



Colistin and Kanamycin Together in Association with *Coridothymus capitatus* to Enhance their Antimicrobial Activity and Fight Multidrug-Resistance Pathogens

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Abstract: It should be remembered that bacteria continue to spread and develop new types of resistance, so further actions are needed to deal with antibiotic resistance. As a result, antibacterial drugs have become less effective, resulting in the accelerated discovery of available alternative treatments, including essential oils. The aim of this work was to intensify and promote the action of two antibiotics, kanamycin, and colistin, to fight antibiotic resistance thanks to the action of essential oil obtained from the flowers of *Coridothymus capitatus* grown on the Iblei mountains. To this end, a comparison of biological and chemical assays was carried out. The results showed a broad antimicrobial power of the essential oil itself and a great synergistic activity in combination with Kanamycin and Colistin against multidrug-resistant bacteria. These combinations increased the range of antibiotics, leading us to speculate that it could be incorporated into new pharmaceutical formulations for therapies of infections caused by increasingly dangerous bacteria. Antibiotic resistance represents an ever-greater danger to human health. This work re-evaluates the use of colistin and kanamycin thanks to the synergistic action found with the addition of a natural substance to pave the way for new therapeutic strategies.

Keywords: Thymus essential oil; multidrug resistance bacteria; 3D checkerboard assay; Kanamycin A; Colistin.

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1. Introduction

The International Organization for Standardization defines Essential Oils (EOs) as products obtained from raw vegetable material by distillation, mechanical process or dry distillation [1]. Biological properties of EOs have been known since a long time. Indeed, numerous studies in the literature confirm their action (*in vitro* and *in vivo*) [2-6]. These activities can be attributed to a large number of molecules such as terpenoids and phenolic compounds, which show antimicrobial activity even when they are tested in the pure form [7-10] as regard *Coridothymus capitatus* is a plant belonging to the family *Lamiaceae*. Common throughout the Mediterranean area, it prefers sandy, sunny, and exposed to salt: Turkey, Tunisia, Greece, Italy, and Spain [11-14]. Nowadays, thymus essential oils are used in clinical as a supplement to conventional medical therapy and in veterinary medicine for dogs, cats, cattle, and sheep (diluted in sweet almond oil) [15]. In addition, TEO has been shown to have antiparasitic [16, 17], insecticidal [18], and antifungal [19] as well as antibacterial activity.

Moreover, it should be remembered that bacteria continue to spread and develop new types of resistance, so more action is needed to address antibiotic resistance [20]. Even if antibiotics save lives, they can contribute to the development of antibiotic resistance because of their inappropriate use [21, 22]. As a result, antibacterial drugs have become less effective, resulting in an accelerating discovery of available alternative treatments [23], among which bacteriophage therapy [24, 25], combined therapies [26-28], probiotics [29] and essential oils [30-32]. In this regard, most of the essential oils of thyme (*Thymus vulgaris*) have thymol as the main component [4, 33-36]. Instead, the peculiarity of *Coridothymus capitatus* is in the very high percentage of CAR [13]. The published studies of *C. capitatus* essential oil indicated anti-inflammatory and antitumor activities as well as antimicrobial activities [37-39].

The aim of this work was to investigate the effectiveness *in vitro* of the variety of *Coridothymus capitatus* EO. For this purpose, the interaction of TEO with two drugs was simultaneously assessed by two and three-dimensional checkerboard assays. Growth inhibition curves for each strain, both with the TEO alone and with the combinations, were generated to evaluate the synergistic effect with KAN and/or COL in order to improve the penetration of the antibiotics, enhancing their range of action against multidrug resistant pathogens. Moreover, post-antibiotic effect of the most powerful combination was also investigated.

2. Materials and Methods

2.1. GC-MS analysis.

Flowers of *Coridothymus capitatus* (L.) Richb.f were collected during the flowering period in an area named “*Lauretum-Rosmarinetum*” located in the southwest of the Aleppo pine reserve on Iblei Mountains (Ragusa, Sicily). The TEO was produced and processed (Catania, Sicily) by continuous steam distillation in a 2 meters column without recycling condensation water. The essential oil obtained was dehydrated over Na₂SO₄ and filtrated. TEO density values were estimated in a range between 0.930 – 0.955 g/mL. CAR solution (purity ≥98%) purchased from Sigma was used as positive internal control, and it had a density of 0.976 g/mL at 20°C. An aliquot was used for gas chromatographer and mass spectrometer measurements, as previously described [40].

