Article

Screening of Antibacterial and Antibiofilm Activities of Commercially Available Essential Oils' Different Samples in Comparison to Conventional Antibiotics

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Abstract: Essential oils (EOs) have gained economic importance due to their biological activities, and increasing amounts are demanded everywhere. However, substantial differences between the same essential oil samples from different suppliers are reported due to numerous companies involved in EOs production and the continuous development of online sales. The present study investigates the antibacterial and antibiofilm activities of 2-4 samples of five commercially available essential oils (Oregano, Eucalyptus, Rosemary, Clove, and Peppermint oils) from different manufacturers. Their effects were evaluated *in vitro* on Gram-positive and Gram-negative bacteria (*Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa)*. The antibacterial efficacy (*ABE%*) and antibiofilm efficacy (*ABfE%*) were determined spectrophotometrically at 562 and 570 nm using microplate cultivation techniques. The essential oils' calculated parameters were compared with those of three standard broad-spectrum antibiotics: Amoxicillin/Clavulanic acid (AMC), Gentamycin (GEN), and Streptomycin (STR), active on tested bacteria. The results showed that at the first dilution (D1 = 2.5 mg/mL), all essential oils (EOs) exhibited antibacterial activity against Gram-positive and Gram-negative bacteria. On *S. aureus* and *E. coli*, EOs had considerable antibacterial effects (*ABE* = 79.70—92.80%, respectively, 71.30 – 94.00%). The highest antibacterial effects of commercially available EOs were against *P. aeruginosa* because all exhibited a significant antibiofilm activity. Their antibiofilm efficacy intensively decreased on *E. coli* and *S. aureus.* Generally, the samples with different manufacturers of the same EO showed similar effects. Only Clove and Peppermint oils samples displayed a higher variability associated with active metabolites' different contents, maybe due to various zones of harvesting raw material, numerous technologies involved in EOs obtaining processes, and complex interactions between components.

Keywords: Origani aetheroleum; Eucalypti aetheroleum; Rosmarini aetheroleum; Caryophylli aetheroleum; Menthae aetheroleum; antibacterial activity; antibiofilm effectiveness

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1. Introduction

Essential oils are highly concentrated plant derivatives defined based on their physicochemical properties [1]. The EOs' chemical composition includes phenolic compounds, terpenes, terpenoids, phenylpropanoids, and other aliphatic and aromatic constituents. European Pharmacopoeia defines EOs as odorous products, usually of complex composition, obtained from a botanically defined plant raw material by steam distillation, dry distillation, or a suitable mechanical process without heating [2]. Generally, EOs have high contents (20-70%) of two or three major phytoconstituents; other compounds are quantified in trace concentrations [3].

Currently, substantial growth in the EOs' general use worldwide has been reported in various domains: the food industry, fragrances, aromatherapy, and cosmetics, personal care, spa and relaxation, home care, and healthcare (pharmaceuticals and nutraceuticals) [4]. Especially, aromatherapy (implying professionals – aromatherapists - and individual customers) has gained extensive applications in numerous countries. The pharmacological potential of EOs has been extensively explored: ranging from antioxidant [5][6], anti-inflammatory [7][8], immunomodulatory [9][10], antinociceptive [11][12], antiulcer [13][14], anticancer [15][16], insecticidal [17][18], larvicidal [19][20], anthelmintic [21][22], antiviral [23][24], and antimicrobial [25][26] properties.

Therefore, the global market for essential oils is anticipated to have a continuous expansion, with regional differences. For example, in the USA, its size is expected to be approximately double in 2030 than in 2021. The USA Food and Drug Administration (FDA) divides essential oils into three categories:

- Cosmetics – Products intended to clean the body (except for soap).
- Household items/Other - Fragrance products, like scented candles, household cleaners, and air fresheners.
- Drugs – Products intended for therapeutic use that can treat or prevent various diseases or affect the body structure or function [27] [28][29]."

The essential oils from doTERRA (Pleasant Grove, Utah, USA) [30] are commercialized with the label CPTG (Certified Pure Therapeutic Grade). However, FDA did not regulate essential oils as foods or dietary supplements; it means that any essential oil product cannot be marketed with the following mention: "it intended to treat, prevent, cure or mitigate any disease or other health condition" – even when there is scientific research to back up the validity of the claims.

In Europe, the EOs industry growth is promoted by the European Federation of Essential Oils (EFEO) [31]. Currently, EFEO is discussing with the European Commission and the EU Parliament to amend or introduce legislation concerning essential oils. Contrariwise, due to numerous bioactivities, the European Medicinal Agency (EMA) considers essential oils as herbal preparations and also as active pharmaceutical ingredients (API) in two groups of herbal products [32]:

- Herbal medicinal products (HMPs), both for human and veterinary use.
- Traditional herbal medicinal products (THMPs) for human use.

Thus, EMA established rigorous quality documentation for all manufacturers, and competent national authorities can refer to one unique set of information concerning registered EOs as HMPs/THMPs when evaluating marketing applications [33][34]. In 2022, EMA revised the "Guideline on specifications: test procedures and acceptance criteria for herbal substances, herbal preparations, and herbal medicinal products/traditional herbal medicinal products" [35]. The quality guidelines when essential oils are used as APIs of HMPs, an analytical characterization of the raw material is required. Moreover, other tests (according to the Ph. Eur. monograph Herbal Drugs) must be performed on the essential oils [32]. According to GMP standards, the manufacturing process is another point,

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implying the quality of water used for the EOs distillation from fresh plants. The composition of essential oils should be within the Ph. Eur. monograph limits.

The documentation regarding the quality of EO must contain all manufacturers. It is difficult to achieve all documents [32] when implied farmers or very small companies are in the manufacturing process.

Therefore, substantial differences could be recorded between the same essential oil samples from different suppliers due to a lack of regulation, numerous companies involved in EOs production, and continuous development of online sales. Thus, Iordache et al. measured the metal content of 34 plant essential oils from various manufacturers [36] and identified Hg levels over six times higher than Ph. Eur. permissible limits in Peppermint oil. Vargas Jentzsch et al. investigated 19 samples of commercial clove essential oil. They found that two samples were adulterated by the addition of benzyl alcohol and a third by the addition of vegetable oil [37]. Recently, Pierson et al. tested 31 EO samples purchased online by evaluating their compliance with ISO standards; they found that more than 45% of the samples did not pass the test, and more than 19% were diluted with solvents as propylene and dipropylene glycol, triethyl citrate, or vegetal oil [38].

Numerous authors investigated the pharmacological activities of commercially available EOs, especially antimicrobial effects [39][40][41] [42]. In a previous study, Brun et al. investigated the antimicrobial effects of commercially available Tea Tree essential oils, finding that only five out of ten samples had significant antimicrobial activity [43]. The present study aims to explore the antibacterial and antibiofilm effectiveness of five commercially available essential oils—well-known for their phytotherapeutic applications —against Gram-positive and Gram-negative bacteria. The novelty of the present study consists of a different design. We checked various Romanian markets (including online suppliers) and selected only four that concomitantly provided the essential oils' chemical composition. Then, 2-4 samples of each essential oil from different manufacturers were evaluated, comparing their antibacterial and antibiofilm effects with conventional antibiotics and correlating the data obtained with the bioactive secondary metabolites content. In addition, a complex statistical analysis supports our results.

2. Results

2.1. Antibacterial and Antibiofilm Activity on S. aureus

The percentual values of antibacterial and antibiofilm efficacy of essential oils against Gram-positive bacteria (*S. aureus*) tested, compared to standard antibiotics, are displayed in **Figure 1.**

Figure 1A shows that, at 50 µg/mL, AMC has very good antibacterial efficacy (*ABE* > 90%), while both aminoglycosides exhibit a good one (*ABE* > 85%). All EOs have significant antibacterial activity against *S. aureus* at 2.5 mg/mL. OEO and CEO1 proved very good antibacterial potential, like the AMC one, and higher than GEN and STR. CEO3, PEO3 and PEO4 have the lowest ABE% (< 80%). *S. aureus* sensitivity commonly decreases directly proportional to EOs concentration. Only a few EOs recorded a good antibacterial effect at all D1, D2, and D3 dilutions: EEOs and REO1. The anti-staphylococcal effect can slowly diminish at progressive dilutions (as in the case of REO2) or intensely decrease (OEOs, CEOs, PEO1-2). PEO3-4 exhibited antibacterial efficacy only at 2.5 mg/mL. As an overview, we could appreciate that the MIC value for all EOs tested is higher than 2.5 mg/mL. In addition, no significant differences between the antibacterial efficacy of the tested samples of each EO at D1 were recorded (**Figure 1A**).

