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Short communication

Syzygium aromaticum (clove) and *Thymus zygis* (thyme) essential oils increase susceptibility to colistin in the nosocomial pathogens *Acinetobacter baumannii* and *Klebsiella pneumoniae*

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ABSTRACT

The discovery of new antibiotics that are effective against *Acinetobacter baumannii* and *Enterobacterales*is a research priority. Several essential oils (EOs) have displayed some antimicrobial activity and could potentially act as antibiotic adjuvants. Research in this area aims to develop new therapeutic alternatives to treat infections caused by these pathogens.

MICs of different EOs were determined against *A. baumannii* and *Klebsiella pneumoniae*. Combined disk diffusion tests and checkerboard assays were used to study the synergy between the EOs and antibiotics. The fractional inhibitory concentration index (FIC_{index}) was calculated in order to categorize the interaction. Time-kill assays were also performed.

The EOs that displayed the highest levels of antimicrobial activity were clove (*Syzygium aromaticum* L.) and thyme (*Thymus zygis* L.). Combined disk diffusion tests and checkerboard assays revealed synergy between these EOs and colistin. Addition of either clove or thyme EO decreased the MIC of colistin by 8- to 64-fold and 8- to 128-fold in the colistin-resistant *A. baumannii* and *K. pneumoniae* strains, respectively ($FIC_{index} \leq 0.5$, synergy). MICs were also reduced in the colistin-susceptible strains. Time-kill assays also indicated the strong activity of the combined therapy. In summary, the use of clove or thyme EO in combination with colistin could improve the efficacy of the antibiotic and significantly reduce the concentrations needed to inhibit growth of *A. baumannii* and *K. pneumoniae*.

1. Introduction

The World Health Organization has recently published a list of priority pathogens for which research and development of new antibiotics is urgently needed. Carbapenem-resistant *Acinetobacter baumannii* and *Enterobacterales*(also 3rd generation cephalosporin-resistant), in particular *Klebsiella pneumoniae*, are classified as of critical priority [1]. The use of old off-patent antimicrobials as a last resort for treating these multidrug-resistant pathogens is becoming increasingly common. Colistin was initially rejected for use due to its high toxicity and challenging pharmacokinetic/pharmacodynamic properties; however, this antibiotic has been reintroduced in clinical practice [2].

The combined use of antibiotics and other non-antibiotic compounds, known as antibiotic adjuvants, is a common strategy used to enhance the activity of antibiotics and thus increase the susceptibility of resistant strains of bacteria. Essential oils (EOs) are aromatic volatile liquids extracted from plants and consist of up to 100 secondary metabolites, mainly terpenes and terpenoids, which are composed of 2–3 major components and some trace elements. Some EOs and their constituents have been shown to display antibacterial activity, by disrupting the cell membrane, interfering in ion transport or causing enzymatic alterations, among other effects. Some examples of combinations that have shown significant synergistic activity against multidrug resistant bacteria are rosewood EO and gentamicin, against

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A. baumannii [3], savory EO and chloramphenicol, against *K. pneumoniae* [4] and thyme EO and ciprofloxacin, against *Pseudomonas aeruginosa* [5]. EOs could therefore potentially be used as antibiotic adjuvants to help restore the effectiveness of antibiotics against multidrug resistant bacteria [6,7].

The aims of the present study were to identify EOs that are good candidates for use as antibiotic adjuvants and to determine whether combined antibiotic/adjuvant therapy improves the antibiotic activity against the multidrug resistant pathogens *A. baumannii* and *K. pneumoniae.*

2. Materials/methods

2.1. Bacterial strains, essential oils and antimicrobial agents

A previously characterized collection of colistin-susceptible and colistin-resistant isolates of *A. baumannii* and *K. pneumoniae* was evaluated in the present study. The strains of *A. baumannii* evaluated were reference strains ATCC 17978 and ATCC 19606, their isogenic colistin-resistant mutants ATCC 19606Δ*lpxC* and ATCC 19606*pmrB*, and the colistin-susceptible and colistin-resistant isogenic pairs ABRIM/ABRIM*pmrB* and AB248/AB249*pmrB*. The strains of *K. pneumoniae* included were reference strains ATCC 700603 and ATCC 700721, the colistin-susceptible and colistin-resistant isogenic pairs Kp HUAC1/Kp HUAC2, the colistin-susceptible clinical isolates MCE010, MCE066 and MCE455, and the colistin-resistant clinical isolates MCE009 and MCE372. The underlying mechanisms of resistance to colistin are detailed in Table 1. Strains were routinely grown at 37 °C in solid and liquid Luria-Bertani (LB) medium and stored at -80 °C in LB broth containing 10 % glycerol. Where necessary, the medium was supplemented with colistin.

