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# *In Vitro* Antimicrobial Activity of Selected Essential Oils Against Clinically Relevant MDR Bacterial Pathogens, Including ESKAPE Species

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## Abstract

**Background/Objectives:** Essential oils (EOs) are volatile, biologically active compounds naturally extracted from aromatic plants. Known for their broad-spectrum antimicrobial properties, EOs are gaining increasing attention as potential alternative or complementary agents in clinical practice, especially in the face of rising antibiotic resistance. EOs act through multiple mechanisms, including disruption of microbial membranes, interference with enzyme activity, and inhibition of quorum sensing pathways, making them promising antimicrobial candidates against multidrug-resistant organisms (MDROs). **Methods:** In this study, the *in vitro* antimicrobial activity of seven essential oil batches, including *Eucalyptus globulus*, *Lavandula hybrida*, *Melaleuca alternifolia*, *Pelargonium graveolens*, *Coriandrum sativum*, *Cymbopogon winterianus*, and *Cinnamomum zeylanicum*, was evaluated against five reference strains and ten clinical strains mostly belonging to ESKAPE species, plus *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, and *Burkholderia cepacia* complex. **Results:** The tested EO batches displayed heterogeneous antimicrobial activity: *Cinnamomum zeylanicum* exhibited the strongest and broadest antibacterial effect, showing consistently low minimum inhibitory concentration (MIC) values across all tested strains, including carbapenemase-producing and non-fermenting Gram-negative bacteria. The remaining oils showed moderate-to-limited activity, particularly against Gram-negative strains. The two *Klebsiella pneumoniae* strains producing either KPC or NDM carbapenemases, and the *Pseudomonas aeruginosa* PAO-1 strain, showed the highest MIC values for all EOs, except for *C. zeylanicum*, for which MICs were 0.125 and 0.25% (*v*/*v*), respectively. **Conclusions:** The consistently low MIC values of the *C. zeylanicum* batch against the panel of tested strains suggested a potential for this essential oil and for its bioactive constituents as promising candidates against several MDROs.

**Keywords:** essential oils; antimicrobial activity; multidrug-resistant pathogens; ESKAPE species; bacterial resistance; natural antimicrobials; clinical isolates

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## 1. Introduction

The rapid and continuous expansion of antimicrobial resistance represents one of the greatest challenges for modern medicine, with dramatic consequences for morbidity, mortality, and the sustainability of healthcare systems worldwide [1]. The emergence of multidrug-resistant (MDR) bacterial strains resistant to multiple classes of antibiotics, including last-resort agents, has drastically reduced therapeutic options, highlighting the urgent need to identify new molecules capable of overcoming extant resistance mechanisms [2–4].

In recent years, bioactive constituents of plant extracts have gained increasing attention due to their potential antimicrobial properties [5], which could help to curb the excessive use of broad-spectrum antibiotics and consequently to limit the emergence of new resistances. Among these, essential oils (EOs) have received particular interest [5].

EOs are complex mixtures of organic compounds produced as secondary metabolites and extracted from various plant parts, including flowers, buds, stems, seeds, bark, roots, and leaves. They play key ecological roles, contributing to plant defense through their antibacterial, antifungal, insecticidal, and antiviral activities [6]. In fact, many EOs exhibit well-documented antimicrobial activity [7]. They contain a wide range of bioactive constituents, including terpenes, phenols, and aldehydes, which are known to inhibit the growth of numerous pathogens [8]. The chemical complexity of EOs confers a multi-target mode of action, ranging from alteration of cell membranes to interference with essential metabolic pathways [6].

In this study, we evaluated the antimicrobial activity of several EOs against a collection of bacterial strains of clinical relevance, including multidrug-resistant organisms (MDROs) representative of some ESKAPE species and other difficult-to-treat Gram-negative species.

## 2. Materials and Methods

### *2.1. Essential Oils*

The EOs investigated in this study were derived from *Eucalyptus globulus* (eucalyptus, EG), *Lavandula hybrida* (lavender, LH), *Melaleuca alternifolia* (tea tree, MA), *Pelargonium graveolens* (geranium, PG), *Coriandrum sativum* (coriander, CS), *Cymbopogon winterianus* (java citronella, CW), and *Cinnamomum zeylanicum* (cinnamon, CZ). The EOs were provided by Casalvento farmhouse (Castellina in Chianti, Siena, Italy) and were stored in brown glass bottles at room temperature. Gas chromatography–mass spectrometry (GC-MS) analyses of the tested steam-distilled essential oil batches were carried out by the producer. Operational parameters for GC–MS analysis, plant parts, geographical origins, and main chemical constituents of each oil batch are provided as Supplementary Data (Table S1).

