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Synergistic and Additive Effects of Binary Combinations of Ciprofloxacin with *Origanum vulgare* L. or *Cinnamomum cassia* (L.) J. Presl. Essential Oil Against Medically Relevant Bacteria

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Abstract

Background/Objectives: Bacterial resistance to antimicrobials has become a public health problem worldwide, limiting the effectiveness of conventional treatments. Therefore, combination antimicrobial therapy has been proposed as a strategy to combat multidrug-resistant bacteria. This study aimed to investigate the antibacterial effects of ciprofloxacin (CIP) in binary combinations with *Cinnamomum cassia* (L.) J. Presl. (CEO) and *Origanum vulgare* L. (OEO) essential oils against *Acinetobacter baumannii*, *Klebsiella quasipneumoniae*, *Pseudomonas paraeruginosa*, *Staphylococcus aureus*, and methicillin-resistant *S. aureus* (MRSA). **Methods**: The hemolytic activity was evaluated for the compounds individually and when combined. The antimicrobial activity was performed by broth dilution in double-antimicrobial gradient and time-kill assays. **Results**: The CEO–CIP and OEO–CIP combinations demonstrated hemolytic activity in human erythrocytes of 4.04 to 3.35% and 3.39 to 2.29%, respectively, and presented synergistic, additive, and indifferent antibacterial effects. The CEO–CIP combination significantly reduced CIP minimal inhibitory concentration from 256 to 0.125 µg/mL against MRSA. OEO–CIP was bactericidal against *P. paraeruginosa* and *S. aureus*; meanwhile, the CEO–CIP combination showed bactericidal activity against *P. paraeruginosa*, but reduced *S. aureus* viability by only 1.83 log after 24 h. **Conclusions**: These findings demonstrate the combination of CEO–CIP against *A. baumannii*, *K. quasipneumoniae*, *P. paraeruginosa*, *S. aureus*, and MRSA, in addition to hemolytic activity below 5% at the tested concentrations. Although limited to in vitro conditions, these results highlight the combination's therapeutic potential and emphasize the necessity for further investigation into its mechanisms of action and in vivo toxicity.

Keywords: *Origanum vulgare* L.; *Cinnamomum cassia* (L.) J. Presl.; multidrug-resistant bacteria; antibacterial combination; hemolytic assay

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1. Introduction

Antimicrobial resistance (AMR) is one of the most serious threats to human health at present [1]. Previous studies estimated that, by 2050, antimicrobial-resistant infections could be responsible for more than 10 million deaths annually if effective control measures and novel therapeutic alternatives were not implemented [2]. More recently, Naghavi et al. [3] projected more than 39 million cumulative deaths attributable to AMR worldwide between 2025 and 2050. This scenario has negative consequences for socioeconomic development by overloading health systems, which increases treatment-related costs, including hospitalization and medication. Furthermore, bacterial resistance can also affect international trade and livestock farming, potentially causing a more severe economic impact than that seen during the 2008 financial crisis [4].

Among the pathogens associated with AMR, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were responsible for approximately 72% of the 4.95 million deaths attributable to multidrug-resistant bacterial infections in 2019 [5]. Additionally, *Klebsiella quasipneumoniae*, which belongs to the *K. pneumoniae* complex, and *Pseudomonas paraeruginosa*, a novel species similar in environmental spectrum to *P. aeruginosa* and notably resistant to last-line antibiotics [6], have also raised concern regarding their resistance to antibiotics [7,8]. These microorganisms have an enormous negative impact on human health, causing infections in skin, soft tissues, burns, and wounds, as well as diseases, such as pneumonia, secondary meningitis, endocarditis, chronic rhinosinusitis, bacteremia, and sepsis [9–12]. The therapeutic management of these infections is further complicated by the increasing prevalence of resistant phenotypes, including methicillin-resistant *S. aureus* (MRSA), which is associated with limited treatment options and substantial morbidity and mortality [13]. Thus, the development of alternative strategies capable of restoring or enhancing the activity of currently available antimicrobials remains an important research priority [14].

In response to this challenge, the antibacterial potential of essential oils stands out; these are natural, complex, liquid, and volatile compounds generally produced by the secondary metabolism of aromatic plants [15]. Essential oils have been used for centuries for different purposes, such as food preservation, perfumes and cosmetic fragrances, and medicinal treatments for various human diseases [16]. About three hundred essential oils can be found on the market for incorporation into several products for food and health purposes, since they are safe at small dosages and under specific conditions [17,18]. Previous studies have shown that *Cinnamomum cassia* (L.) J. Presl. (CEO) and *Origanum vulgare* L. (OEO) essential oils exhibit broad-spectrum antibacterial activity, including against multidrug-resistant strains [19–26]. Both oils have also been evaluated by the Flavor and Extract Manufacturers Association Expert Panel and are included among the flavoring substances recognized as safe for their use in foods [27,28], which may facilitate the development and future application of these essential oils as alternative antimicrobial agents.

Despite their excellent antimicrobial activity, essential oils may present limitations, including poor solubility in certain solvents and strong odor or flavor, depending on their chemical composition [29–31]. Combining essential oils with conventional antimicrobials is a strategy to overcome these limitations, as their distinct mechanisms of action may enhance antimicrobial efficacy. In addition, such combinations may accelerate the antimicrobial effect compared with the individual compounds and/or allow lower concentrations of the agents to be used [32–37].

Ciprofloxacin (CIP), a second-generation fluoroquinolone, has been used as first-line treatment in hospital and clinical settings due to its action against Gram-positive and Gram-negative bacteria [36,38]. Despite its widespread use, increasing resistance to fluoroquinolones has been reported among clinically important pathogens, including *A. baumannii*, *Escherichia coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, and *Salmonella enterica* serovar Corvallis [39–44]. Consequently, strategies capable of enhancing CIP activity or reducing the concentration required for antibacterial effects are of particular interest.

The specific binary combination of *C. cassia* (L.) J. Presl. with CIP against *A. baumannii* ATCC 19606, *P. paraeruginosa* ATCC 9027, *K. quasipneumoniae* ATCC 700603, and *S. aureus* ATCC 6538 strains has not been previously characterized regarding its concomitant evaluation of combination interactions, time-kill, and the erythrocyte hemolytic assay. In contrast, synergistic antibacterial effects have been reported for *O. vulgare* essential oil when combined with CIP against *P. paraeruginosa* ATCC 9027 [45] and *K. pneumoniae* ATCC 13883 [46]. However, these studies have focused on demonstrating the existence of antibacterial interaction, providing limited information regarding the activity of different essential oil and CIP combinations across clinically relevant Gram-positive and Gram-negative bacteria, together with their kinetic of action behavior, and hemolytic activity in erythrocytes.