In particular, GC-MS analysis was carried out using an Bruker Scion SQ (SCION Instruments, Livingston UK), fitted ZB-5HT Inferno capillary column (30m x 0.25mm, i.d. 0.25 µm; Zebron™ Inferno™, Phenomenex, USA). The injection volume was 1 µL, and the temperature of the injector was 250°C. The oven temperature was programmed as follows: it was held at 60°C for 3 min, increased to 150°C at 3°C/min and then, held at 380°C for 3 min. Mass spectra were obtained using the electron impact (EI+) mode at 70eV with an ion source temperature of 230 °C. Mass spectra were recorded in the 50-1200 m/z range. Finally, the structures of molecules were identified by computerized matched in NIST10 spectral library.

2.2. MIC and MBC assays.

MDR bacteria were used to evaluate antibacterial activity of essential oil and combinations: both Gram-positive bacteria (*Enterococcus faecalis*, *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*) (collection of the Laboratory of Applied Microbiology, Department of Biomedical and Biotechnological Sciences, Università degli Studi di Catania). These MDRs, previously isolated from abscess exudates surgically

eliminated in hospitals, were chosen precisely because they can best represent an example of bacteria that are difficult to eradicate. The strains were designated as MDR by International standard definitions for acquired resistance, as described by Magiorakos *et al.* [41]. MDR was defined as acquired non-susceptibility to at least one drug in three or more antimicrobial categories. The antibiotics used to assess their level of resistance/sensitivity were summarized in Table S1.

According to the guidelines of CLSI M100 [42], MIC values were determined by the microdilution method. The assay was performed in 96-wells polystyrene plates (Corning® 96 Well Microplates), with CAMHB medium (Cationic Adjusted Muller Hinton Broth; Oxoid). Briefly, a bacterial suspension of 0.5 McFarland was made for each strain under examination and the dilutions in broth were prepared so as to obtain the final concentration of 10^4 - 10^5 CFU/mL.

Stock solution for TEO was prepared with EtOH to a final range concentrations of 1.0% v/v - 0.001% v/v. EtOH maximum concentration was 1.0% (v/v), and MH broth with 1.0% EtOH was included as growth control. CAR was used as an internal comparative control. Microplates were incubated at 37°C overnight, and MIC value ($\mu\text{g/mL}$) was defined as the lowest concentration, which inhibited the visible growth of the bacterial strains. All determinations were performed six times.

Minimum Bactericidal Concentration (MBC) was performed after that MIC assay has been completed. Briefly, the dilution representing the MIC and two of the more concentrated dilutions were plated on MHA and enumerated to determine viable colonies after incubation at 37°C overnight [43]. The MBC is the lowest concentration that demonstrates a pre-determined reduction (99.9%) in CFU/mL when compared to the MIC [43].

2.3. Growth inhibition curves analysis.

Growth inhibition curves analysis was tested for the pathogens, as previously described [44]. Briefly, a bacterial suspension of 0.5 McFarland (1.5×10^8 CFU/mL) was prepared after an overnight subculture in Mueller-Hinton broth, and a series of dilutions were prepared to obtain 1.5×10^5 CFU/mL. The range concentration tested for TEO was 4715-189 $\mu\text{g/mL}$. Inoculated 96-well polystyrene plates were incubated aerobically with shaking at 37°C for 24 hours, and OD600 measurements (Model 680 Microplate Reader, Bio-Rad) were made any 30 minutes. All measurements were repeated six different times.

2.4. Evaluation of double/triple combinations *in vitro*.

Each strain was tested with double and triple combination of TEO, KAN, and COL. This evaluation was performed as described by El-Azizi [45]. MIC values of the combination were calculated with respect to the most potent antibiotic, single or in double combination. As previously described [45], to assess the antibiotic combinations therapy *in vitro*, an interaction code (IC) was created for each combination: for any 2-fold increase or decrease in the MICs values, a numerical value was assigned. Based on this value, the result (interaction type, IT) is defined as Antagonism if IC is ≤ -2 or less, Indifferent if $-1 < \text{IC} < +1$, Synergism $\text{IC} \geq +2$. Triple combination follows same rules but with respect to the most potent double combination.