The antibacterial properties of the following EOs were evaluated: tea tree, oregano, thyme, citronella, coconut, rosemary, clove, lavender and eucalyptus (for details of the characteristics see Table S1). Antibiotics representative of those usually used to treat *A. baumannii* and *K. pneumoniae* infections were selected for study: ceftazidime, sulbactam, amikacin, colistin (Sigma-Aldrich, USA), imipenem (Actavis, USA), meropenem and tigecycline (Pfizer, USA) (Table S2).

2.2. Synergistic interactions between essential oils and antibiotics

Minimum inhibitory concentrations (MICs) of the nine EOs were determined for the *A. baumannii* ATCC 17978 and *K. pneumoniae* ATCC 700603 reference strains, according to CLSI criteria [8]. Each EO was initially diluted 1:1 in DMSO. Serial dilutions of the different EOs were prepared in Mueller-Hinton II cation-adjusted broth (Sigma, Madrid, Spain) and added to the wells of 96-well microtitre plates. The EOs that displayed the highest levels of activity against the *A. baumannii* ATCC 17978 and *K. pneumoniae* ATCC 700603 strains were further tested against the strains included in Table 1.

Combined disk diffusion tests and checkerboard assays were used to study the synergy between the EOs displaying the highest level of antimicrobial activity and the aforementioned antibiotics.

In the combined disk diffusion tests, antibiotic disks were impregnated with 20 μL of each EO dissolved in 10 % DMSO plus 0.5 % v/v Tween 80 [9]. The disks were placed on Mueller-Hinton agar plates previously spread with strains *A. baumannii* ATCC 17978 and *K. pneumoniae* ATCC 700603 and incubated at 37 °C for 16 − 18 h. A difference of ≥ 4 mm in the inhibition zone of the antibiotic in the presence/absence of the EO was considered to indicate probable synergy. Independent assays were performed in triplicate.

Combinations showing synergy in disk diffusion assays were further analyzed in checkerboard assays with all the strains listed in Table 1. Serial dilutions of the EOs and antibiotics were prepared in Mueller-Hinton II cation-adjusted broth and added to 96-well plates. The microplates were incubated at 37 °C for 18 − 24 h.

The synergy was evaluated using the fractional inhibitory concentration index (FIC_{index}), calculated as follows: $FIC_{index} = FIC_{EO} + FIC_{antibiotic}$ = MIC [$EO_{antibiotic}$]/[EO] + MIC [$antibiotic_{EO}$]/[antibiotic]. The FIC data were interpreted according to the following criteria: $FIC_{index} \leq 0.5$, synergy; $FIC_{index} > 0.5$-4, no interaction [10].

2.3. Time-kill curves

Time-kill curve analysis was performed with ATCC 17978, ATCC 19606*pmrB* and AB249*pmrB A. baumannii* strains and ATCC 700603, Kp HUAC2 and MCE009 *K. pneumoniae* strains to evaluate the bactericidal activity of the most active antibiotic/EO combinations. Isolates were grown in 96-well microplates at 37 °C, with shaking, in an Epoch-2 Microplate Spectrophotometer (BioTek Instruments, Winooski, VT, USA). Aliquots of 1×10^4 CFU/mL were inoculated into Mueller-Hinton II cation-adjusted broth and tested in the presence of i) sub-MIC and MIC of EO alone, ii) sub-MIC and MIC of colistin alone, iii) sub-MIC of colistin in combination with sub-MIC of EO, and iv) no antibiotic or EO (as a control). Bacterial counts (CFU/mL) were determined after 0, 2, 4, 7 and 24 h, by plating strains onto Mueller-Hinton agar plates and

Table 1
Laboratory strains and clinical isolates used in the study.