Considering the known effects of the CEO and OEO on microorganisms but the limited investigation of the antibacterial effects, time of action and hemolytic activity of these compounds with CIP, this study aims to evaluate the hemolytic activity of *C. cassia* (L.) J. Presl. and *O. vulgare* L. essential oils in binary combinations with CIP, and their potential as antimicrobials against clinically relevant Gram-positive and Gram-negative bacteria, including MRSA. The bacterial species included in this study are priority pathogens because of their increasing resistance to antimicrobial agents. As a result, these microorganisms are considered priority targets for the discovery and development of new therapeutic strategies [1].

2. Results

2.1. Hemolytic Activity of Compounds

The compounds alone, the compounds at their minimal inhibitory concentration (MIC) values for the standard bacterial strains, and the OEO–CIP and CEO–CIP combinations did not reach the concentration required to reduce cell viability by 50% compared to the untreated control (CC_{50}) (Figure 1). The CC_{50} of CIP was not found, since its highest tested concentration (8 µg/mL) showed 3.49% hemolysis to human red blood cells (RBCs). The CC_{50} of OEO ranged from 2.962 to 5.925 mg/mL (15.78% and 72.06%, respectively), which was close to its MIC range of 0.740–2.962 mg/mL. In contrast, CEO exhibited a CC_{50} between 13.312 and 26.625 mg/mL (8.67% and 55.18%, respectively), which was more than four times higher than its MIC range of 0.416–3.328 mg/mL. The chosen concentrations of antimicrobials and the percentages of their hemolytic activity are indicated in the Supplementary Materials, Table S1.

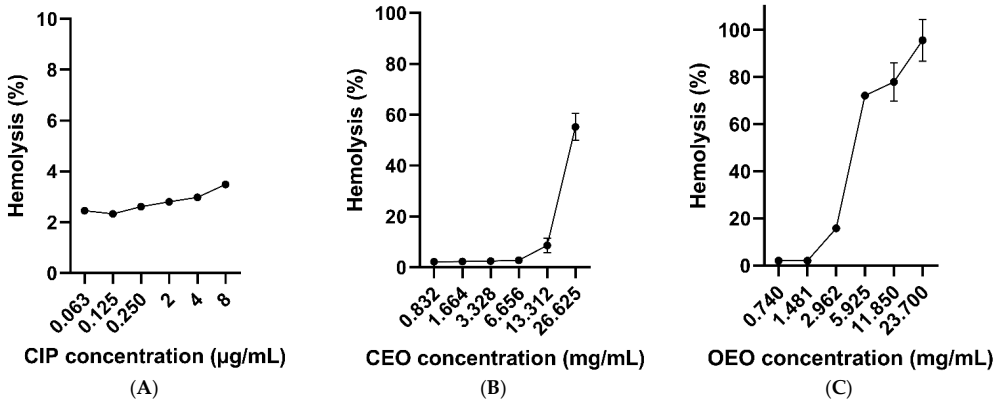


Figure 1. Hemolytic activity of different concentrations of ciprofloxacin (CIP), cinnamon essential oil (CEO), and oregano essential oil (OEO) individually. (**A**) Human erythrocytes exposed to CIP alone ranging from 0.063 to 8 µg/mL. (**B**) Human erythrocytes exposed to CEO alone ranging from 0.832 to 26.625 mg/mL. (**C**) Human erythrocytes exposed to OEO alone ranging from 0.740 to 23.700 mg/mL. Triton-X at 1% was used as a positive control, indicating 100% hemolytic activity. Values of hemolysis percentages are the mean ± standard deviation.

The CEO–CIP and OEO–CIP combinations showed slightly higher percentages of hemolysis in RBCs than the respective compounds evaluated individually at specific concentrations (Table 1). For CEO–CIP, this difference was observed at combinations of 0.125 µg/mL CIP with 0.832 or 1.664 mg/mL CEO and at 0.063 µg/mL CIP with 1.664 mg/mL CEO ($p < 0.05$, Tukey's test). For OEO–CIP, a higher hemolytic activity was observed at the combination of 0.125 µg/mL CIP and 1.481 mg/mL OEO ($p < 0.05$, Tukey's test). All the combined treatments showed no CC_{50} in RBCs at tested concentrations, especially at MIC.

Table 1. Hemolytic activity at different concentrations of ciprofloxacin (CIP, µg/mL) in binary combinations with cinnamon (CEO, mg/mL) and oregano (OEO, mg/mL) essential oils.

Combination	Compound Concentrations		Hemolysis (%)
	CIP (µg/mL)	Essential Oil (mg/mL)	
CIP + CEO	0.125	1.664	4.04 ± 0.04
	0.063	1.664	3.90 ± 0.13
	0.125	0.832	3.35 ± 0.58
	0.031	0.832	3.72 ± 0.09
	0.500	0.416	3.69 ± 0.13
	0.250	0.416	3.64 ± 0.24
CIP + OEO	0.125	1.481	3.39 ± 0.18
	0.063	1.481	2.94 ± 0.21
	0.125	0.740	2.72 ± 0.12
	0.031	0.740	2.29 ± 0.50
	0.500	0.370	2.75 ± 0.18
	0.250	0.370	2.65 ± 0.14

CEO: cinnamon essential oil; CIP: ciprofloxacin; OEO: oregano essential oil; ±: standard deviation. Triton-X at 1% was used as positive control, indicating 100% hemolytic activity. Ciprofloxacin and essential oil concentrations correspond to concentrations used in respective binary combinations.

Based on these erythrocyte hemolysis results, CEO alone exhibited significantly lower erythrocyte hemolysis than OEO at the highest concentrations tested ($p < 0.05$, Tukey's test), leading the present investigation to focus on CEO.

2.2. Minimal Inhibitory Concentration (MIC) of Compounds Individually

CIP, CEO, and OEO inhibited the growth of all bacterial strains evaluated. Table 2 presents the results of the antibacterial activity assay. The MIC values ranged from 0.125 to 256 µg/mL for CIP, from 0.416 to 3.328 mg/mL for CEO, and from 0.740 to 2.962 mg/mL for OEO.

Table 2. Minimum Inhibitory Concentration of ciprofloxacin, cinnamon essential oil, and oregano essential oil individually.

Bacterial Strains	CIP	CEO	OEO
	µg/mL	mg/mL	
A. baumannii ATCC 19606	1	0.832	0.740
K. quasipneumoniae ATCC 700603	1	0.416	1.481
P. paraeruginosa ATCC 9027	0.250	3.328	2.962
S. aureus ATCC 6538	0.125	3.328	2.962
MRSA 2115	256	1.664	–
MRSA 2117	256	1.664	–
MRSA 2119	256	1.664	–

CEO: cinnamon essential oil; CIP: ciprofloxacin; MRSA: methicillin-resistant *S. aureus*; OEO: oregano essential oil; (–) not evaluated. The concentrations are rounded representations of an exact twofold dilution series.