2.5. Two and three-dimensional checkerboard assay.

Checkerboard assays were performed in order to evaluate synergistic effects between TEO + KAN and TEO + COL [46, 47]. The procedure was performed as followed: TEO was diluted in order to obtain different concentrations, and separately, drugs were diluted in the same way. Briefly, on a microplate, TEO has been diluted by factor 2 starting from column 10 up to 2, and the same in another microplate for the antibiotic KAN or COL, column 1 contained only MH broth. 50 µl of the TEO dilutions were transferred from column 10 to column 10 of a new microplate, and each dilution until column 2. From column 10 of antibiotic 50 µl were transferred to row A of the microplate, which already contained TEO, and each dilution until row G; nothing was added in row H. A schematic representation is described in Fig. 1A. Range values used for KAN were chosen as defined by CLSI [42]. The interpretation of the results was carried out by calculating the index of fractional inhibitory concentration (FICI) [48].

$$FICI = a/a' + b/b' = FICa + FICb$$

where a = MIC of TEO in the presence of KAN or COL; a' = MIC of TEO alone; b = MIC of KAN or COL in the presence of TEO; b' = MIC of KAN or COL alone.

For FIC values lower than 0.5 the combination of antibiotics is defined as synergistic, between 0.5 and 4 is defined as indifferent, and for values > 4, antagonist.

For the “three-dimensional checkerboard assays,” was performed the procedure described by Stein et al. [49]. A schematic representation is described in Fig. 1B. In this case, the FICI for triple antibiotic combination was calculated as follows:

$$FICI = a/a' + b/b' + c/c' = FICa + FICb + FICc$$

where a = MIC of TEO in the presence of KAN and COL; 'a' = MIC of TEO alone; b = MIC of KAN in the presence of TEO and COL; 'b' = MIC of KAN alone; c = MIC of COL in the presence of TEO and KAN; 'c' = MIC of COL alone.

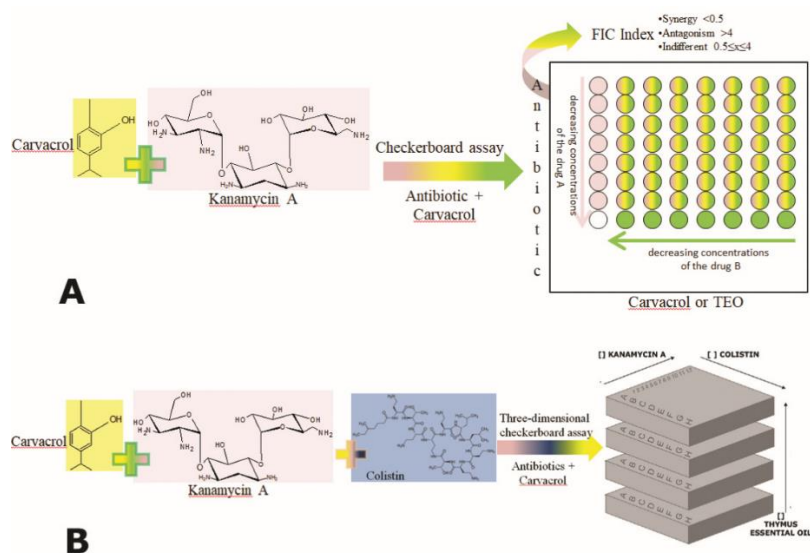


Figure 1. Schematic representation of checkerboard assay to evaluate synergistic effects among two (A) or three (B) different molecules [49]. Adapted from an open access article.

2.6. Determination of the post antibiotic effect (PAE).

The determination of PAE was performed as previously described [50, 51]. Briefly, exponentially growing bacteria in MH broth were adjusted to a concentration of 1.5×10^5 CFU/mL. Drugs and the combination of drugs were added to culture broth at concentrations <https://biointerfaceresearch.com/>

equal to MIC for the tested strains. After incubation of the samples at 37°C for 2.0 h in a shaking water-bath, drugs were removed by centrifugation (8.500 g, 20 min, 4°C) and free-drugs MH broth was added. Exposed and control cultures were incubated at 37°C, and the number of CFU/mL was determined at time 0, post-wash, any 2 hours throughout 24 hours. The PAE was determined according to equation $PAE = T - C$ as the difference in time required by test (T) and control (C) cultures for a 1 log increase in colony count [52]. All experiments were performed six times.