Bacterial strain	Description	Reference
A. baumannii		
ATCC 17978	*A. baumannii* reference strain, completely sequenced. Colistin susceptible.	ATCC[a]
ATCC 19606	*A. baumannii* type strain, completely sequenced. Colistin susceptible.	ATCC
ATCC 19606Δ*lpxC*	Isogenic derivative mutant of ATCC 19606; 84-bp deletion within the *lpxC* gene. Colistin resistant.	[20]
ATCC 19606*pmrB*	Isogenic derivative mutant of ATCC 19606; single amino acid substitution (Ala227Val) in PmrB. Colistin resistant.	[20]
ABRIM	*A. baumannii* clinical isolate. Colistin susceptible.	[20]
ABRIM*pmrB*	Isogenic derivative mutant of ABRIM; single amino acid substitution (Asn353Tyr) in PmrB. Colistin resistant.	[20]
AB248	*A. baumannii* clinical isolate. Colistin susceptible.	[21]
AB249*pmrB*	Isogenic clinical isolate derivative of AB248; single amino acid substitution (Pro233Ser) in PmrB. Colistin resistant.	[21]
K. pneumoniae		
ATCC 700603	*K. pneumoniae* reference strain, completely sequenced. Colistin susceptible.	ATCC
ATCC 700721	*K. pneumoniae* reference strain, completely sequenced. Colistin susceptible.	ATCC
Kp HUAC1	*K. pneumoniae* clinical isolate. Colistin susceptible.	Present study
Kp HUAC2	Isogenic derivative mutant of Kp HUAC1. Clinical isolate, single amino acid substitution (Thr157Pro) in PmrB. Colistin resistant.	Present study
MCE009	*K. pneumoniae* clinical isolate, 1056-bp insertion within the *mgrB* gene. Colistin resistant.	Present study
MCE010	*K. pneumoniae* clinical isolate. Colistin susceptible.	Present study
MCE066	*K. pneumoniae* clinical isolate. Colistin susceptible.	Present study
MCE372	*K. pneumoniae* clinical isolate; single amino acid substitution (Thr157Pro) in PmrB. Colistin resistant.	Present study
MCE455	*K. pneumoniae* clinical isolate. Colistin susceptible.	Present study

[a] American Type Culture Collection.

Table 2
MICs of colistin, clove and thyme essential oils in each monotherapy. MICs of the colistin-clove/colistin-thyme combinations. Values of FIC_{index} for the combinations against different *A. baumannii* and *K. pneumoniae* isolates.

Strain	MIC of clove EO (mg/L)					MIC of thyme EO (mg/L)				
	Colistin	Clove	$Colistin_{Clove}$ [a]	$Clove_{Colistin}$	FIC index	Thyme	$Colistin_{Thyme}$	$Thyme_{Colistin}$	FIC index	
A. baumannii										
ATCC 17978	1	1024	0.06	256	0.31 Synergy	1024	0.03	128	0.155 Synergy	
ATCC 19606	1	1024	0.12	256	0.37 Synergy	1024	≤0.03	128	0.155 Synergy	
ATCC 19606Δ*lpxC*	64	512	2	128	0.281 Synergy	1024	4	64	0.125 Synergy	
ATCC 19606*pmrB*	64	512	4	64	0.188 Synergy	1024	4	64	0.125 Synergy	
AB248	1	2048	0.06	256	0.185 Synergy	1024	0.06	256	0.31 Synergy	
AB249*pmrB*	512	2048	8	256	0.141 Synergy	1024	8	128	0.141 Synergy	
ABRIM	0.5	1024	0.06	256	0.37 Synergy	512	0.12	64	0.37 Synergy	
ABRIM*pmrB*	64	1024	8	256	0.375 Synergy	512	≤0.5	128	0.25 Synergy	
K. pneumoniae										
ATCC 700603	1	2048	0.12	256	0.245 Synergy	1024	0.25	256	0.5 Synergy	
ATCC 700721	2	1024	0.25	256	0.375 Synergy	512	≤0.03	256	0.52 No interaction	
Kp HUAC1	1	1024	0.25	64	0.312 Synergy	1024	0.5	256	0.75 No interaction	
Kp HUAC2	16	1024	1	128	0.187 Synergy	1024	0.5	128	0.155 Synergy	
MCE009	64	1024	2	128	0.156 Synergy	256	8	64	0.375 Synergy	
MCE010	2	1024	0.25	256	0.375 Synergy	512	0.5	64	0.375 Synergy	
MCE066	2	1024	0.5	128	0.375 Synergy	512	0.5	128	0.5 Synergy	
MCE372	64	2048	8	256	0.25 Synergy	512	8	128	0.375 Synergy	
MCE455	1	1024	0.25	256	0.5 Synergy	256	0.5	128	1 No interaction	