Figure 1B shows that all standard antibiotics and EOs have antibiofilm activity at D1 and D2. At D1, AMC shows a moderate *AbfE*, and both aminoglycosides report a

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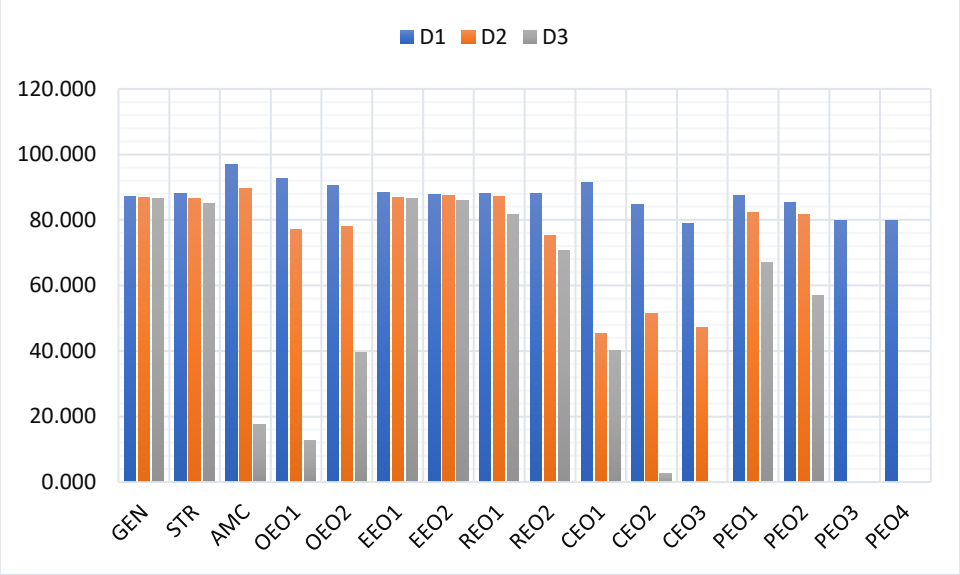
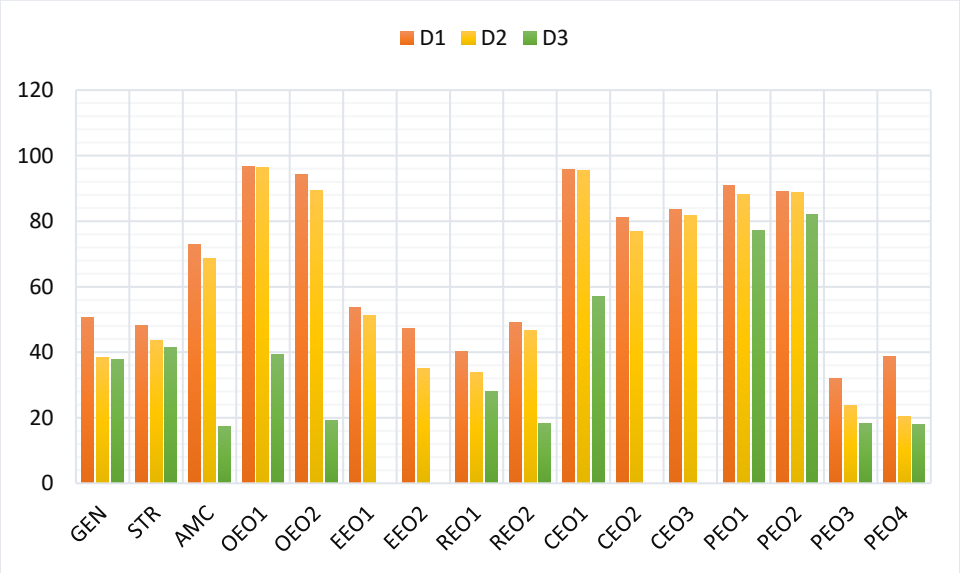
satisfactory one. For EOs, it decreases from "very good" (OEOs, CEO1 and PEO1) to good (CEO2-3 and PEO2), moderate (EEO1), and satisfactory (EEO2, REOs, PEO3-4). The highest differences were recorded in the case of PEO's four samples. Moderate differences in *ABfE* values at D1 were also registered in CEOs and EEOs (**Figure 1B**).



A



B

Figure 1. Antibacterial (**A**) and Antibiofilm efficacy (**B**) of essential oils and antibacterial drugs against *S. aureus:* very good efficacy: ≥ 90%, good efficacy: 75—89%, moderate efficacy: 50—74%, satisfactory: 25—49% and unsatisfactory: 0—24%. GEN — Gentamicin; STR —Streptomycin, AMC — Amoxicillin&Clavulanic acid; OEO1-2—Oregano essential oil from two different manufacturers; EEO1-2—Eucalyptus essential oil from two different manufacturers; REO1-2 —Rosemary essential oil from 2 different manufacturers; CEO1-3—Clove essential oil from three different manufacturers; PEO1-4—Peppermint essential oil from 4 different manufacturers. For EOs, D1 = 2.5 mg/mL, D2 = 0.25 mg/mL and D3 = 0.025 mg/mL. For standard antibiotics, D1 = 50 µg/mL, D2 = 5 µg/mL, and D3 = 0.5 µg/mL.

2.2. *Antibacterial and Antibiofilm Activity on E. coli*

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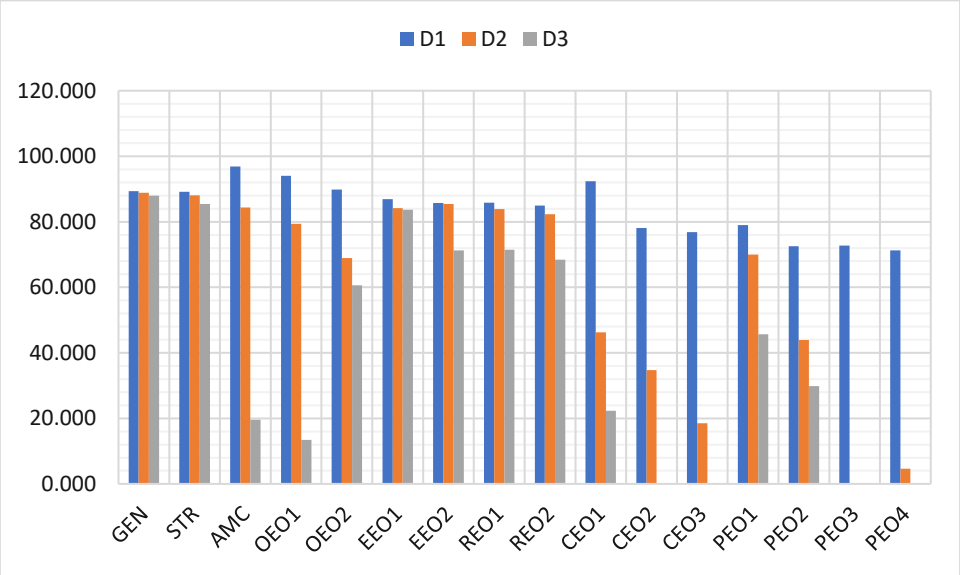
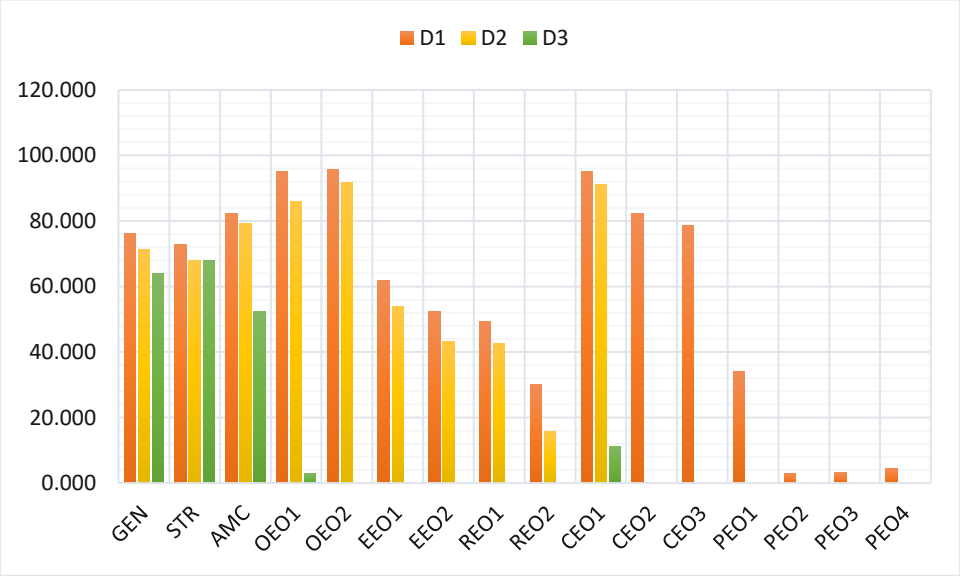
The percentual values of antibacterial and antibiofilm efficacy of essential oils against Gram-negative bacteria *E. coli*, compared to standard antibiotics, are displayed in **Figure 2. Figure 2A** shows that at D1 (= 50 µg/mL), AMC exhibits a very good *ABE*, while both aminoglycosides display a good one (*ABE* < 90%). All EOs inhibited *E. coli* strains growing at 2.5 mg/mL; the antibacterial effect decreased from "very good" (OEO1 and CEO1) to moderate (PEO2-4). Most EOs recorded good inhibitory effects against *E. coli* (**Figure 2A**).



A



B

Figure 2. Antibacterial (**A**) and Antibiofilm efficacy (**B**) of essential oils and antibacterial drugs against *E. coli:* very good efficacy: ≥ 90%, good efficacy: 75—89%, moderate efficacy: 50—74%, satisfactory: 25—49% and unsatisfactory: 0—24%. GEN — Gentamicin; STR —Streptomycin, AMC — Amoxicillin&Clavulanic acid; OEO1-2—Oregano essential oil from two different manufacturers; EEO1-2—Eucalyptus essential oil from two different manufacturers; REO1-2 —Rosemary essential oil from 2 different manufacturers; CEO1-3—Clove essential oil from three different manufacturers; PEO1-4—Peppermint essential oil from 4 different manufacturers. For EOs, D1 = 2.5 mg/mL, D2 = 0.25 mg/mL, and D3 = 0.025 mg/mL. For standard antibiotics, D1 = 50 µg/mL, D2 = 5 µg/mL, and D3 = 0.5 µg/mL..