2.7. Statistical analysis.

Data were summarized using the mean (standard deviation; SD). All results and graphs were generated using GraphPad® Prism ver. 6 software.

3. Results and Discussion

3.1. GC-MS analysis.

GC-MS analysis of TEO (0.943 g/cm³) has allowed the identification of 16 compounds representing 97.33% of the total oil composition. Among these, eight had a percentage greater than 1%. The aromatic fraction (Carvacrol, p-Cymene, γ -Terpinene) constituted 86.85% of the oil composition, while the terpenoid fraction appeared to be 10.48 % (Table 1). Chemical analysis performed on TEO obtained from the flowers of *Coridothymus capitatus* (L.) Richb.f grown in *Lauretum-Rosmarinetum* located in the southwest of the Aleppo pine reserve on Iblei mountains (Ragusa, Sicily) showed a very peculiar chemotype. In fact, as reported in Table 1, TEO had a great CAR content (73.04 %) and no thymol in its phenolic fraction. These results have led us to investigate the antibacterial activity of this EO using CAR solution ($\geq 98\%$) as an internal comparative control. The complete characterization of *Thymus capitatus* var. *coridothymus* essential oil was reported in Table S2. Moreover, Figure S1 showed pertinent chromatogram.

Table 1. Chemical composition of TEO ^a.

Method of extraction	Major components ^b (%)	
Continuous steam distillation	p-Cymene	9.48
	γ -Terpinene	4.33
	Carvacrol	73.04
	Total aromatic fraction	86.85
	β -Thujene	1.16
	α -Pinene	1.20
	β -Myrcene	1.37
	Terpinolene	1.70
	β -Caryophyllene	5.05
	Terpenoid fraction	10.48
	Total identified	97.33

^a Data shown were provided by the manufacture

^b percentage greater than 1 % of the total of identified

3.2. MIC and MBC assays.

Antibacterial activity of TEO, CAR and KAN is shown in Table 2. As regards MIC values of TEO, they were equal to 754 μ g/mL for all strains tested, while MBC values were equal to MIC values except for *Pseudomonas aeruginosa* where MBC was one dilution lower (1509 μ g/mL). MIC values for CAR were equal to 782 μ g/mL, except for *Pseudomonas aeruginosa* where a higher MIC value (3126 μ g/mL) was reported. For CAR solution, MBC

values were one dilution lower than MIC values. Therefore, *Pseudomonas aeruginosa* was the least susceptible of all bacterial strains tested. Despite the difference in CAR content (TEO 73.4%, CAR \geq 98%), the two substances tested had a very similar antibacterial activity when tested alone.

MIC values of KAN and COL complied with those provided by CLSI for Broth Microdilution Method [42]. Moreover, data from literature confirmed limited inhibitory activity of KAN: MIC values against *E. coli* and *E. faecalis* between 64-1 μ g/mL were reported by Fass [53], while *P. aeruginosa*, *K. pneumoniae* and *A. baumannii* were resistant [54, 55].

Table 2. Antimicrobial susceptibility test: MIC and MBC values of TEO, CAR, KAN and COL (expressed as μ g/mL).

Strains	TEO		CAR		KAN	COL
	MIC	MBC	MIC	MBC	MIC	MIC
<i>Enterococcus faecalis</i>	754	754	782	1563	64	256
<i>Staphylococcus aureus</i>	754	754	782	1563	8	256
<i>Escherichia coli</i>	754	754	782	1563	4	4
<i>Pseudomonas aeruginosa</i>	754	1509	3126	1563	512	32
<i>Klebsiella pneumoniae</i>	754	754	782	1563	512	4
<i>Acinetobacter baumannii</i>	754	754	782	1563	512	32

3.3. Growth inhibition curves analysis.

The growth rates of strains grown with TEO compared to positive control curves were shown in Figure 2: all strains were susceptible up to 754 μ g/mL, confirming the MIC values obtained by the broth dilution assay. Moreover, a strong reduction was observed at the concentration of $\frac{1}{2}$ MIC: *A. baumannii* and *K. pneumoniae* showed a reduction of 99.90% and 99.20%, respectively. The other strains exhibited a reduction of almost 50% compared to the positive control, except for *S. aureus*, which showed a lower reduction (-30.78%). The growth rates of strains with combinations of TEO and KAN showed strong synergistic activity against *A. baumannii*. Indeed, the growth curves of *A. baumannii* with 1024 μ g/mL of KAN was comparable to the positive control. The combination KAN 16 μ g/mL - TEO 1509 μ g/mL caused a partial inhibition of growth (-52.40%), while the immediately higher combination (KAN 32 μ g/mL - TEO 189 μ g/mL) resulted in complete bacterial inhibition (Fig. 3A).