[a] $Colistin_{Clove}$ and $Colistin_{Thyme}$, colistin MICs in the presence of clove and thyme EOs; $Clove_{Colistin}$ and $Thyme_{Colistin}$, clove and thyme EOs MICs in the presence of colistin.

incubating at 37 °C for 24 h. All experiments were performed in triplicate. A reduction of ≥2 log CFU/mL caused by the combination antibiotic/EO relative to antibiotic alone was considered to indicate a synergistic interaction [11].

3. Results and discussion

The antimicrobial activity of nine EOs against *A. baumannii* ATCC 17978 and *K. pneumoniae* ATCC 700603 was tested by broth microdilution. Clove and thyme EOs displayed the highest levels of activity. The antimicrobial activity of clove and thyme EOs was then tested against all the *A. baumannii* and *K. pneumoniae* strains included in the study (Table 1). Clove EO yielded MICs between 512 and 2048 mg/L, whereas thyme EO yielded MICs between 256 and 1024 mg/L (Table 2).

Combined disk diffusion tests were performed with both clove and thyme EOs in combination with the antibiotics (ceftazidime, sulbactam, amikacin, colistin, imipenem, meropenem and tigecycline) and reference bacterial strains *A. baumannii* ATCC 17978 and *K. pneumoniae* ATCC 700603. Screening by disk diffusion tests revealed synergy between both of the EOs and colistin against both bacterial strains (Fig. S1). Interestingly, no synergy was detected between these EOs and the other antibiotics.

Subsequent checkerboard assays performed with all the *A. baumannii* and *K. pneumoniae* strains revealed that the addition of clove and thyme EOs to colistin significantly reduced the MIC of the antibiotic. In addition, synergistic colistin-EO interactions were observed for all the colistin-resistant strains ($FIC_{index} \leq 0.5$, Table 2). Clove EO decreased the colistin MIC by 8- to 64-fold in the colistin-resistant *A. baumannii* strains and by 8- to 32-fold in the colistin-resistant *K. pneumoniae* strains. Similarly, thyme EO decreased the colistin MIC by 16- to 128-fold in the colistin-resistant *A. baumannii* strains and by 8- to 32-fold in the colistin-resistant *K. pneumoniae* strains. Interestingly, a reduction in the colistin MICs was also observed in the colistin-susceptible strains, and a synergistic interaction was detected with all of the colistin-susceptible strains tested, except for *K. pneumoniae* strains ATCC 700721, Kp HUAC1 and MCE455 with the colistin/thyme EO combination. The MICs and checkerboard results for all the studied strains are shown in Table 2 and supplementary Figs. S2 and S3.