### *2.2. Bacterial Strains*

The bacterial strains used for testing the activity of EOs included 5 ATCC reference strains (methicillin-resistant *Staphylococcus aureus* ATCC 33591, vancomycin-resistant *Enterococcus faecalis* ATCC 51299, carbapenem-resistant KPC-3-producing *Klebsiella pneumoniae* ATCC BAA-2814, *Acinetobacter baumannii* ATCC 17978, *Pseudomonas aeruginosa* PAO-1 ATCC 15692) and 10 strains of clinical origin representative of clinically relevant MDR pathogens, with some belonging to ESKAPE species ([9–16], Table 1), including a methicillin- and linezolid-resistant *S. aureus* strain (AOUC-0915); a vancomycin-resistant *Enterococcus faecium* strain (333/27194); an NDM-1-producing *K. pneumoniae* strain resistant to cefiderocol (271/22216); an OXA-48-*like*-producing *Escherichia coli* strain (311/25445); a *Proteus mirabilis* strain co-producing OXA-48 and NDM (378/30873); a *Serratia marcescens* strain with an ESBL profile (376/30730); two *Stenotrophomonas maltophilia* strains resistant to trimethoprim/sulfamethoxazole (385/31448 and 351/28640); and two intrinsically multidrug-resistant strains of *Achromobacter xylosoxidans* (265/29789) and *Burkholderia cepacia* complex (246/20216),

respectively. Clinical strains, not previously reported, were identified by MALDI-TOF MS (MALDI Biotyper® Sirius System, Bruker, Germany) and screened for resistance genes using real-time PCR (Allplex™ Entero-DR Assay, Seegene, Republic of Korea). Antimicrobial susceptibility testing was performed by broth microdilution using MICRONAUT-S ITGN or ITGP lyophilized MIC plates (E1-184-100 or E1-185-100, MERLIN Diagnostika, Bornheim, Germany). Detailed susceptibility profiles of the 10 clinical strains are provided in Supplemental Table S2.

### *2.3. In Vitro Susceptibility Testing to EOs*

The solubilization of EOs in the culture medium was carried out in the presence of the surfactant Tween 80 10% (*v*/*v*), followed by sonication (120 W for 30 min at room temperature (Citizen Scale YJ 5120-1, Aczet Pvt. Ltd., Mumbai, India), as previously described [17]. The 10% (*v*/*v*) Tween 80 concentration was selected after preliminary evaluations at 0.5% and 10% (*v*/*v*) to yield a stable suspension and to remain lower than the 16% (*v*/*v*) concentration reported in a recent protocol for determining the MICs of EOs [18]. The stability of the stock EO 10% emulsions following sonication was confirmed visually by monitoring for phase separation, creaming, or droplet coalescence immediately post-preparation and throughout the 24 h incubation period at 37 °C. The MICs of EOs for bacterial strains were determined by the broth microdilution method according to the ISO 20776 guidelines [19]. Briefly, bacterial strains were grown overnight at 35 ± 1 °C for 18 h on Columbia blood agar plates (Thermo Fisher Scientific, Basingstoke, UK), and isolated colonies were resuspended in sterile saline to a turbidity of 0.5 McFarland. The suspension was then diluted in cation adjusted Mueller–Hinton Broth (CAMHB, Becton Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 10% (*v*/*v*) Tween 80 (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), and 50 µL of bacterial suspension was inoculated in the well of microtiter plates (Microtest 96-well plate, Sarstedt, Germany) at a final concentration of ~5 × $10^5$ CFU/mL, containing serial dilutions of the EOs in CAMHB supplemented with 10% (*v*/*v*) Tween 80. Tested EO concentrations ranged between 8% and 0.03% *v*/*v*. The final testing volume was 100 µL, and the final Tween 80 concentration was 10% (*v*/*v*). A growth control (CAMHB + inoculum, without EO) and a vehicle control (CAMHB + 10% Tween 80 + inoculum, without EO) were included for each strain to exclude any antimicrobial activity of 10% (*v*/*v*) Tween 80 [20]. A negative control (CAMHB + EO, without inoculum) was added as a sterility control in the experiments. The minimal inhibitory concentration (MIC) of EOs was defined as the lowest concentration inhibiting visible bacterial growth after 18 ± 2 h of static incubation at 35 ± 1 °C in microplates sealed to minimize evaporation and cross-contamination. MIC values were expressed as % (*v*/*v*) of the EO. For each EO, susceptibility testing was performed in three independent biological experiments. Results differing by no more than one 2-fold dilution were considered acceptable, and the modal MIC value was reported. In cases of discordant results (defined as values differing by more than one 2-fold dilution), the assay was repeated in a technical triplicate, and the modal value of the MIC results was reported.