The combinations CAR+KAN showed no significant synergistic activity. In the same way, the combinations of CAR+COL showed no synergistic activity. Instead, the combination TEO+COL showed great activity, overall on *P. aeruginosa* and *A. baumannii*, with a reduction of growth of 99.80% and 99.90% respectively at the following concentration COL 0.50 μ g/mL – TEO 189 μ g/mL (Fig. 3B).

Finally, the triple combination KAN+COL+TEO caused a total inhibition of growth for *E. coli*, *P. aeruginosa* and *A. baumannii* at the following concentration: 0.12 COL – 2.0 KAN – 47.12 TEO μ g/mL.

3.4. Two and three-dimensional checkerboard assay.

Combinations of KAN+TEO, COL+TEO, and control combinations of KAN+CAR and COL+CAR were tested against the same bacteria. FIC Index values were reported in Table 3. Although TEO and CAR solution showed similar antibacterial activity when tested alone, their behavior in combination with the drugs had shown significant differences. The results of the checkerboard assay KAN+TEO and COL+TEO demonstrated how these combinations had a great synergic activity against some pathogenic bacteria. In an outstanding way, FICI of

KAN+TEO against *A. baumannii* was equal to 0.28 and 0.26 for *S. aureus*, meanwhile, *E. faecalis* showed a FICI slightly higher (0.37). Moreover, FICI values of 0.56, 0.75, and 1.00 were estimated for *K. pneumoniae*, *E. coli*, and *P. aeruginosa*, respectively, showing indifference for this kind of molecules combination.

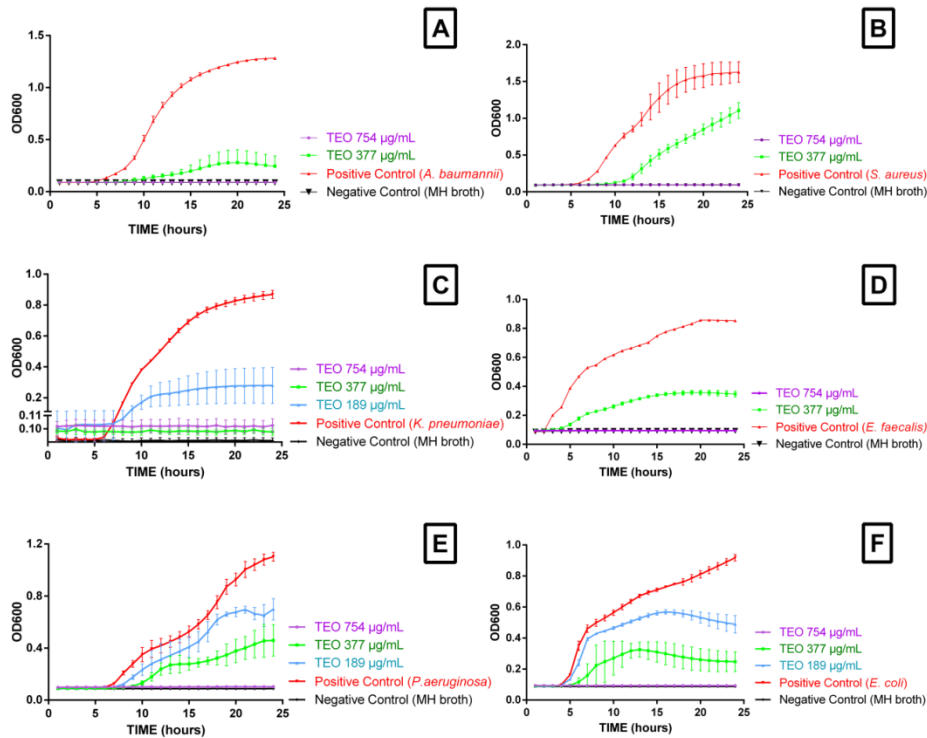


Figure 2. Growth curves inhibition of (A) *A. baumannii*, (B) *S. aureus*, (C) *K. pneumoniae*, (D) *E. faecalis*, (E) *P. aeruginosa* and (F) *E. coli* incubated at 37°C aerobically overnight with MIC and ½ MIC values of TEO. OD600 measurements were done any 30 minutes (Model 680 Microplate Reader, Bio-Rad).