The antibiofilm activity of standard antibiotics and EOs differs significantly at D1 (**Figure 2B).** AMC and GEN show a high *ABfE*, while STR has a moderate one. However,

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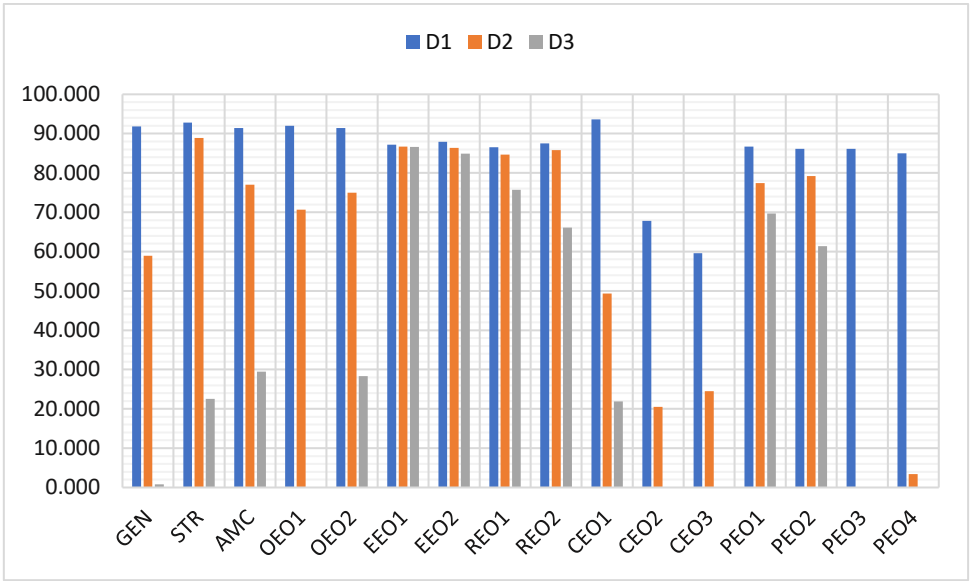
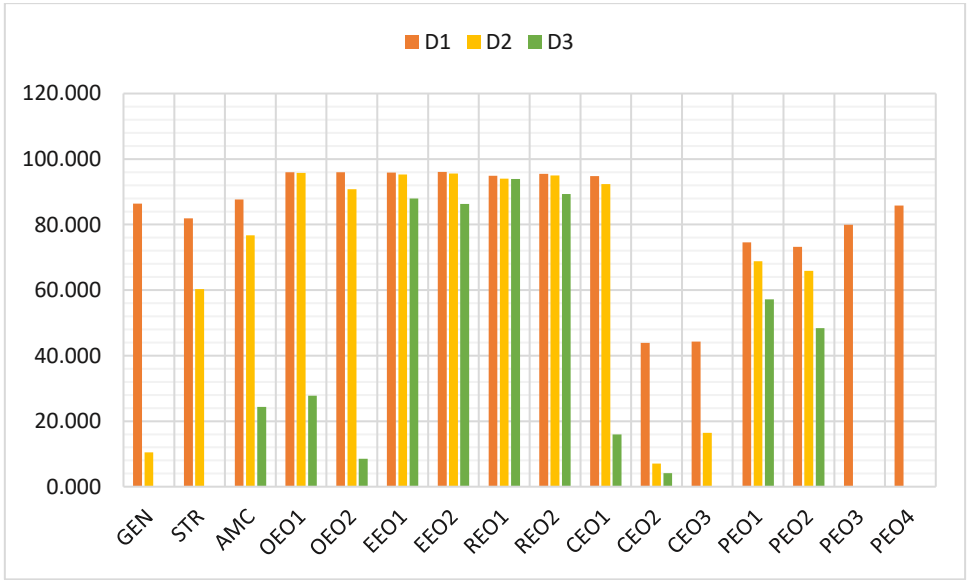
both OEOs and CEO1 show a very good inhibition of *E. coli* biofilm formation, while CEO2-3 have a good one. **Figure 2B** also shows that EEOs have a moderate antibiofilm effect, followed by REOs and PEO1 with satisfactory *ABfE* values. PEO2-4 recorded the lowest inhibitory activity on *E. coli* biofilm formation.

2.3. *Antibacterial and Antibiofilm Activity on P. aeruginosa*

The percentual values of antibacterial and antibiofilm efficacy of essential oils against *P. aeruginosa*, compared to standard antibiotics, are displayed in **Figure 3.**



A



B

Figure 3. Antibacterial (**A**) and Antibiofilm efficacy (**B**) of essential oils and antibacterial drugs against *P. aeruginosa;* very good efficacy: ≥ 90%, good efficacy: 75—89%, moderate efficacy: 50—74%, satisfactory: 25—49% and unsatisfactory: 0—24%. GEN — Gentamicin; STR —Streptomycin, AMC — Amoxicillin&Clavulanic acid; OEO1-2—Oregano essential oil from two different manufacturers; EEO1-2—Eucalyptus essential oil from two different manufacturers; REO1-2 —Rosemary essential oil from 2 different manufacturers; CEO1-3—Clove essential oil from 3 different manufacturers; PEO1-4—Peppermint essential oil from 4 different manufacturers. For EOs, D1 = 2.5 mg/mL, D2 =

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0.25 mg/mL and D3 = 0.025 mg/mL. For standard antibiotics, D1 = 50 µg/mL, D2 = 5 µg/mL, and D3 = 0.5 µg/mL.

Figure 3A shows that all standard antibiotics exhibit very good inhibitory activity on *P. aeruginosa* strains growing at 50 µg/mL (*ABE* > 90%), AMC having a lower one than both aminoglycosides. At 2.5 mg/mL, both OEOs and CEO1 reveal an antibacterial efficacy similar to standard antibiotics, while CEO2-3 reported a moderate one. All the other EOs displayed good antibacterial efficacy against *P. aeruginosa.* Only between CEO samples (CEO1 and CEO2-3) were registered significant differences at D1.

Figure 3B evidences the same differences in the antibiofilm activity of CEO1 compared to CEO2-3. At D1, all antibiotics show good antibiofilm activity, while AMC recorded the highest *ABfE* value. On *P. aeruginosa,* most EOs reported the most increased antibiofilm effects (compared with *E. coli* and *S. aureus*). Therefore, 7 EO samples (OEOs, EEOs, REOs, and CEO1) showed a very good *ABfE.* On the other hand, minimal differences were registered between PEOs (PEO3-4 have a good antibiofilm effect, while PEO1-2 prove a moderate one). Substantial differences were observed in CEOs, CEO2-3 showing the lowest antibiofilm efficacy on *P. aeruginosa.*

2.4. *Data Analysis*

Data regarding antibacterial and antibiofilm effectiveness for all standard antibiotics and essential oils at the first dilution (D1) are displayed in **Figure 4.**

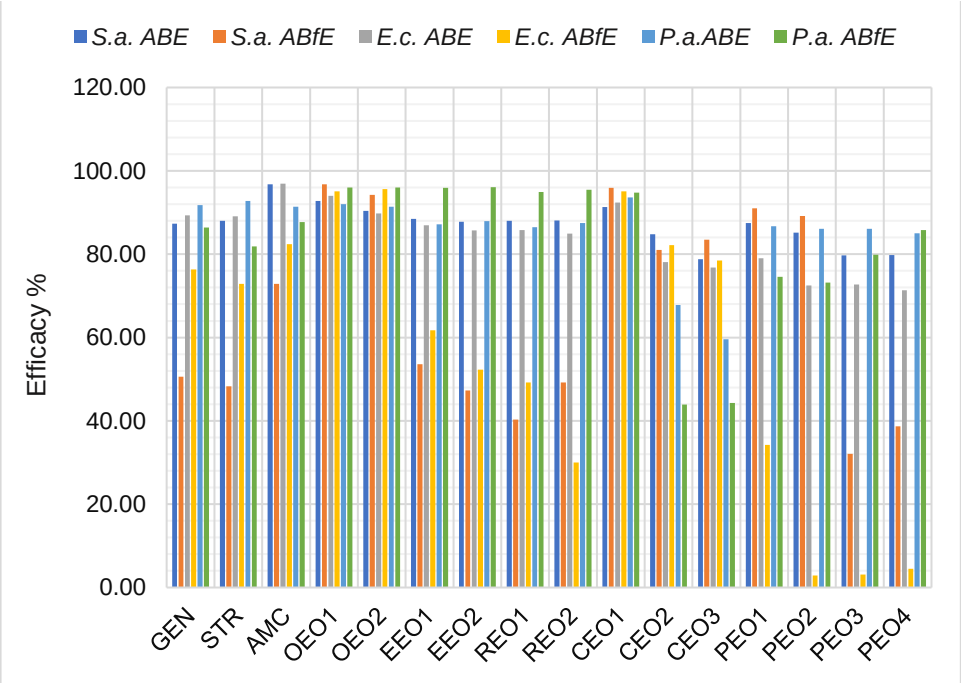

Figure 4. Antibacterial and Antibiofilm efficacy of essential oils and antibacterial drugs against Gram-positive and Gram-negative bacteria; very good efficacy: ≥ 90%, good efficacy: 75—89%, moderate efficacy: 50—74%, satisfactory: 25—49% and unsatisfactory: 0—24%. GEN — Gentamicin; STR —Streptomycin, AMC — Amoxicillin&Clavulanic acid; OEO1-2—Oregano essential oil from two different manufacturers; EEO1-2—Eucalyptus essential oil from two different manufacturers; REO1-2 —Rosemary essential oil from 2 different manufacturers; CEO1-3—Clove essential oil from three different manufacturers; PEO1-4—Peppermint essential oil from 4 different manufacturers; *S.a.—S. aureus, E.c.—E. coli, P.a.—P. aeruginosa, ABE*—Antibacterial efficacy, *ABfE*—Antibiofilm efficacy.