## 3. Results

The seven EO batches showed markedly different antimicrobial activities against the panel of tested strains (Table 1). Overall, the tested batch of CZ oil was consistently the most active EO against both Gram-positive and Gram-negative bacteria.

Among Gram-positive cocci, CZ exhibited potent inhibitory activity, with the lowest MICs recorded in the study. Both methicillin-resistant *S. aureus* (MRSA) (*mecA*$^+$) ATCC 33591 and the MRSA clinical isolate also resistant to linezolid (*mecA*$^+$, *cfr*$^+$, *poxtA*$^+$) were inhibited at 0.06% *v*/*v*, while both vancomycin-resistant enterococci (VRE) showed

somewhat higher CZ MICs (0.5% and 0.125%, respectively). The tested batches of CW, LH, PG, CS, and MA showed an overall lower activity (MIC range, 0.5–2%) against the two MRSA and the two VRE, while the activity of EG was even lower (MIC range, 1–4%).

Among Enterobacterales, CZ exhibited strong activity against all tested strains, including those exhibiting carbapenem resistance. OXA-48-producing *E. coli* showed a very low CZ MIC (0.06%), while KPC- and NDM-producing *K. pneumoniae* exhibited CZ MICs of 0.125%. Notably, the cefiderocol-resistant NDM-producing *K. pneumoniae* showed the highest MICs for all EOs (MA = 2%, others =≥ 8%), except for CZ. Other EOs showed extremely heterogeneous activity among the tested strains (Table 1).

CZ also exhibited remarkable activity against the tested Gram-negative non-fermenters, with MICs ranging between ≤0.03% for *S. maltophilia* and 0.25% for *P. aeruginosa* PAO-1.

Overall, the highest MICs were observed with CW (4–>8%) for all strains except *S. maltophilia* (0.5–1%). The other EOs showed lower MICs when tested against *S. maltophilia* (0.25–2%) compared to the strains of other species (1–>8%) (Table 1).

**Table 1.** Minimal inhibitory concentrations (MICs) of EOs on a collection of Gram-negative and Gram-positive strains. Essential oils tested: *Eucalyptus globulus* (EG), *Lavandula hybrida* (LH), *Coriandrum sativum* (CS), *Melaleuca alternifolia* (MA), *Pelargonium graveolens* (PG), *Cinnamomum zeylanicum* (CZ), and *Cymbopogon winterianus* (CW).