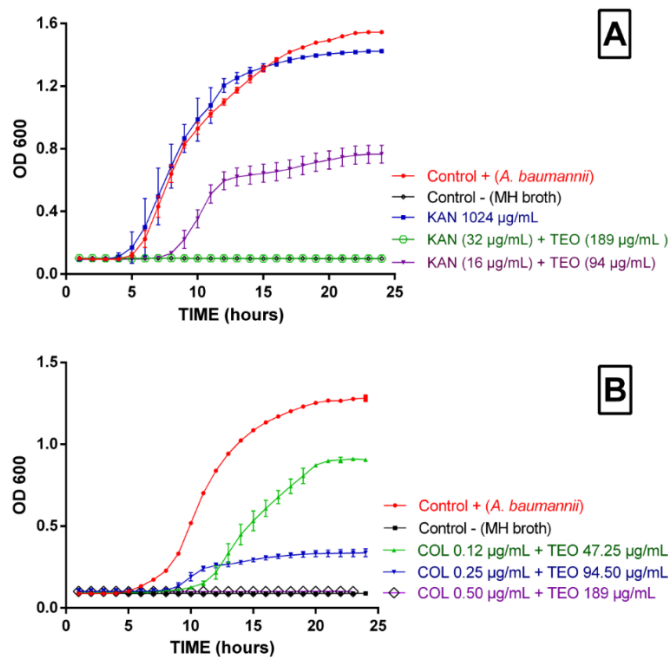


Figure 3. A) Growth curves of *Acinetobacter baumannii* incubated at 37°C with KAN/TEO combinations 32/189 µg/mL and 16/94 µg/mL respectively, for 24 hours. OD600 measurements were done any 30 minutes (Model 680 Microplate Reader, Bio-Rad, Milan, Italy). B) Growth curves of *Acinetobacter baumannii* incubated at 37°C with COL/TEO combinations 0.12/47.25 µg/mL, 0.25/94.50 µg/mL and 0.50/189 µg/mL for 24 hours. OD600 measurements were done any 30 minutes (Model 680 Microplate Reader, Bio-Rad).

Instead, FICI of COL+TEO was 0.26 for *P. aeruginosa* and *A. baumannii*. FICI value of 0.37 was showed for *K. pneumoniae*. For the remaining bacterial strains, the FICI values were greater than 0.5, showing indifference to the combination of the two molecules. Finally, FICI for the triple combination showed a great synergistic activity with a significant lowering of active concentrations for all strains (0.03-0.38) except for *E. faecalis*, where the FICI value showed indifference (0.65).

As regard the checkerboard assay of KAN+CAR, results showed FICI values in a range of 0.62-1.03 showing indifference behavior for all bacteria tested. Moreover, indifference behavior was showed for the combination COL+CAR (FICI values range of 0.75 – 2.00). These differences could be explained by the mechanism of action of phenols contained in TEO. In fact, the doses of TEO below the MIC value could lead to an alteration of bacterial membranes’ physical structure with its expansion and destabilization and an increased fluidity, which would increase passive permeability of the bacterial membrane thus enhancing the entrance of the drugs inside the bacterial cell [56].

Table 3. Checkerboard assays of KAN+TEO and COL+TEO combinations, and control combinations KAN+CAR and COL+CAR.

Bacterial strains	FIC Index				
	KAN + TEO	KAN + CAR	COL + TEO	COL + CAR	KAN + COL + TEO
<i>E. faecalis</i>	0.37	0.75	2.12	1.50	0.65
<i>S. aureus</i>	0.26	1.03	0.53	2.00	0.38
<i>E. coli</i>	0.75	0.75	0.75	1.00	0.06
<i>P. aeruginosa</i>	1.00	0.63	0.26	0.75	0.03
<i>K. pneumoniae</i>	0.56	0.63	0.37	1.00	0.38
<i>A. baumannii</i>	0.28	0.62	0.26	1.50	0.07

3.5. Evaluation of double/triple combinations in vitro.

KAN+TEO, COL+TEO, and KAN+COL+TEO synergisms were also assessed with another kind of assay such as “the double/triple combination *in vitro* therapy” described by El-Azizi [45]. The results of these combinations were shown in Table 4. When the interaction code (IC) was less than +2 the interaction was classified as indifferent (I). KAN+TEO: ICs for *K. pneumoniae* and *P. aeruginosa* were equal to +1. Meanwhile it was equal to 0 for *E. coli*.