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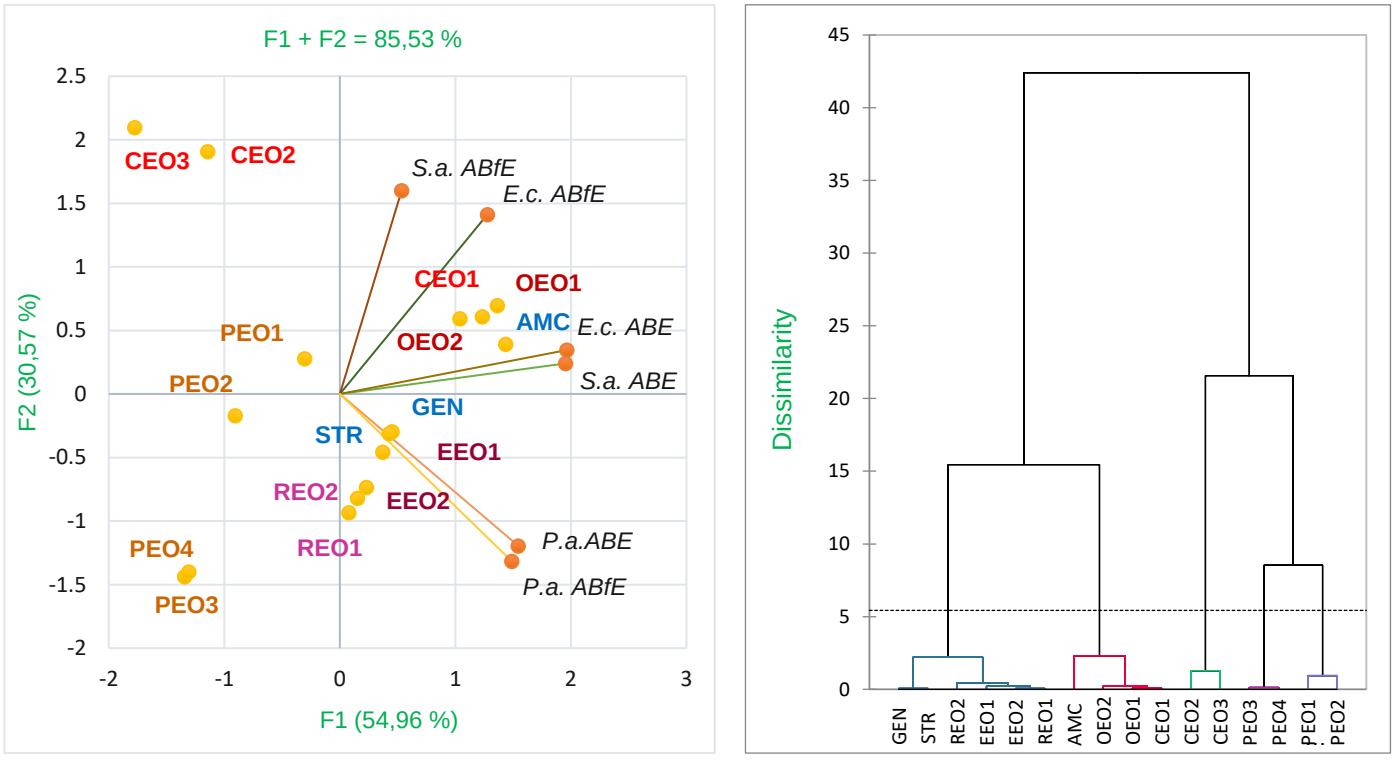
Principal Component Analysis (PCA) was used to evaluate the correlation between the antibacterial and antibiofilm efficacy of EOs on Gram-positive and Gram-negative bacteria (**Figure 5A**) and compared it to standard antibiotics.

The correlation matrix from Supplementary Material and **Figure 5A** highlight a strong correlation, statistically significant, between antibacterial and antibiofilm effects against both Gram-negative bacteria, *P. aeruginosa* ($r = 0.878$, $p < 0.05$) and *E. coli* ($r = 0.772$, $p < 0.05$). On *S. aureus*, both effects are poorly correlated ($r = 0.359$, $p > 0.05$).

However, for all EOs and standard antibiotics, the antibacterial activity against *S. aureus* is considerably associated with that against *E. coli* ($r = 0.895$, $p < 0.05$) and moderately with an inhibitory effect against *P. aeruginosa* strains growing ($r = 0.628$, $p < 0.05$). Antibacterial effects against Gram-negative bacteria also show a moderate correlation ($r = 0.878$, $p < 0.05$). All data are statistically significant ($p < 0.05$). Generally, antibiofilm activities on all bacteria tested are poorly correlated.

The registered data from Results are summarized in **Figure 5A**, evidencing the place of essential oils and standard antibiotic drugs reported to both *ABE* and *ABfE* against all bacteria tested.

In a simplified manner, the dendrogram obtained by Agglomerative Hierarchical Clustering (AHC) from **Figure 5B** and Supplementary Material shows how standard antibiotics and EO samples act similarly. **Figure 5B** shows that PEO1 acts similarly to PEO2, PEO3 to PEO4, and CEO2 to CEO3. On the other hand, both OEOs have similar effects; at D1, they act similarly to AMC. The same observation is available on EEO1-2 and REO1-2, their activities being more closely to Aminoglycosides (STR and GEN).



A B

Figure 5. A. PCA-Biplot displays the antibacterial and antibiofilm efficacy of essential oils and antibacterial drugs against Gram-positive and Gram-negative bacteria. **B.** AHC-Dendrogram. GEN — Gentamicin; STR —Streptomycin, AMC — Amoxicillin&Clavulanic acid; OEO1-2—Oregano essential oil from two different manufacturers; EEO1-2—Eucalyptus essential oil from two different manufacturers; REO1-2 —Rosemary essential oil from 2 different manufacturers; CEO1-3—Clove essential oil from three different manufacturers; PEO1-4—Peppermint essential oil from 4 different

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manufacturers; *S.a.*—*S. aureus, E.c.*—*E. coli, P.a.*—*P. aeruginosa, ABE*—Antibacterial efficacy, *ABfE*—Antibiofilm efficacy.

Knowing each EO's chemical composition (**Figure 6**), the correlations between bioactive constituents and antibacterial and antibiofilm effects were analyzed to explain the differences between the corresponding samples.

Figure 6. Chemical constituents of all EOs' samples: **A**. Eucalyptus Oil (EEO1 and EEO2), Rosemary Oil (REO1 and REO2), Oregano Oil (OEO1 and OEO2); **B.** Clove oil (CEO1-3); **C.** Peppermint Oil (PEO1-4).

Therefore, the PCA-Biplot from **Figure 7** shows the correlation between the previously mentioned variable parameters for 3 EOs: EEO, OEO, and REO.