2.3. Minimal Inhibitory Concentration (MIC) of Compounds in Binary Combinations

The binary combinations of CEO–CIP and OEO–CIP showed significantly lower MIC values than the respective individual treatments, resulting in synergistic, additive, or indifferent antibacterial interactions (Tables 3 and 4, respectively). For *P. paraeruginosa* ATCC 9027, *S. aureus* ATCC 6538, and the three MRSA strains, the MIC of CIP was significantly reduced ($p < 0.05$, Tukey's test) when combined with CEO. Similarly, the MIC of CIP was significantly reduced ($p < 0.05$, Tukey's test) in combination with OEO against *K. quasipneumoniae* ATCC 700603, *P. paraeruginosa* ATCC 9027, and *S. aureus* ATCC 6538. The chosen concentrations of the combinations against the standard strains and the three MRSA isolates are indicated in the Supplementary Materials, in Tables S2 and S3, respectively.

Table 3. Minimum Inhibitory Concentration of ciprofloxacin (CIP, µg/mL) in combination with cinnamon essential oil (CEO, mg/mL).

Bacterial Strains	CIP		CEO		FICI (Interaction)
	MIC_C	Fold Decrease	MIC_C	Fold Decrease	
A. baumannii ATCC 19606	0.500	2×	0.416	2×	1 (A)
K. quasipneumoniae ATCC 700603	0.500	2×	0.416	0×	1.500 (I)
P. paraeruginosa ATCC 9027	0.063	4×	1.664	2×	0.750 (A)
S. aureus ATCC 6538	0.016	8×	0.832	4×	0.374 (S)
MRSA 2115	0.125	2048×	0.832	2×	0.5005 (A)
MRSA 2117	0.125	2048×	0.832	2×	0.5005 (A)
MRSA 2119	0.125	2048×	0.832	2×	0.5005 (A)

CEO: cinnamon essential oil; CIP: ciprofloxacin; FICI: fractional inhibitory concentration index; MIC_C: MIC values of each compound combined; MRSA: methicillin-resistant *S. aureus*. Fold decrease describes how much the MIC of both compounds in combination was reduced in comparison to the MIC of the same compounds individually (MIC values of compounds alone are shown in Table 2). The unrounded concentrations tested were used to calculate both fold reductions and FICI values. The FIC index was interpreted as follows: ≤0.5, synergy; >0.5 to 1, addition; >1 to <4, indifference; and ≥4, antagonism. (S) Synergistic interaction. (A) Additive interaction. (I) Indifferent.

Table 4. Minimum Inhibitory Concentration of ciprofloxacin (CIP, µg/mL) in combination with oregano essential oil (OEO, mg/mL).

Bacterial Strains	CIP		OEO		FICI (Interaction)
	MIC_C	Fold Decrease	MIC_C	Fold Decrease	
A. baumannii ATCC 19606	0.500	2×	0.370	2×	1 (A)
K. quasipneumoniae ATCC 700603	0.250	4×	0.185	8×	0.375 (S)
P. paraeruginosa ATCC 9027	0.125	2×	1.481	2×	1 (A)
S. aureus ATCC 6538	0.016	8×	0.740	4×	0.375 (S)

CIP: ciprofloxacin; FICI: fractional inhibitory concentration index; MIC_C: MIC values of each compound combined; OEO: oregano essential oil. Fold decrease describes how much the MIC of both compounds in combination was reduced in comparison to the MIC of the same compounds individually (MIC values of compounds alone are shown in Table 2). The unrounded concentrations tested were used to calculate both fold reductions and FICI values. The FIC index was interpreted as follows: ≤0.5, synergy; >0.5 to 1, addition; >1 to <4, indifference; and ≥4, antagonism. (S) Synergistic interaction. (A) Additive interaction.

2.4. Time-Kill Curve

For the Gram-negative strain *P. paraeruginosa* ATCC 9027 (Figure 2) and the Gram-positive strain *S. aureus* ATCC 6538 (Figure 3), treatment with OEO at MIC (2.962 mg/mL) values resulted in no detection of viable cells after 10 s ($p < 0.05$, Tukey's test). CEO at MIC (3.328 mg/mL) values resulted in a 2.41-log reduction after 5 min of treatment and a bactericidal effect after 15 min for *P. paraeruginosa* ATCC 9027 ($p < 0.05$, Tukey's test). For *S. aureus* ATCC 6538, there was a 1.52-log reduction with 15 min of treatment, followed by a

decrease of 2.78-log in 30 min ($p < 0.05$, Tukey's test). After 1 h of treatment, no viable cells were detected ($p < 0.05$, Tukey's test). The positive control (C+) remained viable throughout the entire experiment, with an increase of 3.32-logs in 24 h for *P. paraeruginosa* ATCC 9027 and 2.93-logs for *S. aureus* ATCC 6538.

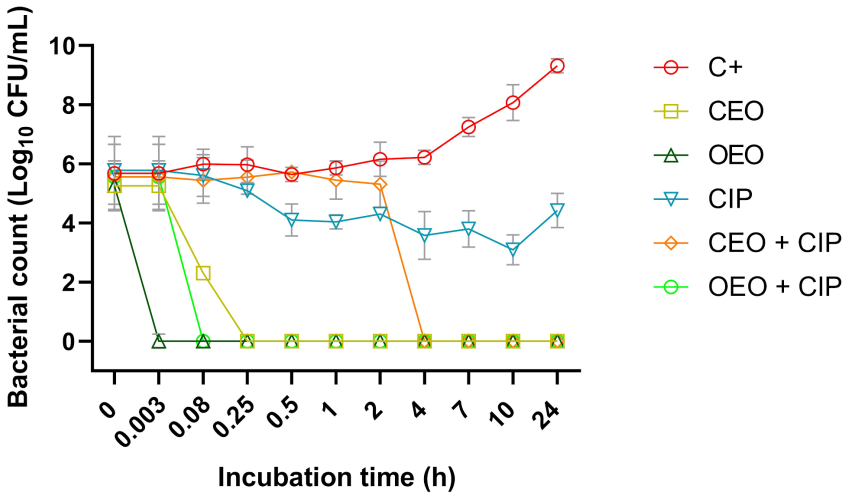


Figure 2. Time-kill curves of *P. paraeruginosa* ATCC 9027 exposed to cinnamon essential oil at 3.328 mg/mL (CEO), oregano essential oil at 2.962 mg/mL (OEO) and ciprofloxacin at 0.250 µg/mL (CIP) individually and in binary combinations (CEO at 1.664 mg/mL plus CIP at 0.063 µg/mL; OEO at 1.481 mg/mL plus CIP at 0.125 µg/mL). Positive control (C+) indicates the bacterial growth, as it does not contain any antimicrobial agent. All data are presented as the mean log10 CFU/mL of three replicates with standard deviation bars for all points.