Table 4. Double and triple combinations assay of TEO with KAN and COL.

Bacterial strains	Double combinations						Triple combination		
	KAN + TEO			COL + TEO			KAN + COL + TEO		
	MIC	IC	IT	MIC	IC	IT	MIC	IC	IT
<i>E. faecalis</i>	16	+2	S	32	+3	S	8	+1	I
<i>S. aureus</i>	0.25	+2	S	8	+5	S	4	-1	I
<i>E. coli</i>	4	0	I	1	+2	S	0.06	+4	S
<i>P. aeruginosa</i>	256	+1	I	0.5	+6	S	0.06	+3	S
<i>K. pneumoniae</i>	256	+1	I	0.5	+3	S	0.5	0	I
<i>A. baumannii</i>	32	+2	S	0.5	+6	S	0.12	+2	S

Moreover, as for the results obtained by checkerboard assay, *E. faecalis*, *S. aureus*, and *A. baumannii* showed ICs equal to +2, further the combination showed synergism (S) against the strains just mentioned. COL+TEO: this combination showed prodigious results for *P. aeruginosa* and *A. baumannii* with an IC value of +6, for *S. aureus* with an IC value of +5 and for *E. faecalis* and *K. pneumoniae* strains with an IC of +3. Finally, *E. coli* had an IC value of +2. Therefore, the double combination COL+TEO showed synergism for all strains tested.

The triple combination KAN+COL+TEO undergoes the benefits of the two double combinations leading to further synergistic action for some strains: *E. coli*, *P. aeruginosa*, and *A. baumannii*. In contrast, for the strains *E. faecalis*, *S. aureus*, and *K. pneumoniae* the combination showed indifference in comparison to the double combinations separately.

3.6. Determination of the post antibiotic effect (PAE).

The PAE results were summarized in Table 5, and Figure 4 depicts the PAE of TEO at MIC concentration for all strains studied. In particular, PAE observed for *A. baumannii* was equal to 5.5 h (Fig.4A), for *S. aureus* was equal to 8.5 h (Fig. 4B), instead, *K. pneumoniae* showed a PAE of 9.5 and, *P. aeruginosa* of 13.5 h (Fig. 4D and F, respectively). *E. faecalis* showed a PAE of 15.5 h (Fig. 4C), and finally, it was not possible to detect PAE for *E. coli* (≥ 24 h, Fig 4E). Moreover, all strains showed a PAE value of ≥ 24 h for the triple combination at MIC concentration value.