| Species | Strain Name | Reference | Main Acquired Resistance Gene (S) | EG % *v*/*v* | LH % *v*/*v* | CS % *v*/*v* | MA % *v*/*v* | PG % *v*/*v* | CZ % *v*/*v* | CW % *v*/*v* |
|---|---|---|---|---|---|---|---|---|---|---|
| *S. aureus* | ATCC 33591 | [9] | *mecA* | 4 | 1 | 1 | 2 | 2 | 0.06 | 1 |
| *S. aureus* | AOUC-0915 (53/4250) | [10] | *mecA*, *cfr*, *poxtA* | 1 | 1 | 1 | 1 | 2 | 0.06 | 1 |
| *E. faecium* | 333/27194 | This study | *vanA* | 1 | 0.5 | 0.5 | 0.5 | 0.5 | 0.125 | 2 |
| *E. faecalis* | ATCC 51299 | [11] | *vanB* | 2 | 2 | 2 | 2 | 1 | 0.125 | 2 |
| *E. coli* | 311/25445 | This study | $bla_{\text{OXA-48-}like}$ | 2 | 2 | 1 | 1 | 2 | 0.06 | 8 |
| *K. pneumoniae* | ATCC BAA-2814 | [12] | $bla_{\text{KPC-3}}$ | 8 | >8 | >8 | 2 | 4 | 0.125 | >8 |
| *K. pneumoniae* | 271/22216 | [13] | $bla_{\text{NDM-1}}$ | 8 | 8 | 8 | 2 | >8 | 0.125 | >8 |
| *S. marcescens* | 376/30730 | This study | - | 8 | 2 | 2 | 2 | 1 | 0.125 | 2 |
| *P. mirabilis* | 378/30873 | This study | $bla_{\text{NDM}}$ + $bla_{\text{OXA-48-}like}$ | 2 | 1 | 2 | 2 | 1 | 0.06 | 2 |
| *S. maltophilia* | 385/31448 | This study | - | 0.5 | 1 | 1 | 0.5 | 2 | ≤0.03 | 1 |
| *S. maltophilia* | 351/28640 | [14] | $bla_{\text{VIM-1}}$ | 2 | 0.25 | 0.5 | 0.5 | 0.5 | ≤0.03 | 0.5 |
| *A. baumannii* | ATCC 17978 | [15] | - | 2 | 2 | 2 | 1 | 4 | 0.06 | 1 |
| *P. aeruginosa* | PAO-1 (ATCC 15692) | [16] | - | 4 | >8 | 4 | 4 | >8 | 0.25 | 8 |
| *A. xylosoxidans* | 265/29789 | This study | - | 2 | 1 | 1 | 2 | 1 | 0.06 | 1 |
| *B. cepacia* | 246/20216 | This study | - | 2 | 2 | 2 | 1 | 1 | 0.06 | 4 |

A heatmap visualization (Figure 1) allowed rapid comparison of the activity profiles of all EOs across the tested MDR pathogens. CZ showed the highest antibacterial activity (brightest green, lowest MICs) across all the tested strains.

