} = (MIC_{A\ in\ combination} / MIC_{A\ alone}) + (MIC_{B\ in\ combination} / MIC_{B\ alone})$

$FICI_{ABC} = (MIC_{A\ in\ combination} / MIC_{A\ alone}) + (MIC_{B\ in\ combination} / MIC_{B\ alone}) + (MIC_{C\ in\ combination}/MIC_{C\ alone})$

FICI values were interpreted as follows: FICI ≤ 0.5 —synergism (S); 0.5 < FICI < 1 —partial synergism (PS); FICI = 1 —additive effects (Add.); 1 < FICI ≤ 4 —indifference (I); FICI > 4 —antagonism (Ant.) [25].

4.4. Data Analysis

The differences between the combination groups were determined using Box&Whisker Plots from Microsoft 365 Excel® v.2023 (Microsoft Corporation, Redmond, WA, USA) [29]. The statistically significant values were marked in Figure 3 with superscripts [32]. The Kruskal Wallis samples comparison, and the correlations between variable parameters examined through Principal Component Analysis [34] were performed with XLSTAT 2023.1.4. by Lumivero (Denver, CO, USA) [35].

The statistical significance was established at $p < 0.05$ [36].

5. Conclusions

The present research performed the GS-MS phytochemical screening and the antibacterial activity evaluation of five commercially available essential oils: Lavender Essential Oil, Clove Essential Oil, Oregano Essential Oil, Eucalyptus Essential Oil, and Peppermint Essential Oil. The antibacterial effects on Gram-positive (*S. aureus*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria were evaluated on each EO alone, five binary EO combinations, and 15 triple combinations with conventional antibiotics (Tetracycline, Neomycin, and Bacitracin). Two different techniques of diffusimetric antibiogram adapted from the Kirby Bauer method were used, and data recorded were correlated with the main phytochemicals of each EO. The analysis of interactions between EOs revealed one synergic binary combination (OEO+CEO) against *P. aeruginosa,* another partial synergy (EEO+PEO) against *S. aureus*, and another additive one (CEO+PEO) against *E. coli.* Four partially synergistic triple combinations with antibiotic drugs (TET and NEO) were revealed against *S. aureus* (CEO+PEO+TET and EEO+PEO+NEO) and *E. coli* (CEO+PEO+TET and EEO+PEO+TET). At the same time, BAC was involved only in Indifferent or antagonistic triple associations with EOs. Our results could enrich the scientific database through an appreciable diversity of tested combinations regarding EOs and conventional selected antibiotics. Further research could investigate the inhibitory effects of these combinations against other bacteria and even various pathogenic fungi. Moreover, the antibacterial effects of other potential double and triple different EO combinations of these five essential oils could be explored to find the most effective ones.

Supplementary Materials: The following supporting information can be downloaded at: Preprints.otg.