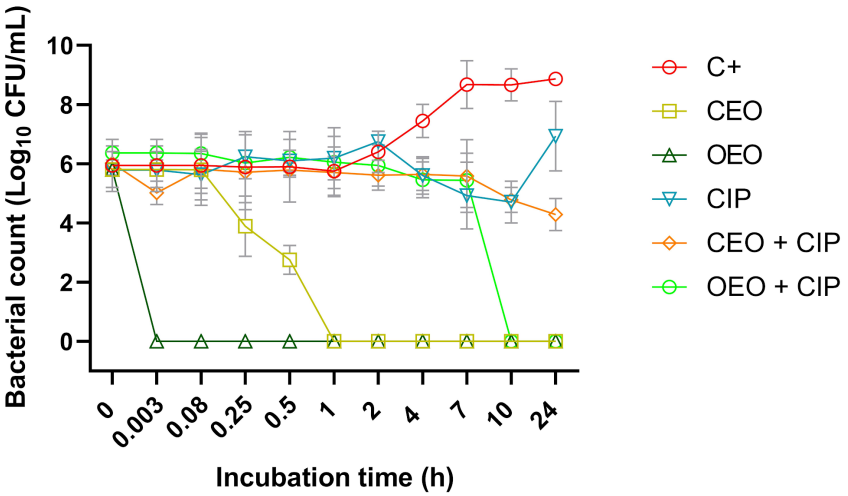


Figure 3. Time-kill curves of *S. aureus* ATCC 6538 exposed to cinnamon essential oil at 3.328 mg/mL (CEO), oregano essential oil at 2.962 mg/mL (OEO) and ciprofloxacin at 0.125 µg/mL (CIP) individually and in binary combinations (CEO at 0.832 mg/mL plus CIP at 0.016 µg/mL; OEO at 0.740 mg/mL plus CIP at 0.016 µg/mL). Positive control (C+) indicates the bacterial growth, as it does not contain any antimicrobial agent. All data are presented as the mean log10 CFU/mL of three replicates with standard deviation bars for all points.

All treatments (CIP, CEO, OEO, CEO–CIP, and OEO–CIP) evaluated in the time-kill assay showed a significant difference ($p < 0.05$, Tukey's test) when compared with the bacterial growth control in terms of CFU/mL. When treatments were compared with one another using a two-way ANOVA test, all differences were significant ($p < 0.05$, Tukey's test), except when the CFU/mL reduction in CIP alone was compared to the CEO–CIP combination against *S. aureus* ATCC 6538.

CIP at its MIC value did not eliminate *P. paraeruginosa* ATCC 9027 (0.250 µg/mL) and *S. aureus* ATCC 6538 (0.125 µg/mL). After 1 h of treatment, the bacterial population of *P. paraeruginosa* decreased by 1.89-log. For *S. aureus*, there was a reduction of 0.93-log after 7 h of incubation. Although there was a reduction in the bacterial populations, both tested strains exhibited an increase in growth after 10 h of treatment.

The combination of CIP and OEO led to a faster reduction in CFU/mL than individual treatment with CIP ($p < 0.05$, Tukey's test). Against *P. paraeruginosa* ATCC 9027, the combination of the two compounds at additive MIC (0.125 µg/mL and 1.481 mg/mL for CIP and OEO, respectively) values showed bactericidal activity after 5 min of treatment. The combination at the synergistic MIC values (0.016 µg/mL for CIP and 0.740 mg/mL for OEO) against *S. aureus* ATCC 6538 led to a total reduction in the viable cells after 7 h of treatment.

Against *P. paraeruginosa* ATCC 9027, the combination of CIP and CEO at additive MIC values (0.063 µg/mL and 1.664 mg/mL, respectively) resulted in complete elimination of detectable viable cells after 2 h ($p < 0.05$, Tukey's test), but the combination at synergistic MIC values (0.016 µg/mL and 0.832 mg/mL for CIP and CEO, respectively) for *S. aureus* ATCC 6538 caused a 1.83-log decrease in cell population in 24 h.

3. Discussion

This study showed that *C. cassia* and *O. vulgare* essential oils have potent antibacterial activity, in agreement with previous studies [21,25,47–49]. In this study, CEO was antibacterial against the MRSA isolates, with an MIC of 1.664 mg/mL, and with MIC values for the other bacteria from 0.416 to 3.328 mg/mL (Table 2), similar to the results found in the literature. Hossan et al. [48] reported for CEO an MIC value of 1.5 mg/mL against *P. aeruginosa* ATCC 1544. Campini et al. [25] reported an MIC of 1 mg/mL for *S. aureus* ATCC 6538, while Vasconcelos et al. [23] reported a CEO MIC of 0.28 mg/mL for a clinical isolate of *K. pneumoniae*.

To the best of our knowledge, no studies have been previously characterized regarding the concomitant evaluation of the antibacterial effects of *C. cassia* (L.) J. Presl. and *O. vulgare* L. essential oils against *K. quasipneumoniae* ATCC 700603. Therefore, in the absence of species-specific evidence, previous studies involving *K. pneumoniae* were considered for contextual comparison. Although *K. quasipneumoniae* and *K. pneumoniae* are distinct species, they belong to the *K. pneumoniae* complex, share similar phenotypic and biochemical characteristics, and are often clinically indistinguishable [7,50,51]. Likewise, *P. aeruginosa* was considered as a reference for comparison, since studies investigating *P. paraeruginosa* in the context of the present study are limited. Nevertheless, previous studies have reported that *P. paraeruginosa* isolates share similar pathogenic characteristics with the most common *P. aeruginosa* phylogroups and exhibit a comparable environmental distribution [6,8]. Additionally, the strains *K. quasipneumoniae* ATCC 700603 and *P. paraeruginosa* ATCC 9027 were formerly designated *K. pneumoniae* K6 and *P. aeruginosa* ATCC 9027, respectively, before being reclassified based on genomic and phylogenomic evidence [52,53].

For OEO, the MIC values obtained in our study ranged from 0.740 to 2.962 mg/mL (Table 2), which were compatible with the literature results. Scandorieiro et al. [49] reported MIC values of 0.74, 1.48, and 2.38 mg/mL against *A. baumannii* ATCC 19606, *K. pneumoniae* ATCC 10031, and *S. aureus* ATCC 25923, respectively. Torabian Kakhki et al. [21] reported an MIC of 1.28 mg/mL against *P. paraeruginosa* ATCC 9027. Different values from those obtained in this study were reported by Amaral et al. [22], who described an MIC of 1.75 mg/mL for the same *A. baumannii* strain. This difference may be related to the use of Tween 80 as a surfactant [54].