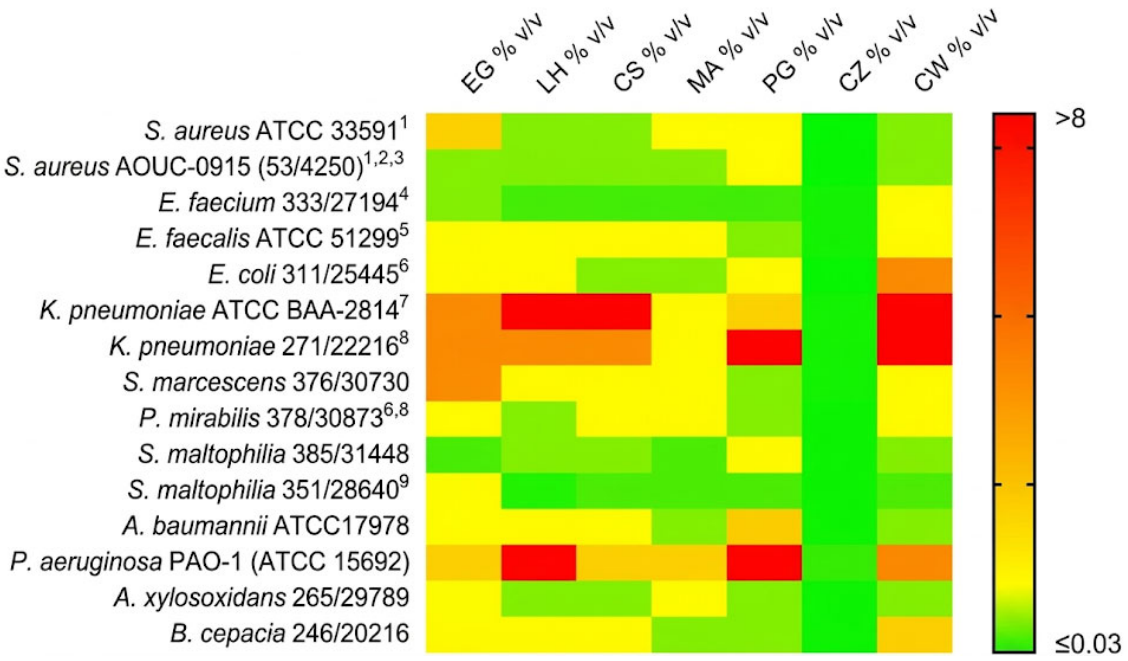



**Figure 1.** Heatmap of the antimicrobial activity of seven essential oil batches against the multidrug-resistant bacterial collections. Colors represent MIC values expressed in % *v*/*v* and transformed as log10 for visualization purposes: green/brighter colors correspond to lower MICs (higher activity), while red/darker colors indicate higher MICs (lower activity). Off-scale MIC values of '≤0.03' and '>8' were set to 0.03 and 16, respectively. The heatmap was constructed using GraphPad Prism (v 8.0). Essential oils tested: *Eucalyptus globulus* (EG), *Lavandula hybrida* (LH), *Coriandrum sativum* (CS), *Melaleuca alternifolia* (MA), *Pelargonium graveolens* (PG), *Cinnamomum zeylanicum* (CZ), and *Cymbopogon winterianus* (CW). Bacterial species and resistance profiles are indicated on the y-axis. Resistance determinants associated with each isolate are indicated as superscripts: *mecA* [1], *cfr* [2], *poxtA* [3], *vanA* [4], *vanB* [5], $bla_{\text{OXA-48-like}}$ [6], $bla_{\text{KPC}}$ [7], $bla_{\text{NDM}}$ [8], $bla_{\text{VIM}}$ [9].

## 4. Discussion

Thanks to their ability to affect multiple cellular processes critical for microbial survival, EOs represent promising candidates as antimicrobial agents or for potential use in combination with conventional antibiotics [21,22]. Although the antibacterial properties of several EOs have been previously reported [6,22], knowledge on their efficacy against clinically relevant MDROs, including members of the ESKAPE group, remains limited, and they have often been tested only with diffusion methods [23]. In this study, we assessed the antimicrobial activity of seven EO batches against a collection of MDROs, including some ESKAPE pathogens carrying different resistance determinants of major global concern, using a broth microdilution method.

The tested EOs exhibited heterogeneous antimicrobial activity against the panel of multidrug-resistant Gram-positive and Gram-negative strains. Overall, CZ showed the strongest and broadest activity across species, displaying consistently low MIC values. This broad-spectrum efficacy is consistent with previous studies highlighting the strong antimicrobial properties of cinnamon-derived oils, mainly attributed to cinnamaldehyde and eugenol [24–26]. These compounds are known to disrupt membrane integrity, induce leakage of intracellular contents, and interfere with essential metabolic pathways [5]. Indeed, *mecA*-, *cfr*-, *poxtA*-, *vanA*/B, $bla_{\text{KPC}}$-, $bla_{\text{VIM}}$-, and $bla_{\text{NDM}}$-positive strains were inhibited at CZ concentrations as low as ≤0.03–0.25% (*v*/*v*). Moreover, non-fermenting Gram-negative pathogens such as *S. maltophilia*, *A. baumannii*, and *P. aeruginosa*, which represent major therapeutic challenges, were also inhibited by CZ at MIC ≤0.25% (*v*/*v*). The very low MIC values for *S. maltophilia* (≤0.03%) and *A. baumannii* (0.06%) are particularly noteworthy, as

both species commonly exhibit resistance to most antimicrobial classes [14,27]. Therefore, our study supports the potential of CZ as an alternative antimicrobial against difficult-to-treat bacteria and reinforces the importance of further investigating cinnamon oil and its main components, cinnamaldehyde and eugenol, for possible clinical applications.

As for the other EOs, our findings confirmed that EOs differ markedly in their antimicrobial potential, allegedly depending on their chemical composition and the intrinsic resistance mechanisms of the target microorganisms, as observed also by other authors [28–30]. Their mechanism of action has yet to be fully elucidated, but it has been hypothesized to be due to loss of membrane integrity and function [5].

In our study, the MA batch exhibited interesting antimicrobial activity, ranking second among the tested EOs in broad-spectrum antibacterial activity against the Gram-positive and Gram-negative MDR bacteria tested. Although the MIC value (4%) against the *P. aeruginosa* PAO-1 strain was the highest observed in our panel, previous studies reported both bactericidal activity and antibiofilm properties of tea tree oil against this pathogen [31,32]. The antimicrobial activity of tea tree oil was likely dependent on the synergistic action of its constituent compounds, mainly terpinen-4-ol and terpineol (also detected in the batch tested in this study), whose lipophilicity and hydroxyl groups facilitate efficient cellular membrane penetration and biological interaction [33].

Finally, CS and LH showed good activity against the tested MRSA and VRE. Previous studies associated the antimicrobial activity with their major component, linalool, targeting the bacterial cytoplasmic membrane and the glutathione pathway [34–36].