In the time-kill curves, six isolates were selected on the basis of the demonstrated synergy between colistin and both EOs in checkerboard assays. The isolates comprised three *A. baumannii* (one colistin-susceptible and two colistin-resistant phenotype) and three *K. pneumoniae* (one colistin-susceptible and two colistin-resistant phenotype). The synergistic effects of colistin plus clove and thyme EOs were clearly observed in the time-kill curves (Fig. 1 and Table 3). In the *A. baumannii* strains tested (ATCC 17978, ATCC 19606*pmrB* and AB249*pmrB*), colistin, clove EO and thyme EO at 1 x MIC decreased the bacterial load at 24 h by 6.6–9.3, 9.0–9.6 and 8.9–9.3 $\log_{10}$ CFUs/mL respectively, relative to the bacterial load in the control cultures. Similarly, the sub-MIC combinations of colistin/clove EO and colistin/thyme EO decreased bacterial loads by 5.5–8.6 and 8.3–9.6 $\log_{10}$ CFUs/mL respectively, relative to the control cultures. A synergistic effect was also observed in all *K. pneumoniae* strains tested (ATCC 700603, Kp HUAC2 and MCE009). Colistin, clove EO and thyme EO at 1 x MIC decreased bacterial loads by 6.4–8.7, 8.7–9.4 and 5.1–9.1 $\log_{10}$ CFUs/mL, respectively, relative to control cultures. Sub-MIC concentrations of the colistin/clove EO and colistin/thyme EO combinations decreased bacterial counts by 4.1–7.0 and 6.2–6.6 $\log_{10}$ CFUs/mL respectively.

Clove EO is traditionally used to treat burns and wounds and is also used as a treatment/analgesic in dental care. Several studies have shown that clove EO has antimicrobial, antifungal, antiviral, anticancer and anti-inflammatory properties [12]. Thyme EO has historically been used as a culinary ingredient and as a food preservative, as well as in medicine. Similarly to clove EO, many studies associate thyme EO with antifungal and antibacterial properties [13].

To date, reliable information on the use of EOs or antibiotic/EO combinations to treat *A. baumannii* and *K. pneumoniae* infections is scarce. In relation to *A. baumannii*, synergy between different EOs (*e.g.* coriander and longbeak eucalyptus EOs) and some antibiotics (*e.g.* aminoglycosides, tetracyclines, fluoroquinolones and chloramphenicol) has been observed. In *K. pneumoniae,* synergy has also been observed between thyme EO and gentamicin, ciprofloxacin, pristinamycin or cefixime [5] and between lemon thyme EO and chloramphenicol, among other combinations [14,15]. However, no synergy between colistin and any EOs has previously been described in relation to these multidrug resistant pathogens (for further details, see review by P. Aelenei et al.) [15]. The interaction between EOs and antibiotics has been further studied in other multidrug-resistant pathogens, such as *P.aeruginosa*, in which synergy has been observed between different EOs