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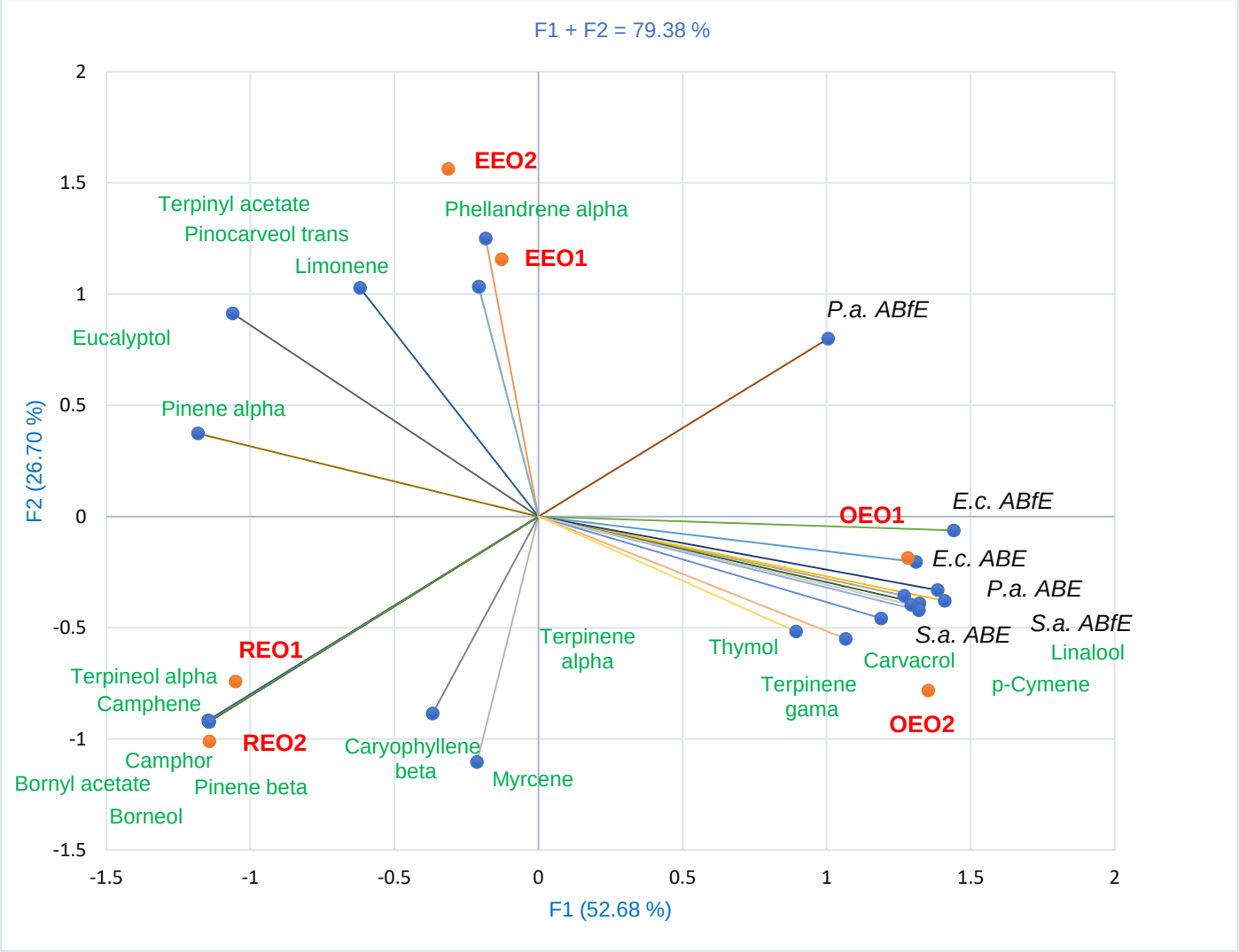

Figure 7. PCA-Biplot displaying the correlations between bioactive constituents and antibacterial and antibiofilm effects on Gram-positive and Gram-negative bacteria in each OEO, EEO, and REO sample. OEO1-2—Oregano essential oil from two different manufacturers; EEO1-2—Eucalyptus essential oil from two different manufacturers; REO1-2 —Rosemary essential oil from 2 different manufacturers; *S.a.—S. aureus, E.c.—E. coli, P.a.—P. aeruginosa, ABE*—Antibacterial efficacy, *ABfE*—Antibiofilm efficacy.

All three EOs contain *p*-cymene. EEOs and REOs have eucalyptol (1,8-cineol), pinene alpha, and limonene, while in EEOs and OEOs, terpinene gamma was quantified. In this EOs group, the Correlation Matrix from Supplementary Material evidence strong and statistically significant correlations between several secondary metabolites and antibacterial and antibiofilm effects.

Antibacterial effects on *S. aureus* and *E. coli* are substantially correlated with antibiofilm ones ($r = 0.927$, $r = 0.898$, $p < 0.05$). On *P. aeruginosa*, both activities are moderately correlated ($r = 0.591$, $p > 0.05$).

From EOs constituents, carvacrol and linalool displays the highest correlation with previously mentioned activities, except *ABfE* on *P. aeruginosa* (carvacrol: $r = 0.985$, $r = 0.959$, $r = 0.974$, $r = 0.887$, $r = 0.965$, $p < 0.05$; linalool: $r = 0.991$, $r = 0.943$, $r = 0.980$, $r = 0.870$, $r = 0.952$, $p < 0.05$).

P-cymene is significantly correlated with *ABE* against *P. aeruginosa* and *ABfE* against *S. aureus* and *E. coli* ($r = 0.830$, $r = 0.886$, $r = 0.886$, $p < 0.05$) and moderately with *S.a. ABE* and *E.c. ABE* ($r = 0.680$, $r = 0.687$, $p > 0.05$). Similarly, terpinene gamma shows good and

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moderate correlation with all 5 previously mentioned effects ($r = 0.788$, $r = 0.799$, $r = 0.750$, $r = 0.539$, $r = 0.539$, $p > 0.05$).

Eucalyptol and alpha-pinene evidence a high negative correlation with antibacterial and antibiofilm effects on *S. aureus* and *P. aeruginosa* ($r = –0.813$, $r = –0.861$, $r = –0.828$, $r = –0.830$, $r = –0.885$, $p < 0.05$). Camphor, pinene beta, camphene, bornyl-acetate, terpineol alpha, and borneol are significantly negatively correlated only with *P.a. ABfE:* $r = –$ [0.849 – 0.898], $p < 0.05$. With the other activities (excepting *S.a. ABE*), they report a moderate negative correlation: $r = –$ [0.560 – 0.738], $p > 0.05$.

Thymol and terpinene alpha are moderately correlated with *ABfE* on *S. aureus* and *E. coli* and *ABE* against *P. aeruginosa* ($r =$ [0.555 - 0.727], $p > 0.05$).

Figure 7 also shows the place of each OEO, EEO, and REO correlated to chemical composition and antibacterial and antibiofilm activities, with both samples of each EO having similar properties without significant differences.

Figure 8 reveals a good and moderate correlation between bioactive constituents and CEOs' antibacterial and antibiofilm effectiveness. Therefore, beta-caryophyllene has a good correlation with *ABE* against *S. aureus* ($r = 0.758$, $p > 0.05$) and moderate ones with *ABE* against *P. aeruginosa* and *ABfE* against *E. coli* ($r = 0.559$, $r = 0.543$, $p > 0.05$). Eugenol is moderately correlated with *S. aureus* and *P. aeruginosa* biofilm inhibition ($r = 0.638$, $r = 0.516$, $p > 0.05$), and eugenyl acetate with antibacterial activity against *S. aureus* ($r = 0.609$, $p > 0.05$).

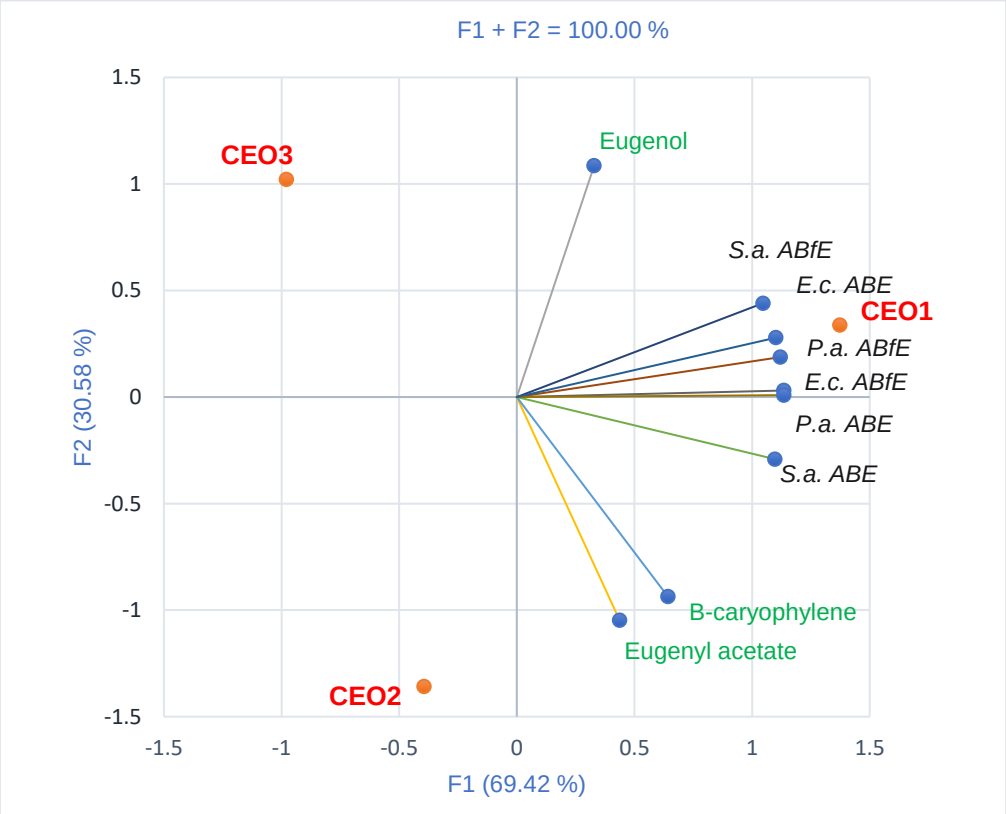

Figure 8. PCA-Biplot displaying the correlations between bioactive constituents' content and antibacterial and antibiofilm effects on Gram-positive and Gram-negative bacteria in each CEO sample. CEO1-3—Clove essential oil from three different manufacturers; *S.a.—S. aureus, E.c.—E. coli, P.a.—P. aeruginosa, ABE*—Antibacterial efficacy, *ABfE*—Antibiofilm efficacy.