While *in vitro* studies on essential oils in the microbiological field are increasing in number, research into their possible clinical applications is limited, and even more so is the analysis of their pharmacodynamic and pharmacokinetic properties in humans. Pharmacokinetic data in animals showed that terpenes were well absorbed after oral, transdermal, or inhalational administration and were largely metabolized in the liver into secondary metabolites, which were excreted mainly in the urine and to a lesser extent in the feces [37]. In humans, the most consistent data concerns the essential oil of *E. globulus* and its main component, 1,8-cineole [38,39], and to a lesser extent, linalool (the main component of the essential oil of *L. hybrida*) [40,41]. Despite the strong *in vitro* potency of CZ, limited pharmacokinetic data are available, mostly from animal models. Its primary constituent, cinnamaldehyde, undergoes rapid hepatic metabolism into cinnamic acid and hippuric acid, resulting in a low plasma half-life of the free compound after oral administration [42]. Careful consideration is therefore required before the possible *in vivo* application can be studied. First of all, the tissue and plasma concentrations detected for the main oil compounds or their secondary metabolites in humans are in the order of ng/mL, whereas the *in vitro* MICs of these oils are in the order of μg/mL. At present, studies on lipid-based nano/microscale delivery systems appear to be the most promising options to increase the bioavailability of the essential oils for *in vivo* application, although their clinical application in humans is still distant and unstudied [43]. A further consideration stems from the fact that, while the *in vitro* antibiotic activity of essential oils as a whole or of their main constituents is well documented, that of their secondary metabolites is scarce and not systematically studied. Secondary metabolites are the compounds most commonly measured in plasma or urine after oral administration; therefore, their *in vitro* antibiotic properties should be investigated to allow a more informed *in vivo* application. Despite the limitations outlined above, clinical studies exist that examine the effect of essential oils in the treatment of infections, mostly involving topical applications (e.g., cutaneous or mucosal); however, these data remain very limited in number and methodological quality [44]. Altogether, the *in vivo* use of essential oils in the microbiological field requires further pharmacokinetic and safety studies, as well as further investigation into the antimicrobial activity of the main metabolites excreted in

urine and detectable in plasma after oral administration. At the same time, their possible topical application represents a promising area of research.

Our study provided an *in vitro* evaluation of the activity of several different EOs against a panel of clinically relevant bacterial pathogens, including some difficult-to-treat Gram-positive and Gram-negative members of ESKAPE species. However, this study also has some limitations that should be acknowledged: (i) the antimicrobial activity of the EOs was evaluated against a relatively small panel of representative strains, limiting the generalizability of the results across species and resistance mechanisms; (ii) the potential cidality of active compounds was not evaluated; (iii) interactions of EOs with conventional antimicrobial agents in terms of synergism, indifference, or antagonism of antimicrobial activity were not evaluated; (iv) the mechanism of antimicrobial action of EOs at the molecular level was not investigated. Investigation of these aspects was beyond the scope of this initial scoping work, but could be the subject of future studies with the most promising compounds identified in this work, such as CZ. Additionally, the identification of the main constituents responsible for antimicrobial activity will be essential to improve reproducibility, ensure batch-to-batch consistency, reduce the variability associated with the use of the complex EO mixture, and progress into clinical development.

## 5. Conclusions

These findings suggest that CZ could represent a promising strategy to tackle some clinically relevant antimicrobial-resistant pathogens and contribute to the development of new antimicrobial agents and strategies. Further research is needed to broaden the panel of species tested, assess safety profiles and potential synergistic effects with conventional treatments, and evaluate pharmacokinetic properties in view of potential clinical development.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antibiotics15111071/s1, Table S1. Major constituents identified by GC-MS in steam-distilled essential oil batches tested for their antimicrobial activity in this study; Table S2. Phenotypic and molecular profile of clinical isolates included in this study.

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**Institutional Review Board Statement:** Not applicable. The study utilized anonymized clinical strains collected during routine diagnostic activities, along with reference strains from public culture collections. No human subjects, patient personal data, or clinical interventions were directly involved in this research.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study, including replicate-level minimum inhibitory concentration (MIC) determinations and batch-specific GC–MS analytical profiles, are available on reasonable request from the corresponding author.

**Conflicts of Interest:** Roberto Spampatti and Tommaso Pelagatti, employed by the drugstore "Farmacia Tili", provided the essential oils, produced at the farmhouse "Casalvento". Neither Farmacia Tili nor Casalvento had any role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results. All other authors declare no conflicts of interest relative to this research.

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