Time-kill assays showed that OEO at MIC values eliminated all cell populations immediately (10 s) in the two bacterial strains evaluated (Figures 2 and 3) ($p < 0.05$, Tukey's

test). CEO MIC values showed no viable cells after 15 min for *P. paraeruginosa* ATCC 9027 (Figure 2) and 1 h for *S. aureus* ATCC 6538 (Figure 3). Likewise, Scandorieiro et al. [49] reported that when *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 at 10^8 CFU/mL were exposed to OEO (0.6 mg/mL), there were no viable cells after 10 and 30 min, respectively. Sim et al. [55] demonstrated that CEO presented a greater 2-log reduction against *P. aeruginosa* and *S. aureus* cells after treatment.

Studies indicate that Gram-positive bacteria can be more sensitive to the action of essential oils, including their derivatives [47]. In contrast, the results of the present study showed that the Gram-negative bacteria *A. baumannii* ATCC 19606 and *K. quasipneumoniae* ATCC 700603 presented lower MIC values for both essential oils when compared to the MIC values of *S. aureus* ATCC 6538 (Table 2), indicating a greater sensitivity to CEO and OEO. Similar results were also seen by Campini et al. [25], who presented higher MIC values for the *C. cassia* essential oil for *S. aureus* ATCC 6538 (1 mg/mL) than for *S. enterica* subsp. *Enterica* ATCC 10708 (0.13 mg/mL) and *E. coli* ATCC 11229 (0.250 mg/mL).

Our findings broadly align with the previous literature, indicating that both essential oils possess broad-spectrum antibacterial action. These general trends are consistent with observations reported by other authors, who also demonstrated the antimicrobial efficacy of these botanical species [22,23,25,49,56]. According to the manufacturer's technical report, the major components of the CEO used in this study are cinnamaldehyde (80%), cinnamic acid (2%), benzaldehyde (2%), coumarin (2%), and styrene (2%), whereas for OEO, they are carvacrol (72%), γ-terpinene (4.5%), linalool (4%), para-cymene (4%), and thymol (2%). Nevertheless, an important limitation of this study is that the specific experimental batches were not independently characterized by GC-MS, GC-FID, or other analytical methods. Consequently, the reported constituent percentages derive exclusively from the manufacturer's data, rather than measurements performed in our study. This absence of independent characterization limits the exact reproducibility of our findings, constrains direct quantitative comparisons with other investigations that utilize different plant batches, and prevents the precise attribution of the antibacterial activity to individual major or minor constituents. While variations in essential oil composition due to seasonal, geographic, storage, or developmental factors [57,58] highlight the need for independent chemical profiling in future studies, the observed biological properties provide a solid baseline to guide further investigations into the feasibility of these combinations.

In this study, CIP exhibited broad-spectrum antibacterial action against the bacterial strains (0.125–1 μg/mL), inhibiting growth of all tested bacteria (Table 2), in agreement with the literature [45,59–61]. Qi et al. [59] reported CIP MIC values of 1 mg/mL against the same *A. baumannii* strain. Similar values to those obtained in this study for CIP were reported by Cabral et al. [61], who described an MIC of 0.25 mg/mL for *S. aureus* ATCC 6538, and Sena et al. [45], who reported an MIC of 0.2 mg/mL for the same *P. paraeruginosa* ATCC 9027 strain. For contextual comparison, Uddin et al. [60] reported an MIC of 2 mg/mL against *K. pneumoniae* ATCC 23357, providing a reference point for comparison with the present findings for *K. quasipneumoniae*. In addition, the three clinical isolates showed resistance to CIP (256 μg/mL), since the MIC was above 4 μg/mL [62].

The time-kill assays for CIP exhibited bacteriostatic activity, given that there was no reduction of 3-logs or more, with an increase of 1.19-log between 10 and 24 h for *S. aureus* ATCC 6538 (Figures 2 and 3). Sihotang et al. [63] reported that CIP did not show bactericidal activity after 24 h of treatment for *P. aeruginosa* ATCC 27583, with an increase of approximately 1-log after 8 h of treatment. Kang et al. [64] showed that there was no significant reduction in viable bacterial cells after 8 h of treatment with CIP against *S. aureus* ATCC 29213, and no bactericidal activity after 24 h of treatment, findings which were congruent with our results (Figures 2 and 3).

Sena et al. [45] observed additive and synergistic effects of essential oils from plants belonging to the *Lauraceae* and *Lamiaceae* families, which include *C. cassia* and *O. vulgare* plants, respectively, in combination with several antimicrobials, as well as CIP, gentamicin, meropenem, and tetracycline, against *E. coli*, *P. aeruginosa*, and *Enterococcus faecalis*. Other studies have shown synergistic interactions of CIP with other natural alternative compounds, such as piceatannol, a natural product extracted from grape skins [36], and carvacrol [65]. Silva et al. [46] showed a synergistic effect of OEO combined with CIP against *K. pneumoniae*, which is similar to the results found in this study for *K. quasipneumoniae* ATCC 700603 (Table 4), although these findings involve different species and should therefore be interpreted as contextual rather than direct comparisons. These findings demonstrate the combination interaction of *O. vulgare* L. and CIP against *A. baumannii* ATCC 19606 and *S. aureus* ATCC 6538, providing evidence of the additive and synergistic antimicrobial potential of this association against these pathogens.

In the literature, the combination of *Cinnamomum zeylanicum* with gentamicin showed a synergistic interaction against *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, an isolate of *Staphylococcus epidermidis* [35], and multidrug-resistant *A. baumannii* [66]. These findings demonstrate the synergistic, additive, and indifferent effects of the combination interaction between *C. cassia* and CIP against *A. baumannii* ATCC 19606, *P. paraeruginosa* ATCC 9027, *K. quasipneumoniae* ATCC 700603, and *S. aureus* ATCC 6538 strains, including MRSA bacteria (Table 3). Against *K. quasipneumoniae*, the combination showed an indifferent interaction, which may be related to the greater tolerance of this bacterium to CEO, since Gram-negative bacteria are intrinsically less susceptible to many antibacterial agents than Gram-positive ones, due to the presence of an additional outer membrane acting as a barrier for amphipathic compounds [67]; however, further studies are needed to confirm this hypothesis. At the tested concentrations, when CEO was combined with CIP against the three MRSA isolates, the CIP concentration required for bacterial inhibition decreased 2048-fold (Table 3), highlighting the potential of CIP in the presence of CEO against multidrug-resistant *S. aureus*. The initial CIP MIC of 256 μg/mL was consistent with the resistant phenotype of the tested MRSA isolates [62], whereas the presence of the subinhibitory concentration of CEO (0.832 mg/mL, which corresponds to ½ MIC when compared to individual MIC values) represented the lowest observed FICI among the tested pairs and successfully inhibited bacterial growth when combined with the CIP MIC of 0.125 μg/mL (Supplementary Materials, Table S3).