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Moreover, antibacterial and antibiofilm effects are strongly correlated on Gram-negative bacteria (*P. aeruginosa* and *E. coli*, $r = 0.976$, $r = 0.990$, $p > 0.05$), and moderately on *S. aureus* ($r = 0.791$, $p > 0.05$).

Finally, the PCA Biplot shows each CEO sample's place considering these variable parameters; it evidences that CEO1 is usually the most active sample regarding antibacterial and antibiofilm effects. All detailed data are found in Supplementary Material.

Figure 9 reveals a strong and statistically significant correlation between antibacterial and antibiofilm activities in *S. aureus* and *E. coli* ($r = 0.974$, $r = 0.975$, $p < 0.05$) and an appreciable negative correlation in *P. aeruginosa* ($r = -0.856$, $p > 0.05$).

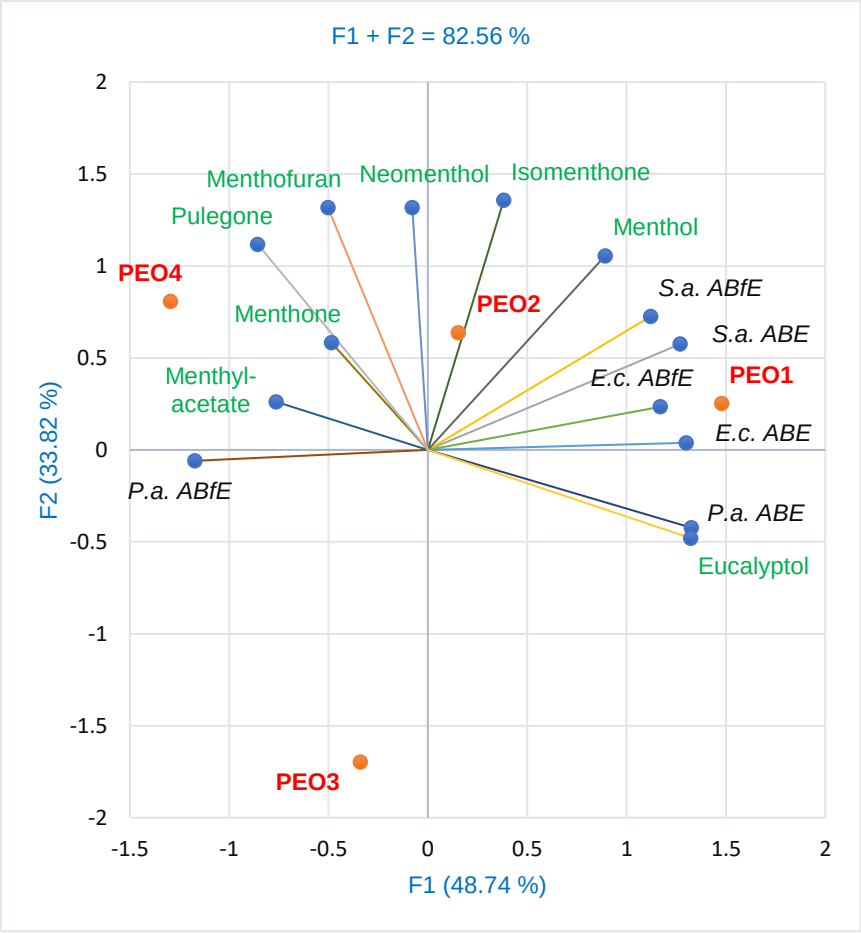

Figure 9. PCA-Biplot displaying the correlations between bioactive constituents' content and antibacterial and antibiofilm effects on Gram-positive and Gram-negative bacteria in each PEO sample; PEO1-4—Peppermint essential oil from 4 different manufacturers; *S.a.—S. aureus, E.c.—E. coli, P.a.—P. aeruginosa, ABE*—Antibacterial efficacy, *ABfE*—Antibiofilm efficacy.

From bioactive constituents, eucalyptol shows a remarkable and significant statistical correlation with antibacterial activity against *P. aeruginosa* ($r = 0.995$, $p < 0.05$). It is also considerably correlated with *ABE* against *E. coli* ($r = 0.830$, $p > 0.05$) and moderately associated with *ABE* against *S. aureus* ($r = 0.716$, $p > 0.05$) and *ABfE* in *S. aureus* and *E. coli* ($r = 0.593$, $r = 0.685$, $p > 0.05$). However, eucalyptol negatively correlates with *ABfE* in *P. aeruginosa* ($r = -0.803$, $p > 0.05$).

Concomitantly, menthol is considerably correlated with both *ABE* and *ABfE* in *S. aureus* ($r = 0.847$, $r = 0.826$, $p > 0.05$) and *E. coli* ($r = 0.680$, $r = 0.754$, $p > 0.05$).

Moderate correlations were evidenced between menthone et pulegone and antibiofilm activity against *P. aeruginosa* ($r = 0.736$, $r = 0.503$, $p > 0.05$); both previously mentioned

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constituents and menthofuran are negatively correlated with *P.a. ABE* (r = –0.579, r = –0.818, r = –0.617, $p > 0.05$).

Isomenthone and neomenthol with *ABfE* on *S. aureus* (r = 0.708, r = 0.550, $p > 0.05$). Moreover, isomenthone is moderately correlated with antibacterial activity against *S. aureus* (r = 0.636, $p > 0.05$).

Menthyl acetate shows a robust negative correlation with *ABE* and *ABfE* against *E. coli* (r = –0.809, r = –0.855, $p > 0.05$), while pulegone reports a moderate one, only with *E.c. ABE* (r = –0.516, $p > 0.05$)

Finally, considering all discussed variable parameters—extensively described in Supplementary Material—**Figure 9** shows the place of each PEO sample, thus explaining all differences between them and supporting the results.

3. Discussion

The present study investigated 5 common essential oils used in phytotherapy [44] with a well-known phytochemical and pharmacological profile. All samples were manufactured by four autochthonous companies and regularly commercialized in pharmacies, pharma markets [45], and online markets.

Four essential oils are registered by European Medicinal Agency (EMA) as HMPs for human and veterinary use [45][46], having a periodically updated monography (Table 1) [47]. Moreover, they have individual monographs in European Pharmacopoeia, indicating the bioactive constituents' concentration limits.

Only Oregano oil is authorized as a feed additive for animal species [48], and these data follow ISO 13171:2016 from International Organization for Standardization, Geneva, Switzerland (Table 1) [49]. Phytogenic feed additives (phytobiotics) are currently used in traditional European animal healthcare [50]. The effect of oregano oil dietary supplementation in poultry on production parameters, intestinal villi height, and broiler breast's antioxidant capacity is well-studied [51].

The antibacterial effects of essential oils investigated in our study are implied in all their therapeutical benefits, as mentioned in Table 1.

Table 1. The main phytoconstituents and applications of essential oils according to Ph. Eur. 10 and the European Medicinal Agency.

	Essential oil name	Botanical name	Main phytoconstituents	Therapeutic area / Applications
1.	*Caryophylli floris aetheroleum* (Clove oil)	*Syzygium aromaticum* (L.) Merr. et L.M. Perry, syn. *Eugenia caryophyllus* (Spreng.) Bullock et S.G. Harrison	• β-caryophyllene: 5 - 14% • Eugenol: 75 – 88% • Eugenyl-acetate: 4 - 15% [52]	Mouth and throat disorders [53]
2.	*Menthae piperitae aetheroleum* (Peppermint oil)	*Mentha x piperita* L.	• Menthol: 30 - 55% • Menthone: 14 – 32% • Isomenthone: 1.5 - 10 % • Menthyl acetate: 2.8 - 10.0 % • 1,8-cineol: 3.5 - 8.0 % • Limonene: 1.0 - 3.5 % • Menthofuran: 1.0 - 8.0 % • Pulegone: ≤ 3.0 % • Carvone: ≤ 1.0 % • Isopulegol: ≤ 0.2 % [52]	Pain and inflammation; Skin disorders and minor wounds; Cough and cold; Gastrointestinal disorders [54]

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3.	*Eucalypti aetheroleum* (Eucalyptus oil)	*Eucalyptus globulus* Labill. *Eucalyptus polybractea* R.T. Baker. *Eucalyptus smithii* R.T. Baker.	• α-pinene: 1 – 9% • β-pinene: < 1.5% • α-phellandrene: < 1.5% • Limonene: 4 -12% • 1,8-cineol: ≥ 70% • Camphor: < 0.1% [52]	Pain and inflammation Cough and cold [56]
4.	*Rosmarini aetheroleum* (Rosemary oil)	*Rosmarinus officinalis* L.	[55] • α-pinene: 18 – 26% • Camphene: 8 – 12% • β-pinene: 2 – 6% • β-myrcene: 1.5 - 5% • Limonene: 2.5 – 5% • 1,8-cineol:16 – 25% • p-cymene: 1 – 13% • Camphor: 13 – 21% • Bornyl acetate: 0.5 – 2.5% • α-terpineol:1– 3.5% • Borneol: 2 – 4.5% • Verbenone: 0.7 – 2.5% [52]	Circulatory disorders; Gastrointestinal disorders [57]
7.	*Origani aetheroleum* (Oregano oil)	*Origanum vulgare* ssp. hirtum (Link) Ietsw.	• Carvacrol: 60 - 80% • p-cymene: 4 - 10% • γ-terpinene: 3 - 9% • Thymol: 0.5 - 5% [49]	Feed additive for certain animal species [48]

3.1. Antibacterial and Antibiofilm Activity of Oregano Oils

The main constituents of OEO are carvacrol and thymol, which have solid pharmacological potential, including antibacterial, anti-inflammatory, and antioxidant activities. OEO could be a broad-spectrum natural antibiotic [58][59]. Both compounds act synergic in combination, having an additive effect [60]. Investigating the antibacterial mechanism against MRSA, Cui et al. proved that OEO affects bacterial wall permeability, leading to an irreversible depletion [61]. It can inhibit bacterial respiratory metabolism (perturbing the tricarboxylic acids cycle) and the expression of MRSA's crucial pathogenic factor PVL. Furthermore, carvacrol can form a chimera with DNA [61], and thymol reduces enterotoxins A, B, and α-hemolysin secreted by *S. aureus* isolates [62]. Other studies confirm both activities (antibacterial and antibiofilm) on *S. aureus* [63], *E. coli* [64], and *P.aeruginosa* [65][66]. Carvacrol exhibits a synergic effect with its biological precursor, *p*-cymene.