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References

1. Dhifi, W.; Bellili, S.; Jazi, S.; Bahloul, N.; Mnif, W. Essential Oils' Chemical Characterization and Investigation of Some Biological Activities: A Critical Review. *Medicines* **2016**, *3*. https://doi.org/10.3390/medicines3040025.
2. Masyita, A.; Mustika Sari, R.; Dwi Astuti, A.; Yasir, B.; Rahma Rumata, N.; Emran, T. Bin; Nainu, F.; Simal-Gandara, J. Terpenes and Terpenoids as Main Bioactive Compounds of Essential Oils, Their Roles in Human Health and Potential Application as Natural Food Preservatives. *Food Chem X* **2022**, *13*, 100217. https://doi.org/10.1016/j.fochx.2022.100217.
3. Manion, C.R.; Widder, R.M. Essentials of Essential Oils. *American Journal of Health-System Pharmacy* **2017**, *74*, e153–e162. https://doi.org/10.2146/ajhp151043.
4. Aziz, Z.A.A.; Ahmad, A.; Setapar, S.H.M.; Karakucuk, A.; Azim, M.M.; Lokhat, D.; Rafatullah, Mohd.; Ganash, M.; Kamal, M.A.; Ashraf, G.M. Essential Oils: Extraction Techniques, Pharmaceutical And Therapeutic Potential - A Review. *Curr Drug Metab* **2018**, *19*. https://doi.org/10.2174/1389200219666180723144850.
5. Yeddes, W.; Ouerghemmi, I.; Hammami, M.; Gadhoumi, H.; Affes, T.G.; Mohamed, S.N.; Aidi-Wannes, W.; Witrowa-Rajchert, D.; Saidani-Tounsi, M.; Nowacka, M. Optimizing the Method of Rosemary Essential Oils Extraction by Using Response Surface Methodology (RSM)-Characterization and Toxicological Assessment. *Sustainability (Switzerland)* **2022**, *14*, 3927. https://doi.org/10.3390/su14073927.
6. Khodaei, N.; Nguyen, M.M.; Mdimagh, A.; Bayen, S.; Karboune, S. Compositional Diversity and Antioxidant Properties of Essential Oils: Predictive Models. *LWT* **2021**, *138*. https://doi.org/10.1016/j.lwt.2020.110684.
7. Piatkowska, E.; Rusiecka-Ziółkowska, J. Influence of Essential Oils on Infectious Agents. *Advances in Clinical and Experimental Medicine* **2016**, *25*.
8. Mutlu-Ingok, A.; Devecioglu, D.; Dikmetas, D.N.; Karbancioglu-Guler, F.; Capanoglu, E. Antibacterial, Antifungal, Antimycotoxigenic, and Antioxidant Activities of Essential Oils: An Updated Review. *Molecules* **2020**, *25*, 4711.
9. Reichling, J.; Schnitzler, P.; Suschke, U.; Saller, R. Essential Oils of Aromatic Plants with Antibacterial, Antifungal, Antiviral, and Cytotoxic Properties - An Overview. *Forsch Komplementarmed* **2009**, *16*.
10. Guimarães, A.C.; Meireles, L.M.; Lemos, M.F.; Guimarães, M.C.C.; Endringer, D.C.; Fronza, M.; Scherer, R. Antibacterial Activity of Terpenes and Terpenoids Present in Essential Oils. *Molecules* **2019**, *24*, 2471. https://doi.org/10.3390/molecules24132471.
11. LaLonde, T.; Bowser, T.; Jadeja, R. Essential Oils as Antimicrobials. *Madridge Journal of Food Technology* **2019**, *4*, 163–169. https://doi.org/10.18689/mjft-1000125.
12. Chouhan, S.; Sharma, K.; Guleria, S. Antimicrobial Activity of Some Essential Oils—Present Status and Future Perspectives. *Medicines* **2017**, *4*, 58. https://doi.org/10.3390/medicines4030058.
13. Yap, P.S.X.; Yiap, B.C.; Ping, H.C.; Lim, S.H.E. Essential Oils, A New Horizon in Combating Bacterial Antibiotic Resistance. *Open Microbiol J* **2014**, *8*, 6–14. https://doi.org/10.2174/1874285801408010006.
14. Upadhyay, R. Essential Oils: Antimicrobial, Antihelminthic, Antiviral, Anticancer and Antiinsect Properties. *J Appl Biosci* **2010**, *36*, 1–22.
15. Sateriale, D.; Forgione, G.; De Cristofaro, G.A.; Facchiano, S.; Boscaino, F.; Pagliuca, C.; Colicchio, R.; Salvatore, P.; Paolucci, M.; Pagliarulo, C. Towards Green Strategies of Food Security: Antibacterial Synergy of Essential Oils from Thymus Vulgaris and Syzygium Aromaticum to Inhibit Escherichia Coli and Staphylococcus Aureus Pathogenic Food Isolates. *Microorganisms* **2022**, *10*, 2446. https://doi.org/10.3390/microorganisms10122446.
16. Cheesman, M.J.; Ilanko, A.; Blonk, B.; Cock, I.E., Developing New Antimicrobial Therapies: Are Synergistic Combinations of Plant Extracts/Compounds with Conventional Antibiotics the Solution? *Pharmacogn Rev* 2017, *11*.
17. Böhme, K.; Barros-Velázquez, J.; Calo-Mata, P.; Aubourg, S.P. Antibacterial, Antiviral and Antifungal Activity of Essential Oils: Mechanisms and Applications. In *Antimicrobial Compounds: Current Strategies and New Alternatives*; 2014; Vol. 9783642404443.
18. Langeveld, W.T.; Veldhuizen, E.J.A.; Burt, S.A. Synergy between Essential Oil Components and Antibiotics: A Review. *Crit Rev Microbiol* **2014**, *40*, 76–94. https://doi.org/10.3109/1040841X.2013.763219.
19. Nguyen, F.; Starosta, A.L.; Arenz, S.; Sohmen, D.; Dönhöfer, A.; Wilson, D.N. Tetracycline Antibiotics and Resistance Mechanisms. *Biol Chem* **2014**, *395*, 559–575. https://doi.org/10.1515/hsz-2013-0292.
20. Jana, S.; Deb, J.K. Molecular Understanding of Aminoglycoside Action and Resistance. *Appl Microbiol Biotechnol* **2006**, *70*, 140–150. https://doi.org/10.1007/s00253-005-0279-0.