The potentiated effects of the combinations observed in this study, which included a 2048-fold reduction in the CIP MIC against MRSA in the presence of CEO at the tested concentrations, may be related to the complementary mechanisms of action of essential oils with conventional antimicrobials [23,24,67]. In this context, according to the manufacturer's report, the major components of the CEO and OEO used in this study were cinnamaldehyde (80%) and carvacrol (72%), respectively. These compounds have been reported to cause structural and functional damage to the bacterial cell membrane [17,23,68,69]. Such effects could increase bacterial membrane permeability, thereby facilitating the intracellular accumulation of CIP, whose antibacterial activity involves inhibition of DNA gyrase [37]. Vasconcelos et al. [24] reported that the *C. cassia* essential oil causes leakage of intracellular proteins by increasing the membrane permeability of Carbapenemase-producing *Serratia marcescens*, allowing polymyxin B to enter the bacterial periplasmic space when in combination. In addition, CEO and OEO may inhibit efflux pumps, either directly or indirectly, through bacterial membrane disruption or dysfunction, as well as by interfering with the synthesis of essential bacterial metabolites, which could increase the intracellular availability of CIP [70]. Synergistic and additive interactions between essential oils and conventional antimicrobials may involve multiple mechanisms of action, offering several

advantages, such as enhancing the activity of the agents involved, reducing the dosage of the antimicrobials when combined, and decreasing the time of action compared to one or both agents alone [36,37,49]. Further mechanistic studies with the CEO–CIP combination are required to determine the specific processes responsible for the observed interactions.

The time-kill assays for the OEO–CIP combination at subinhibitory values showed no detected viable cells after 5 min for *P. paraeruginosa* and 7 h for *S. aureus* (Figures 2 and 3), while the CEO–CIP combination at subinhibitory values showed no detected viable cells after 2 h for *P. paraeruginosa* and a 1.83-log reduction after 24 h for *S. aureus* (Figures 2 and 3). The OEO–CIP combination showed enhanced reduction when compared to CIP treatment individually ($p < 0.05$, Tukey's test), since CIP alone did not eliminate the bacterial populations of *P. paraeruginosa* ATCC 9027 and *S. aureus* ATCC 6538, with growth returning after 10 h (Figures 2 and 3). Comparable results were reported by Vasconcelos et al. [24], who described that the combination of *C. cassia* and polymyxin B against a Carbapenem-producing *K. pneumoniae* exhibited a faster antimicrobial effect than either agent used alone. Similarly, Shi et al. [36] reported that when *S. aureus* ATCC 29213 was exposed to the piceatannol and CIP combination, there was a faster action and superior log reduction compared to CIP alone.

Due to their strong antimicrobial properties, CEO and OEO have potential to be used in combination with conventional antibiotics or as complementary agents in medical products, as well as in food preservation and cosmetic products, among other applications [16]. Nevertheless, their use requires special treatment and storage conditions so that the original chemical profile is not modified by different environmental aspects, such as light, heat, and oxidation. In addition, essential oils also exhibit high volatility, hydrophobicity, flavor, and odor that may limit their uses. These limitations may be overcome by encapsulation of volatile essential oils in various delivery systems, including lipid nanoparticles, liposomes, films, emulsion gels, oil-in-water emulsions, and spray-dried microparticles, which can help preserve their biological activity while improving absorption and controlled delivery. These strategies are particularly relevant for biomedical, pharmaceutical, medical, and cosmetic applications, as they may preserve their pharmacological activities, while reducing potential toxicity and protecting their chemical composition from alterations [71].

Both combinations (CEO–CIP and OEO–CIP) did not show high percentages of erythrocyte hemolysis (3.35% to 4.04%; 2.29% to 3.39%, respectively) at MIC values, since they caused less than 5% hemolysis (CC_{50} of combinations was not found). All the combined concentrations assessed (Table 1) did not cause high degrees of hemolysis, even when they showed additive and synergistic antibacterial effects.

Moreover, CEO, OEO, and CIP did not reach CC_{50} at MIC values for the bacterial strains (Figure 1). CEO exhibited a CC_{50} between 13.312 and 26.625 mg/mL (8.67% and 55.18%, respectively) (Figure 1B), which is still approximately four times the MIC range for the tested standard strains (Table 2). Barros et al. [72] observed that *C. zeylanicum* essential oil can exhibit hemolytic effects on RBCs, depending on the concentration. Notably, within the concentration range evaluated by the authors (5–200 µg/mL), the maximum hemolytic percentage was approximately 2%.

When OEO (Figure 1C) was at inhibitory concentrations, the hemolytic activity ranged from 2.02% to 15.78% (0.740 to 2.962 mg/mL, respectively), but at concentrations above the MIC tested (5.925 to 23.700 mg/mL), OEO showed hemolytic activity higher than CC_{50} (72.06% to 95.6%, respectively). Similar results were found by Scandorieiro et al. [49] for the OEO, considering that the inhibitory activity was lower than CC_{50} (Table 2). Therefore, when compared to CEO (Figure 1B), OEO (Figure 1C) showed a higher percentage of erythrocyte hemolysis at the tested concentrations (Supplementary Table S1). Based on these results, CEO was selected for further evaluation against the clinical MRSA isolates.

CIP (Figure 1A) exhibited low hemolytic activity (2.44% to 3.49%) at concentrations closer to the MIC values for the standard bacterial strains (Table 2). Although the hemolytic activity of CIP was not evaluated at concentrations close to the MIC determined for MRSA (256 μg/mL), CIP is widely used in clinical and hospital settings [36]. According to the U.S. Food and Drug Administration (FDA), CIP is available in oral formulations, including 250 and 500 mg tablets, while recommended adult doses range from 250 to 750 mg, depending on the indication [73]. Nevertheless, because the erythrocyte hemolysis assay was performed using blood from a single healthy human donor, donor-to-donor variability could not be assessed, and the results do not establish general mammalian safety.

This study has several limitations that should be considered when interpreting the findings. The relatively small bacterial panel and predominant use of reference strains limit the generalizability of the findings. In addition, only three clinical MRSA isolates were included, and OEO–CIP was not evaluated against these isolates. Time-kill assays were restricted to two reference strains, limiting assessment across a broader range of isolates. The essential oil batches were not independently characterized by analytical methods, and batch-to-batch compositional variation cannot be excluded. The erythrocyte hemolysis assay used blood from a single healthy human donor, preventing assessment of donor-to-donor variability and limiting extrapolation to broader safety outcomes. Finally, direct mechanistic experiments and in vivo studies were not performed; therefore, the mechanisms underlying the observed antibacterial effects and the in vivo efficacy and safety of these combinations remain to be established.