Moreover, carvacrol and thymol, obtained by chemical synthesis, could be adulterants of oregano oil [49].

In the present study, both OEOs display similar antibacterial and antibiofilm activities, evidencing the highest effects of all EOs investigated. Their inhibitory activity against all bacteria tested is similar to Amoxicillin&Clavulanic acid. Regarding the chemical composition, OEO1 contains all four constituents in the suitable Ph. Eur. limits (Table 1). OEO2 has a lower carvacrol content, but other metabolites in augmented concentrations than

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OEO1: thymol content, 7 times higher; γ-terpinene, 3.5 times higher and p-cymene, 2 times higher. A similar thymol concentration was quantified by Salehi et al. in OEO from Greece [67]. Moreover, another 3 compounds, unmentioned in Ph. Eur., were found in the GC-MS report: linalool, myrcene, and β-caryophyllene, in up to 2% concentration. They could contribute to the antibacterial effects due to complex interaction with the other bioactive metabolites [43].

Our PCA analysis, separately performed on OEOs, reported a strong statistically significant correlation between the bioactive constituents and *ABE*, respectively *ABfE*, thus justifying OEOs' records. Thus, carvacrol and linalool were substantially correlated (r = 0.999, $p < 0.05$) with *ABE* and *ABfE* against *S. aureus* and *P. aeruginosa* and only *ABE* against *E. coli.* In contrast, thymol, linalool, terpinene alpha, and gamma evidenced the same correlation with antibiofilm activity in *E. coli.* For all bacteria tested, MIC > 2.5 mg/mL. Other studies indicated various MIC values: > 3.2 mg/mL against *E. coli* [68], [2—4] mg/mL against *S. aureus,* and 63 mg/mL against *P. aeruginosa* [69].

3.2. Antibacterial and Antibiofilm Activity of Eucalyptus Oils

Bachir et al. [70][71] reported the antibacterial efficacy of *Eucalypti aetheroleum* against *S. aureus* due to its phytoconstituents (1,8–cineol, linalool, β-pinene). Moreover, its bactericidal effect against *E. coli* and *P. aeruginosa* was reported [72]. The EEO's bioactive constituents are responsible for the antibiofilm one due to 1,8-cineol (eucalyptol) [73][74][75]. Therefore, Eucalyptus oil penetrates the biofilm matrix, interfering with the essential constituents' synthesis and the metabolic processes of the biofilm. EEO has synergistic antibacterial activity against Gram-positive bacteria, while against Gram-negative ones, it is additive [76]. 1, 8 cineol obtained by chemical synthesis could be mixed with Eucalyptus oil for adulteration [77].

The present study proved *Eucalypti aetheroleum*'s appreciable antibacterial effectiveness against *S. aureus*, *E. coli*, and *P. aeruginosa* (MIC > 2.5 mg/mL). The EEO's MIC against MRSA varies between 0.032—307 mg/mL [78]. Mulyaningsih et al. [79] evidenced antibacterial activity against *E. coli* with a MIC > 4 mg/mL, while Van et al. reported a median MIC of 27.26 mg/mL against *P. aeruginosa* isolates [80]. However, EEOs recorded a moderate antibiofilm efficacy on *S. aureus* and *E. coli* strains and a substantial one against *P. aeruginosa,* like OEOs. Minimal differences were registered between both tested samples, with EEO1 acting higher than EEO2. Of six metabolites mentioned in the EEO monograph from Ph. Eur. (Table 1), only 4 (1,8-cineol, α-pinene, α-phellandrene, and limonene) appear in the suppliers' GC-MS results. Their concentration in EEO1 is included in regulatory limits. EEO2 has a lower content of eucalyptol (61.45% *vs.* 79.73%) and a 6 times higher concentration. The samples have small contents of other different compounds: myrcene (in EEO1), trans-pinocarveol, terpinyl acetate (in EEO2), and *p*-cymene and γ-terpinene (in EEO1 and EEO2). Both common constituents are considerably correlated with antibacterial/antibiofilm activities. Thus, data analysis indicated a strong statistically significant correlation between eucalyptol, limonene, and *p*-cymene and both antibacterial and antibiofilm activities against *S. aureus* and *E. coli*; at the same time, α-pinene is substantially correlated with *ABE* and *ABfE* against *P. aeruginosa* (r = 0.999, $p < 0.05$).

3.3. *Antibacterial and Antibiofilm Activity of Rosemary Oils*

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Previous studies [68][81][82][83] evidenced the antibacterial effects of *Rosmarini aetheroelum* against *S. aureus* and *E. coli*. Santoyo et al. [84] highlighted the antibacterial efficacy of REO against *P. aeruginosa* due to the bioactive constituents, camphor, borneol, and verbenone. Rosemary oil also inhibits *P. aeruginosa* biofilm formation [85].

Our results show that *Rosmarini aetheroleum* had significant antibacterial and antibiofilm efficacy against *P. aeruginosa*. The antibacterial activity is similar for all Gram-positive and Gram-negative bacteria tested (*ABE* > 80.00%, MIC > 2.5 mg/mL). Other studies reported MIC values of [6.2—25] mg/mL against *S. aureus*, [12.5—25] mg/mL against *E. coli*, and 50 mg/mL against *P. aeruginosa* [86]. REO1 was slightly more active than REO2, but no significant differences were recorded between the two samples' effects. Compared to Ph. Eur. data, REOs have around 2 times higher eucalyptol content (48.10% in REO1 and 42.50% in REO2) and no verbenone. In addition, REO2 contains 4.45% β-caryophyllene. Pinene alpha, beta, camphor, borneol, and camphene were substantially correlated with *ABE* and *ABfE* on *P. aeruginosa* ($r = 0.999$, $p < 0.05$). Eucalyptol has a similar correlation with *ABE* against *E coli;* camphor and camphene are correlated with *ABE* and *ABfE* against *S. aureus;* pinene beta only correlates with *ABfE* on *S. aureus.*

Moreover, the synthetic equivalents of their main components, 1.8 cineole and camphor could be used for Rosemary oil adulteration [87].

3.4. Antibacterial and Antibiofilm Activity of Clove Oils

Xu et al. [88] highlighted the antibacterial efficacy of *Caryophylli aetheroleum* against *S. aureus* (with a MIC value = 0.0625 mg/mL). They hypothesized that the volatile oil destroys the cell wall and membranes, causing loss of vital intracellular materials, resulting in bacterial death. Generally, the MIC values of CEO against *S. aureus* vary in the range of [0.52—1.04] mg/mL [89]. The volatile oil also penetrates the cytoplasmic membrane and inhibits the normal synthesis of DNA and proteins necessary for bacterial growth. Yadav et al. [90] reported the antibiofilm effect of Clove oil on *S. aureus* attributed to eugenol. It inhibits biofilm formation, interrupts intercellular connections, detaches pre-existing biofilms, and kills bacteria in biofilms. Synthetic eugenol is also used for Clove oil adulteration [37].

Burt et al. [91] evidenced the antibacterial efficacy of *Caryophylli aetheroleum* against *E. coli*, The CEO's MIC value belonged to the range of [0.64—1.28] mg/mL [89]. Another study by Kim et al. [92] reported the antibiofilm efficacy of Clove oil against *E. coli* due to eugenol inhibitory activity on biofilm formation.

The CEO's antibacterial efficacy against *P. aeruginosa* is also demonstrated [93], with a MIC of 4.9 mg/mL [94]. Moreover, the antibiofilm activity of Clove oil is due to its main bioactive compounds, eugenol and eugenyl acetate [95].

The present study reports a few differences between the three CEO samples.

Thus, CEO1 showed substantial antibacterial and antibiofilm efficacy against all Gram-positive and Gram-negative bacteria tested (with *ABE* and *ABfE* values > 91.80%)

CEO2 and CEO3 proved good antibacterial and antibiofilm effectiveness against *S. aureus* and *E. coli.* However, significant differences were registered in their effects against *P. aeruginosa,* exhibiting moderate and satisfactory antibacterial and antibiofilm activity.

Generally, their chemical composition corresponds to Ph. Eur.; CEO1 has the highest eugenol content, followed by CEO3 and CEO2. However, CEO3 has the lowest eugenyl acetate concentration and no β-caryophyllene; both constituents are highly and moderately correlated with antibacterial and antibiofilm activities.