21. Suzuki, M.; Yamada, K.; Nagao, M.; Aoki, E.; Matsumoto, M.; Hirayama, T.; Yamamoto, H.; Hiramatsu, R.; Ichiyama, S.; Iinuma, Y. Antimicrobial Ointments and Methicillin-Resistant *Staphylococcus Aureus* USA300. *Emerg Infect Dis* **2011**, *17*, 1917–1920. https://doi.org/10.3201/eid1710.101365.
22. Vorobets, N.M.; Yavorska, H. V Modifications of Agar Diffusion Method to Determination of the Antimicrobial Effect of the Herbal Medicinal Products. *Ukraïns'kij biofarmacevtičnij žurnal* **2016**, *2*, 80–84.
23. Boz, I.; Gille, E.; Necula, R.; Dunca, S.; Zamfirache, M.M. Chemical Composition and Antibacterial Activity of Essential Oils from Five Populations of Thymus Pulegioides L. *Cellulose Chemistry and Technology* **2015**, *49*, 169–174.
24. Semeniuc, C.A.; Pop, C.R.; Rotar, A.M. Antibacterial Activity and Interactions of Plant Essential Oil Combinations against Gram-Positive and Gram-Negative Bacteria. *J Food Drug Anal* **2017**, *25*, 403–408. https://doi.org/10.1016/j.jfda.2016.06.002.
25. Pereira, V.; Dias, C.; Vasconcelos, M.C.; Rosa, E.; Saavedra, M.J. Antibacterial Activity and Synergistic Effects between Eucalyptus Globulus Leaf Residues (Essential Oils and Extracts) and Antibiotics against Several Isolates of Respiratory Tract Infections (Pseudomonas Aeruginosa). *Ind Crops Prod* **2014**, *52*, 1–7. https://doi.org/10.1016/j.indcrop.2013.09.032.
26. Schelz, Z.; Molnar, J.; Hohmann, J. Antimicrobial and Antiplasmid Activities of Essential Oils. *Fitoterapia* **2006**, *77*, 279–285. https://doi.org/10.1016/j.fitote.2006.03.013.
27. van Vuuren, S.; Viljoen, A. Plant-Based Antimicrobial Studies – Methods and Approaches to Study the Interaction between Natural Products. *Planta Med* **2011**, *77*, 1168–1182. https://doi.org/10.1055/s-0030-1250736.
28. Moon, S.-E.; Kim, H.-Y.; Cha, J.-D. Synergistic Effect between Clove Oil and Its Major Compounds and Antibiotics against Oral Bacteria. *Arch Oral Biol* **2011**, *56*, 907–916. https://doi.org/10.1016/j.archoralbio.2011.02.005.
29. Neagu, R.; Popovici, V.; Ionescu, L.E.; Ordeanu, V.; Popescu, D.M.; Ozon, E.A.; Gîrd, C.E. Antibacterial and Antibiofilm Effects of Different Samples of Five Commercially Available Essential Oils. **2023**, *12*, 1191.
30. Luță, E.A.; Biță, A.; Moroșan, A.; Mihaiescu, D.E.; Ghica, M.; Mihai, D.P.; Olaru, O.T.; Deculescu-Ioniță, T.; Duțu, L.E.; Popescu, M.L.; et al. The Influence of Phytosociological Cultivation and Fertilization on Polyphenolic Content of Menthae and Melissae Folium and Evaluation of Antioxidant Properties through In Vitro and In Silico Methods. *Plants* **2022**, *11*, 2398. https://doi.org/10.3390/plants11182398.
31. Hudzicki, J. Kirby-Bauer Disk Diffusion Susceptibility Test Protocol Author Information. *American Society For Microbiology* **2012**, 1–13.
32. Popovici, V.; Bucur, L.; Calcan, S.I.; Cucolea, E.I.; Costache, T.; Rambu, D.; Schröder, V.; Gîrd, C.E.; Gherghel, D.; Vochita, G.; et al. Elemental Analysis and In Vitro Evaluation of Antibacterial and Antifungal Activities of Usnea Barbata (L.) Weber Ex F.H. Wigg from C ă Limani Mountains, Romania. *Plants* **2022**, *11*, 32.
33. Popovici, V.; Matei, E.; Cozaru, G.C.; Bucur, L.; Gîrd, C.E.; Schröder, V.; Ozon, E.A.; Karampelas, O.; Musuc, A.M.; Atkinson, I.; et al. Evaluation of Usnea Barbata (L .) Weber Ex F . H . Wigg Extract in Canola Oil Loaded in Bioadhesive Oral Films for Potential Applications in Oral Cavity Infections and Malignancy. *Antioxidants* **2022**, *11*, 1601.
34. Ivan, I.M.; Popovici, V.; Chițescu, C.L.; Popescu, L.; Luță, E.A.; Ilie, E.I.; Brașoveanu, L.I.; Hotnog, C.M.; Olaru, O.T.; Nițulescu, G.M.; et al. Phytochemical Profile, Antioxidant and Cytotoxic Potential of Capsicum Annuum (L.) Dry Hydro-Ethanolic Extract. *Pharmaceutics* **2024**, *16*, 245. https://doi.org/10.3390/pharmaceutics16020245.
35. Popovici, V.; Bucur, L.; Gîrd, C.E.; Popescu, A.; Matei, E.; Caraiane, A.; Botnarciuc, M. Phenolic Secondary Metabolites and Antiradical and Antibacterial Activities of Different Extracts of Usnea Barbata (L .) Weber Ex F . H . Wigg from Călimani Mountains, Romania. *Pharmaceuticals* **2022**, *15*, 829. https://doi.org/10.3390/ph15070829.
36. Luță, E.-A.; Biță, A.; Moroșan, A.; Mihaiescu, D.E.; Mihai, D.P.; Popescu, L.; Bejenaru, L.E.; Bejenaru, C.; Popovici, V.; Olaru, O.T.; and; et al. Implications of the Cultivation of Rosemary and Thyme (Lamiaceae) in Plant Communities for the Development of Antioxidant Therapies. *Int. J. Mol. Sci.* **2023**, *24*, 11670.