Our results highlight the significant 2048-fold decrease in the CIP concentration required for growth inhibition in the presence of *C. cassia* against MRSA at the tested concentrations, as well as the synergistic and additive effects observed for CIP in combination with *C. cassia* or *O. vulgare* essential oils against *A. baumannii* ATCC 19606, *K. quasipneumoniae* ATCC 700603, *P. paraeruginosa* ATCC 9027, and *S. aureus* ATCC 6538 strains (Tables 3 and 4). The OEO–CIP also showed a bactericidal effect against *P. paraeruginosa* and *S. aureus*; meanwhile, CIP alone showed only bacteriostatic activity. The CEO, CIP, and CEO–CIP and OEO–CIP combinations exhibited less than 5% hemolysis at the MIC values for the bacterial strains. However, despite being below the CC_{50} value, OEO caused 15.78% hemolysis at a concentration of 2.962 mg/mL. Additionally, future research on the same topic as the present study may include an extended bacteria panel, including more multidrug-resistant isolates; both more essential oils and combinations; chemical characterization of the experimental essential oil batches; in-depth studies on the mechanisms of action; and in vivo trials to evaluate the effectiveness and safety of using these combinations.

4. Materials and Methods

4.1. Bacterial Strains

The antimicrobial assays were performed against reference strains from American Type Culture Collection (ATCC) provided by the Laboratory of Basic and Applied Bacteriology of Londrina State University, located in Londrina, Paraná, Brazil. The standard bacterial strains used were *Acinetobacter baumannii* (ATCC 19606), *Klebsiella quasipneumoniae* (ATCC 700603), *Pseudomonas paraeruginosa* (ATCC 9027) and *Staphylococcus aureus* (ATCC 6538). The strains *K. quasipneumoniae* ATCC 700603 and *P. paraeruginosa* ATCC 9027 were formerly designated *K. pneumoniae* K6 and *P. aeruginosa* ATCC 9027, respectively, and were subsequently reclassified based on genomic and phylogenomic evidence [52,53]. Three isolates of MRSA strains (Table 5) were provided by Dr. Eliana Carolina Vespero, Londrina University Hospital, Londrina, Paraná, Brazil. All bacterial samples were stored in Brain Heart Infusion (BHI, Acumedia, San Bernardino, CA, USA) broth containing 30% (*v*/*v*) glycerol (Merck, Rahway, NJ, USA) at −80 °C.

Table 5. Susceptibility of clinical isolates of *Staphylococcus aureus* to antibiotics.

Staphylococcus aureus	BZP	CIP	CLI	ERY	LEV	OXA
2115	R	R	R	R	R	R
2117	R	R	R	R	R	R
2119	R	R	R	R	R	R

BZP: Benzylpenicillin; CIP: ciprofloxacin; CLI: clindamycin; ERY: erythromycin; LEV: levofloxacin; OXA: oxacillin. R: Resistant. The numbers 2115, 2117 and 2119 are strain numbers at Laboratory of Clinical Analysis, Hospital Universitário de Londrina. The test was performed following the CLSI guidelines for antimicrobial disk susceptibility tests.

4.2. Antibacterial Agents

Ciprofloxacin was obtained from Farmabase (São Paulo, Brazil). The stock drug solution was prepared in sterile, deionized water and stirred to ensure complete mixing, reaching a final concentration of 2000 μg/mL.

C. cassia (L.) J. Presl (batch 227) and *O. vulgare* L. (batch 245) were both obtained from Ferquima Industry and Commerce of Essential Oils (São Paulo, Brazil). According to the company technical report, the essential oils were obtained by steam distillation of the plant material. CEO was obtained from the leaves, stems, and bark of plants originating from China, whereas OEO was obtained from the leaves of plants originating from Moldova. The CEO density is 1.065 g/mL and its main components are cinnamaldehyde (80%), cinnamic acid (2%), benzaldehyde (2%), coumarin (2%), and styrene (2%); the OEO density is 0.948 g/mL and the main components are carvacrol (72%), γ-terpinene (4.5%), linalool (4%), para-cymene (4%), and thymol (2%).

For each experiment, individual treatment solutions for CEO and OEO were freshly prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, São Paulo, Brazil; v/v) at a proportion of 1:1. The DMSO maximum concentration in assays was 1.25% (v/v), and it did not show antibacterial action.

4.3. Erythrocyte Hemolytic Assay

Hemolytic activity of CIP, CEO, and OEO was determined according to the method described by Izumi et al. [74], with necessary modifications. Blood, from a single healthy human donor, with voluntary consent obtained and approval from the Human Ethics Committee (CAAE 47661115.0.0000.5231, No. 1.268.019—UEL), was collected in heparinized tubes (Vacuplast, São Paulo, Brazil). Erythrocytes were separated by centrifugation (5500 rpm, 4 °C, 5 min) and were resuspended in phosphate-buffered saline (0.1 M PBS, pH 7.2) at 6% (v/v). PBS was composed of 0.85% (w/v) sodium chloride (Merck, Rahway, NJ, USA), 0.2 M monobasic sodium phosphate (Chemco, São Paulo, Brazil), and 0.2 M dibasic sodium phosphate (Anidrol, São Paulo, Brazil). In 96-well plates, 100 μL of RBCs at 3% was added to 100 μL of PBS with different concentrations of compounds (Supplementary Materials, Table S1), individually and in binary combinations (CIP, CEO, and OEO; CEO–CIP, and OEO–CIP). After 3 h of incubation at 37 °C, supernatants were read at 550 nm to monitor release of hemoglobin. Triton X-100 (Sigma-Aldrich, São Paulo, Brazil) at 1% (v/v) was used as a positive control, representing 100% hemolytic activity, while PBS without antimicrobials was used as the negative control for hemolytic activity. Hemolysis percentage was calculated for each compound concentration according to Equation (1).

$$Hemolysis\ \ (\%) = \left[\frac{(OD550\ treatment - OD550\ negative\ control)}{(OD550\ positive\ control - OD550\ negative\ control)}\right] \times 100, \quad (1)$$

Based on the erythrocyte hemolysis results, where CEO exhibited lower erythrocyte hemolysis than OEO ($p < 0.05$, Tukey's test) at the highest concentrations tested, this specific essential oil was selected for subsequent evaluation against the clinical MRSA isolates.

4.4. Antibacterial Activity of CEO, OEO and CIP Separately

For the evaluation of antibacterial activity from the actives, Minimum Inhibitory Concentration (MIC) was determined following the broth dilution method, according to Clinical and Laboratory Standards Institute guidelines [75] with necessary modifications. The concentration range was 0.031–512 µg/mL for CIP, 0.104–13.312 mg/mL for CEO and 0.370–11.850 mg/mL for OEO. The concentrations are rounded representations of an exact twofold dilution series. As sterility controls, Mueller–Hinton broth (MHB, Difco, Detroit, MI, USA) alone, CIP plus sterile deionized water on MHB, CEO plus DMSO on MHB, and OEO plus DMSO on MHB were used. Untreated bacteria inoculated on MHB alone was evaluated as a growth control.