3.5. Antibacterial and Antibiofilm Activity of Peppermint Oils

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In PEO, the association of menthol, menthone, limonene, neomenthol, carvone, and 1,8-cineol with other minor constituents appears to induce a synergistic antibacterial activity. A recent study [96] evaluated the antibacterial activity of volatile oil obtained from Mentha×piperita L. leaves on MDR strains from hospitalized patients. The authors used bacterial cell lines (ATCC) and isolates of *S. aureus, E coli,* and *P. aeruginosa,* proving PEOs' bactericidal effects against all microorganisms.

Li et al. [97] evidenced that *Menthae aetheroleum* (with a high content of carvone, menthone, isomenthone, neomenthol, menthol, and menthyl acetate) has a significant antibacterial effect against *S. aureus* [98]. All tested samples of Peppermint oil showed appreciable anti-staphylococcal efficacy. Kang et al. [99] showed that PEO inhibits the biofilm of *S. aureus* by altering the permeability and integrity of bacterial cell membranes. Peppermint oil also significantly inhibits biofilm formation and inactivates the mature biofilm [100].

Alamoti et al. [101] proved the antibacterial efficacy of *Menthae aetheroleum* against *E. coli* due to pulegone content.

Peppermint oil also inhibits *P. aeruginosa* [102], showing substantial antibiofilm activity [103][104].

All four *Menthae aetheroleum* samples investigated in the present study had remarkable antibacterial effects against Gram-positive and Gram-negative bacteria, with no significant differences (MIC > 2.5 mg/mL). They recorded the highest *ABE* (> 85.00%) on *P. aeruginosa* and *S. aureus* (*ABE* > 79.70%). On *E. coli,* the PEOs' antibacterial efficacy was good to moderate, in the range of [71.30 – 79.00] %; PEO1 shows the highest effect. Evaluating the antibacterial effect of EO from *Mentha×piperita* L. against MDR bacterial strains, Muntean et al. reported the following MIC values range: [5—20] mg/mL on *S. aureus,* [10—20] mg/mL on *E. coli*, and [20—40] mg/mL on *P. aeruginosa* [96].

Regarding the antibiofilm activity, the PEOs displayed considerable effects on *P. aeruginosa, ABfE* = [73.20 – 85.80] %. On *E. coli*, PEOs registered the lowest effects: *AbfE* = [2.90 – 34.20] %. The most significant differences were highlighted in the antibiofilm efficacy evaluation against *S. aureus.* The obtained data show that PEO1 and PEO2 have a substantial antibiofilm activity *AbfE* = [89.20 – 91.00] %. Concomitantly, PEO3 and PEO4 exhibited a poor antibiofilm effect [32.10 – 38.70] %. These differences could be explained by their bioactive metabolite content. PEO1 and PEO2 have the highest concentrations of menthol, menthyl acetate, isomenthone, and eucalyptol; these constituents considerably correlate with antibacterial and antibiofilm activities. Moreover, synthetic menthol could substitute peppermint in adulterant oil [105].

4. Materials and Methods

4.1. Materials

All chemicals and reagents were on analytical grade. Poly (ethylene glycol)-block-poly (propylene glycol)-block-poly (ethylene glycol) (Poloxamer 407) and Crystal Violet (Gentian Violet) were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany).

Gentamicin® 80 mg/2 ml (GEN) injectable solution was supplied by KRKA (Novo mesto, Slovenia). Antibiotice SA (Iași, Romania) provided Streptomycin (Strevital®) 1 g (STR) powder for an injectable solution and Amoxicillin/Clavulanic acid (Amoxiplus®) 1.2 g (AMC) [106] powder for an injectable solution.

Gram-positive (*S. aureus*) and Gram-negative (*E. coli* and *P. aeruginosa*) bacteria were obtained from the sub-collection of the Experimental Microbiology Laboratory of the "Cantacuzino" National Military Medical Institute for Research and Development, Bucharest. Other recently published studies used these strains for antibacterial activity screenings [107],[108]. Sanimed International Impex SRL (Calugareni, Romania) was the Muller Hinton culture media supplier.

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The laboratory equipment consisted of an EnSight Multimode Plate Reader (PerkinElmer, Waltham, Massachusetts, USA), an adjustable incubator (Memmert GmbH + Co.KG, Büchenbach, Germany), a microplate shaking incubator (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany), microbiological hood class II with laminar flow (Jouan SA, Saint-herblain, Pays de la Loire, France), Evoqua double-water distiller (Evoqua Water Technologies GmbH, Barsbüttel, Germania) and an electronic scale (Ohaus Corporation, Parsippany, NJ, USA). The NUNC™ MaxiSorp™ 96 well plates were supplied from Electron Microscopy Sciences (Hatfield, Pennsylvania, USA).

Five commercially available essential oils were purchased from Romanian markets (2-4 samples for each EO, having different manufacturers, noted with *1, 2, 3*, and *4*):

- *Origani aetheroleum 1, 2* (Oregano essential oil, OEO);
- *Eucalypti aetheroleum 1, 2* (Eucalyptus essential oil, EEO);
- *Rosmarini aetheroleum 1, 2* (Rosemary essential oil, REO);
- *Caryophylli aetheroleum 1. 2, 3* (Clove essential oil, CEO);
- *Menthae aetheroleum 1, 2, 3, 4* (Peppermint essential oil, PEO).

4.2. Antibacterial Activity

The current method was adapted from [109][110]. It involved the cultivation of bacteria in 96-well microplates with Muller Hinton medium with EOs samples and incubation at 37 °C for 24 hours.

4.2.1. Inoculum preparation

The direct colony suspension method (CLSI) was used for preparing the bacterial inoculum. First, bacterial colonies selected from a 24 h agar plate were suspended in an MHA medium. The bacterial inoculum was accorded to the 0.5 McFarland standard, measured at Densimat Densitometer (Biomerieux, Marcy-l'Étoile, France) with around 10^8 CFU/mL (CFU = colony-forming unit).

4.2.2. Sample preparation

The samples were O/W emulsions prepared with an essential oil concentration of 30% w/w; the emulsifier was Poloxamer 407 5% in water, as previously mentioned [111]. Each emulsion was diluted with double distilled water to achieve the final concentration of each EO stock solution (25 mg/mL).

4.2.3.. Standard Antibiotic solutions preparation

All antibiotic drug solutions were prepared with double distilled water, the final stock solution concentration being 0,5 mg/mL.

4.2.4.. Microdillution method

All successive steps were performed in a laminar flow; In 96-well plates, we performed serial dilutions, adapting the protocol described by Gómez-Sequeda et al. [110] and detailed in our recently published study [107]. All well plates were incubated for 24 h at 37 °C. After incubation, the antibacterial efficacy of essential oils was determined by reading the absorbance values using the EnSight Multimode Plate Reader and calculated according to Sandulovici et al. [107].

4.3. Antibiofilm Activity

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The method used was adapted from [112] and detail presented in our recently published article [107]. After incubation, the bacterial biofilm production was evidenced by staining with 0.1% Gentian Violet after removing the culture medium, washing twice with sterile distilled water, and drying at room temperature under airflow. After dye removal, the microplates were dried at 50 °C for 60 minutes. The dye incorporated in bacterial cells that formed the biofilm was solubilized with 95% ethanol for 10 minutes under continuous stirring at 450 rpm.

4.4. Quantification and Interpreting of Antibacterial and Antibiofilm Activities

The antibacterial and antibiofilm effects of essential oils were determined by reading the absorbencies using the EnSight Multimode Plate Reader at 562 and 570 nm, respectively, and calculated according to Sandulovici et al. [107].

The obtained results were compared to standard antibiotics.

Interpretation of antibacterial (*ABE%—bacterial growth inhibition%*) and antibiofilm (*ABfE%—biofilm formation inhibition%*) efficacy was quantified on conventional arithmetic intervals: very good efficacy: ≥ 90%, good efficacy: 75—89%, moderate efficacy: 50—74%, satisfactory: 25—49% and unsatisfactory: 0—24%.

4.5. Data Analysis

The analyses were performed in triplicate. The absorbance values, expressed as a mean of three determinations, were included in the calculation formula detailed in our previous publication [107].

The correlations between variable parameters [113] were examined through principal component analysis [114] performed with XLSTAT 2023.1.4. by Lumivero (Denver, CO, USA); their statistical significance was available when $p < 0.05$ [115].

5. Conclusions

All essential oils and antibacterial drugs exhibited antibacterial efficacy against Gram-positive and Gram-negative bacteria tested.

The most active essential oils were Oregano oils, with a substantial antibacterial and antibiofilm effect on all tested bacteria.

P. aeruginosa was the most susceptible bacteria, all EOs having concomitantly antibacterial and antibiofilm effects. The antibiofilm effectiveness decreases in order: *P aeruginosa, E. coli,* and *S. aureus.*

The highest antibacterial effects of commercially available essential oils were against *P. aeruginosa* because they exhibited a significant antibiofilm activity. Their antibiofilm efficacy intensively decreased on *E. coli* and *S. aureus*.

Generally, the samples with different manufacturers of the same essential oil showed similar activities; only Clove and Peppermint oils showed higher differences, maybe due to different places of harvesting the raw plant material, various technological processes through these essential oils were obtained, and complex interactions between constituents.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Principal Component Analysis.

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