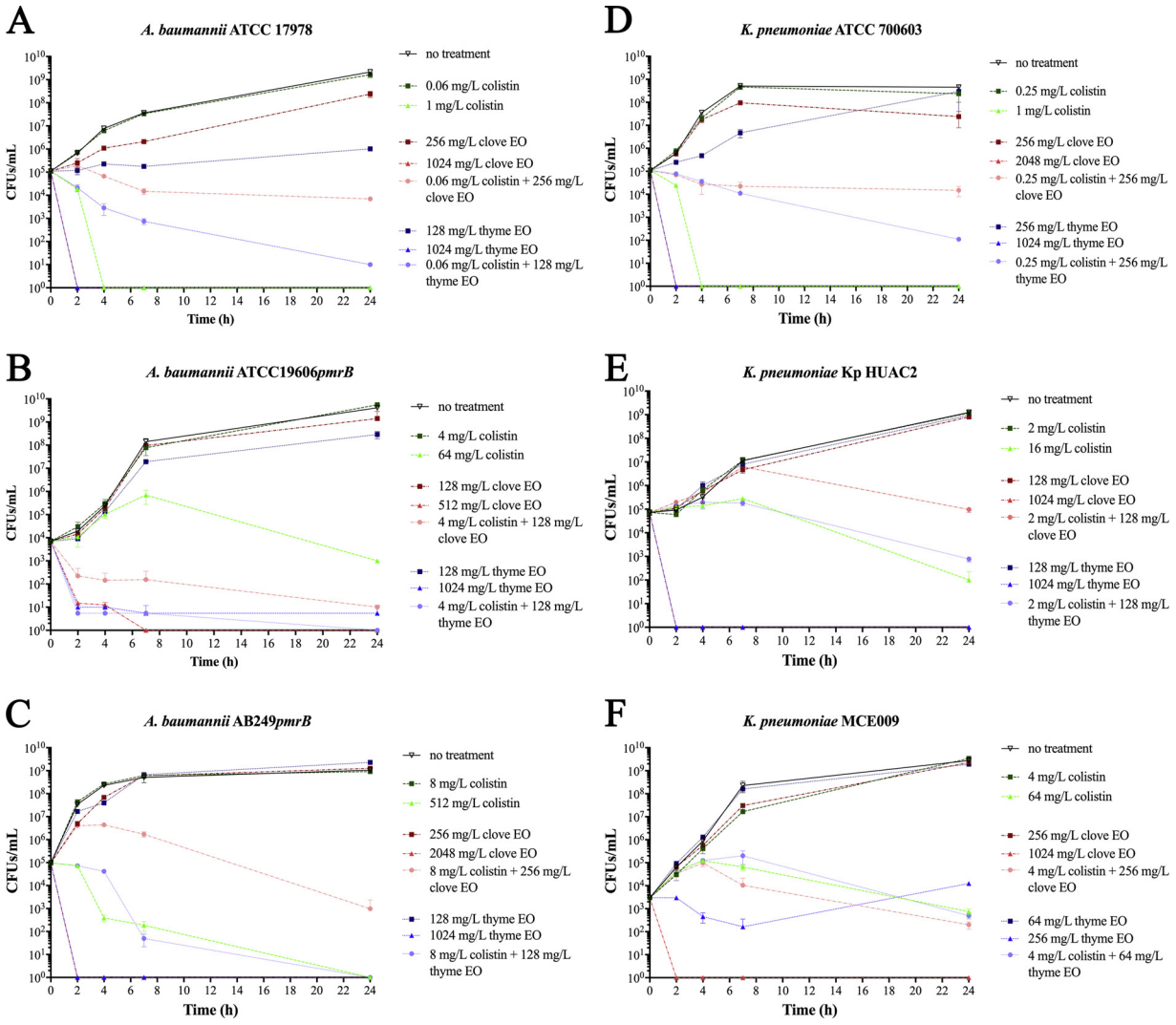

Fig. 1. Time kill curves used to assess synergistic interactions. Total bacterial counts of A) *A. baumannii* ATCC 17978, B) *A. baumannii* ATCC 19606*pmrB*, C) *A. baumannii* AB249*pmrB*, D) *K. pneumoniae* ATCC 700603, E) *K. pneumoniae* Kp HUAC2 and F) *K. pneumoniae* MCE009. The following conditions are represented: the absence of antimicrobials (no treatment), presence of colistin without and with the addition of EOs, and presence of EOs without the addition of colistin.

and antibiotics such as aminoglycosides, tetracyclines, fluoroquinolones and chloramphenicol. Similarly, in *Escherichia coli* synergy between EOs and aminoglycosides, tetracyclines and chloramphenicol, among other antibiotics, has been observed [15]. In Gram-positive bacteria, such as *Staphylococcus aureus*, synergistic interactions between EOs and fluoroquinolones, aminoglycosides, tetracyclines, cefixime and pristinamycin have also been observed [7].

The synergistic effects observed in this study may be largely due to the targets shared by both colistin and clove/thyme EOs. The major components of clove EO are eugenol (a phenylpropene) and caryophyllene (a terpene), and those of thyme EO are p-cymene (terpene) and thymol and carvacrol (terpenoids). Terpenes display low levels of antimicrobial activity. However, terpenoids, which are biochemically modified terpenes with an extra oxygen, display strong antimicrobial activity; the presence of hydroxyl groups enhances the antimicrobial activity [16,17]. Eugenol, thymol and carvacrol are probably the main compounds responsible for the observed synergy with colistin. The activity of these phenols is explained by interactions with the bacterial outer membrane and is correlated with the hydrophobicity of the compounds [17]. Colistin is a polycationic lipopeptide whose initial target is the polyanionic LPS of the Gram-negative cell membrane of bacteria. The amphipathic nature of colistin, with one hydrophilic and one hydrophobic end, is essential for interactions with lipid A, the main component of LPS [18]. Thus, the major components of clove and thyme EOs and colistin probably act together at the cell membrane, interfering in the control of cell permeability and precipitating bacterial death.