For inoculum preparation, isolated colonies of bacterial cultures grown in the Muller–Hinton Agar media (MHA, Kasvi, Paraná, Brazil) were suspended in a saline solution (0.85% sodium chloride, *w*/*v*, Kasvi, Paraná, Brazil) and adjusted to achieve turbidity equivalent to 0.5 McFarland suspension, which corresponds to 1.5×10^8 colony-forming units (CFU)/mL. The inoculum was then diluted 1:100 in MHB to reach 10^6 CFU/mL. From these suspensions, 50 µL was inoculated into the wells of the microplates containing the isolated antimicrobials and the growth control.

After 24 h incubation at 37 °C, the MIC was determined by verifying the lowest concentration capable of inhibiting visible growth. Each assay was performed in triplicate on three separate occasions.

4.5. Antibacterial Binary Combination Assay

To assess the interaction between the CEO–CIP and OEO–CIP combinations, the broth dilution in double-antimicrobial gradient test was performed with necessary modifications [76]. Single colonies of bacterial cultures grown on MHA medium were suspended in saline solution (0.85% sodium chloride, *w*/*v*, Merck, Rahway, NJ, USA) to achieve turbidity equivalent to 0.5 McFarland suspension, corresponding to approximately 1×10^8 CFU/mL. Then, the inoculum was diluted 1:100 in MHB to reach 10^6 CFU/mL. Finally, 50 µL of the bacteria (1:100) was added to each well containing 50 µL of MHB supplemented with the binary combinations (CEO–CIP or OEO–CIP) at subinhibitory concentrations based on the individual MIC values (Supplementary Materials, Tables S2 and S3). The chosen concentrations performed against the MRSA isolates were based on CIP ranges that encompass the susceptibility and intermediate breakpoints defined by the CLSI [62]. The microplates were then incubated at 37 °C for 24 h.

The sterility and growth controls were performed as in the MIC determination assay described previously. The interpretation of the interaction of the binary combinations CEO–CIP and OEO–CIP was determined by calculating the fractional inhibitory concentrations (FICs) of compounds individually according to Equation (2), followed by the fractional inhibitory concentrations index (FICI) calculated using Equation (3) [77]. FICI is interpreted as "synergistic" when $\leq$0.5, as "additive" when >0.5 and $\leq$1, as "indifferent" (no interaction) when >1 and <4, and as "antagonistic" when $\geq$4. All assays were conducted in triplicate on three independent occasions. The CEO–CIP combination was evaluated against all Gram-positive and Gram-negative bacterial strains, including the clinical multidrug-resistant isolates. In contrast, the OEO–CIP combination was evaluated only against ATCC reference strains, due to the higher percentage of erythrocyte hemolysis exhibited by OEO individually during the erythrocyte hemolytic assay.

$$FIC = \frac{MIC_{combination}}{MIC_{individual}} \quad (2)$$

$$FICI = FIC_{EO} + FIC_{CIP} \quad (3)$$

where

FIC_{EO}: fractional inhibitory concentrations for essential oils (CEO or OEO);

FIC_{CIP}: fractional inhibitory concentrations for CIP.

4.6. Time-Kill Assay

The time-kill assay was performed using the viable cell count method with modifications [78]. Five treatment conditions were evaluated: bacterial cultures treated with CIP, CEO, and OEO alone and in binary combinations (CEO–CIP and OEO–CIP), in addition to a bacterial culture without antimicrobial agents, which served as a growth control. Eleven time points were evaluated (0 h, 10 s, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 7 h, 10 h and 24 h). At each time point, triplicate 10 µL aliquots from serial dilutions of treated and untreated cultures in sterile saline solution (0.85% NaCl, *w*/*v*) were transferred to MHA and incubated at 37 °C. Sterile pipette tips were utilized in each dilution step to prevent sample carryover between successive dilutions. The theoretical detection limit of the assay was 100 CFU/mL. After 24 h of incubation, the CFU/mL was determined. The treatments were considered bactericidal if they exhibited at least 3-$\log_{10}$ CFU/mL reduction in relation to the initial inoculum. All assays were conducted in triplicate on three separate occasions using two bacterial strains (*P. paraeruginosa* ATCC 9027 and *S. aureus* ATCC 6538).

4.7. Statistical Analysis

The results were analyzed using the GraphPad Prism version 9.1.1 (GraphPad Software, San Diego, CA, USA). For experiments in which treatment and concentration were evaluated, two-way ANOVA, followed by Tukey's post hoc test, was performed. Results with a *p*-value less than 0.05 were considered statistically significant.

5. Conclusions

This study demonstrates the synergistic, additive, and indifferent interaction of *C. cassia* (L.) J. Presl. essential oil combined with CIP against *A. baumannii*, *K. quasipneumoniae*, *P. paraeruginosa*, and *S. aureus*. Notably, the required CIP concentration for growth inhibition was reduced by 2048-fold at the tested concentrations against MRSA strains, for which new antibiotic alternatives are urgently needed. Moreover, the hemolytic activity of CEO at MICs and the CEO–CIP and OEO–CIP combinations indicated less than 5% of hemolysis on RBCs. In addition, this article also shows the antibacterial combination and kinetic of action of *O. vulgare* L. essential oil with ciprofloxacin. We highlight the great antibacterial activity of the *C. cassia* essential oil when in combination with ciprofloxacin, which significantly reduced the CIP MIC against MRSA, showed antibacterial synergism and additive interaction, had bactericidal activity against *P. paraeruginosa*, and had hemolytic activity at MIC values for the standard strains below the CC_{50}. In conclusion, these findings suggest that combining this essential oil with ciprofloxacin may represent a promising strategy to enhance antibacterial efficacy against multidrug-resistant pathogens. However, further studies are required to elucidate the antibacterial mechanisms and to evaluate their efficacy and safety in vivo before considering potential clinical applications.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/antibiotics15111053/s1, Table S1. Hemolytic activity of ciprofloxacin (CIP, µg/mL), cinnamon (CEO, mg/mL), and oregano (OEO, mg/mL) essential oil alone at different concentrations; Table S2. All tested subinhibitory concentrations based on the individual MIC values for ciprofloxacin (µg/mL) in combination with cinnamon (mg/mL) or oregano essential oil (mg/mL) against the standard strains; Table S3. All tested subinhibitory concentrations based on the individual MIC values for the cinnamon essential oil (mg/mL) in combination with ciprofloxacin (µg/mL) against methicillin-resistant *Staphylococcus aureus*.

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Informed Consent Statement: Informed consent was obtained from the subject involved in the study.

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