The toxicity of colistin is dose-dependent and thus the lower the dose administered, the fewer the adverse reactions. Strategies that allow the doses of the antibiotic to be reduced while maintaining the therapeutic efficacy are therefore required. Colistin is one of the very few antimicrobials available for treating infections caused by carbapenem-resistant strains of *A. baumannii* and *K. pneumoniae*. Unfortunately, an increase in the resistance rates of this antibiotic has been observed in last two decades [19].

The present is the first study of the use of clove and thyme EOs as colistin adjuvants and is also the first to determine the synergistic effects of these compounds on *A. baumannii* and *K. pneumoniae*. The study findings may represent an interesting starting point for designing new combination therapies, mainly against colistin-resistant strains.

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Table 3
MICs of colistin and essential oils, alone or in combination, and bacterial load reduction (mean $\log_{10}$ CFU/mL ± SD) observed in time kill curves, relative to the bacterial load for no treatment.

Strain	Colistin		Clove		Colistin-Clove		Thyme		Colistin-Thyme		
	Concentration	Reduction in bacterial load	Concentration	Reduction in bacterial load	Colistin concentration	Clove concentration	Reduction in bacterial load	Concentration	Reduction in bacterial load	Colistin concentration	Thyme concentration	Reduction in bacterial load
A. baumannii												
ATCC 17978	1 x MIC (1 mg/L)	9.33 ± 0.11	1 x MIC (1024 mg/L)	9.33 ± 0.11	1/16 x MIC (0.06 mg/L)	1/4 x MIC (256 mg/L)	5.49 ± 0.20	1 x MIC (1024 mg/L)	9.33 ± 0.11	1/16 x MIC (0.06 mg/L)	1/8 x MIC (128 mg/L)	8.33 ± 0.11
ATCC 19606*pmrB*	1 x MIC (64 mg/L)	6.62 ± 0.05	1 x MIC (512 mg/L)	9.62 ± 0.05	1/16 x MIC (4 mg/L)	1/4 x MIC (128 mg/L)	8.62 ± 0.05	1 x MIC (1024 mg/L)	8.88 ± 0.75	1/16 x MIC (4 mg/L)	1/8 x MIC (128 mg/L)	9.62 ± 0.05
AB249*pmrB*	1 x MIC (512 mg/L)	9.03 ± 0.05	1 x MIC (2048 mg/L)	9.03 ± 0.05	1/64 x MIC (8 mg/L)	1/8 x MIC (256 mg/L)	6.04 ± 0.24	1 x MIC (1024 mg/L)	9.03 ± 0.05	1/64 x MIC (8 mg/L)	1/8 x MIC (128 mg/L)	9.03 ± 0.05
K. pneumoniae												
ATCC 700603	1 x MIC (1 mg/L)	8.66 ± 0.02	1 x MIC (2048 mg/L)	8.66 ± 0.02	1/4 x MIC (0.25 mg/L)	1/8 x MIC (256 mg/L)	4.48 ± 0.23	1 x MIC (1024 mg/L)	8.66 ± 0.02	1/4 x MIC (0.25 mg/L)	1/4 x MIC (256 mg/L)	6.62 ± 0.08
Kp HUAC2	1 x MIC (16 mg/L)	7.10 ± 0.96	1 x MIC (1024 mg/L)	9.10 ± 0.06	1/8 x MIC (2 mg/L)	1/8 x MIC (128 mg/L)	4.11 ± 0.17	1 x MIC (1024 mg/L)	9.10 ± 0.06	1/8 x MIC (2 mg/L)	1/8 x MIC (128 mg/L)	6.21 ± 0.17
MCE009	1 x MIC (64 mg/L)	6.44 ± 0.28	1 x MIC (1024 mg/L)	9.44 ± 0.15	1/16 x MIC (4 mg/L)	1/4 x MIC (256 mg/L)	6.97 ± 0.31	1 x MIC (256 mg/L)	5.14 ± 0.18	1/16 x MIC (4 mg/L)	1/4 x MIC (64 mg/L)	6.54 ± 0.28

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Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.110606.

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