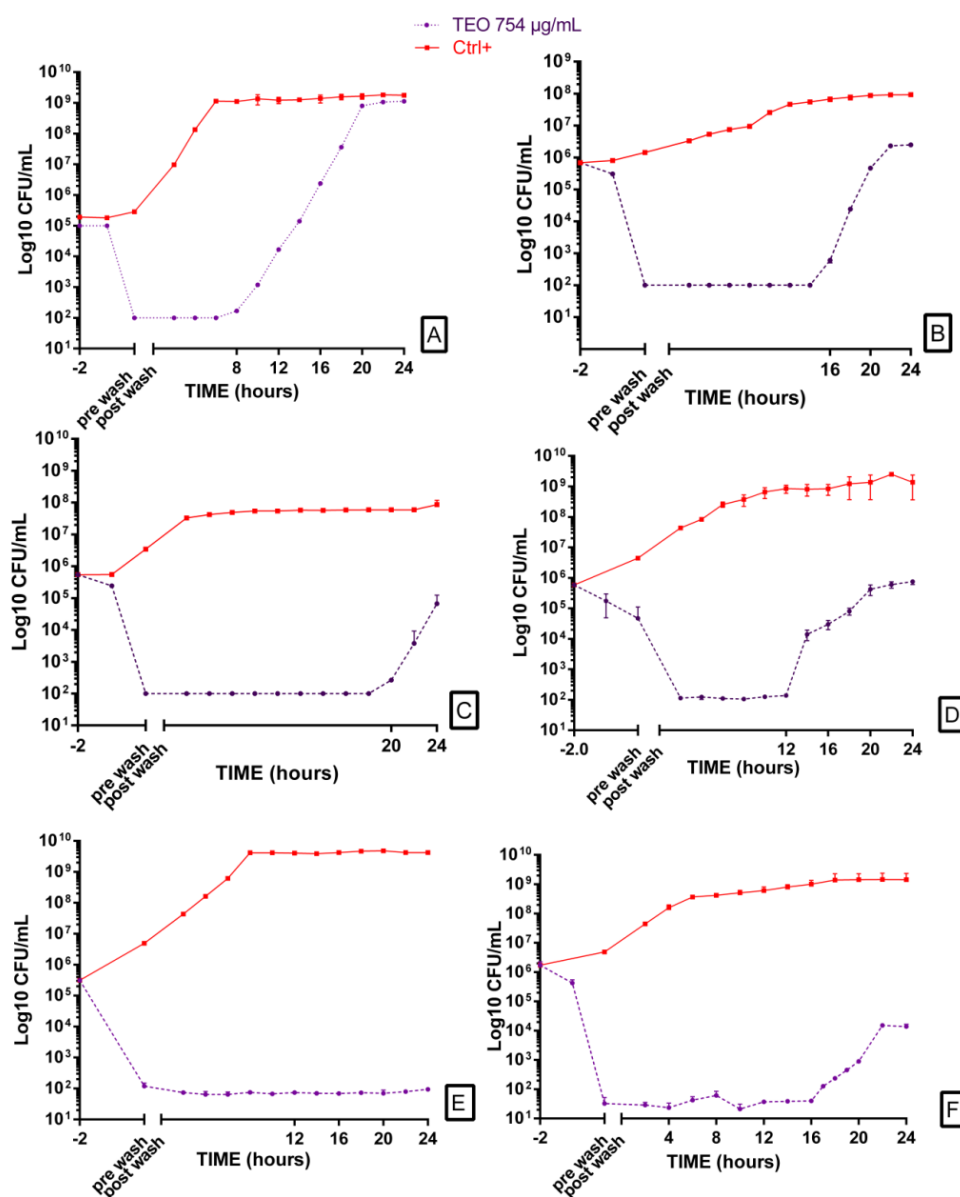


Figure 4. Post antibiotic effect of TEO against Gram-positive and Gram-negative bacteria. TEO at the concentration of MIC and control broth were added to logarithmic phase cultures of (A) *A. baumannii*, (B) *S. aureus*, (C) *E. faecalis*, (D) *K. pneumoniae*, (E) *E. coli* and (F) *P. aeruginosa*.

Table 5. PAE expressed in h by TEO (MIC, 754 µg/mL) and a triple combination at the MIC value.

Thymus essential oil			
Gram positive	PAE	Gram negative	PAE
<i>Enterococcus faecalis</i>	15.5	<i>Escherichia coli</i>	>24
<i>Staphylococcus aureus</i>	8.5	<i>Pseudomonas aeruginosa</i>	13.5
		<i>Klebsiella pneumoniae</i>	9.5
		<i>Acinetobacter baumannii</i>	5.5
Triple combination TEO+KAN+COL			
Gram positive	PAE	Gram negative	PAE
<i>Enterococcus faecalis</i>	>24	<i>Escherichia coli</i>	>24
<i>Staphylococcus aureus</i>	>24	<i>Pseudomonas aeruginosa</i>	>24
		<i>Klebsiella pneumoniae</i>	>24
		<i>Acinetobacter baumannii</i>	>24

Table 6. Comparison between control growth and TEO growth of each strain at 24h (CFU/mL).

	CTRL	TEO 754 µg/mL	Δ%	Adj. p value
<i>E. faecalis</i>	8.63e+07 ± 3.07e+07	6.69e+04 ± 5.73e+04	-99.92	0.0082
<i>S. aureus</i>	9.26e+07 ± 3.33e+06	2.47e+06 ± 2.52e+05	-97.33	1.30E-06
<i>E. coli</i>	4.23e+09 ± 5.77+07	93.33 ± 5.51	-99.99	2.30E-08
<i>P. aeruginosa</i>	1.43e+09 ± 8.88e+08	1.38e+04 ± 2.57e+03	-99.99	0.05
<i>K. pneumoniae</i>	1.36e+09 ± 9.99e+08	7.47e+05 ± 1.37e+05	-99.94	0.078
<i>A. baumannii</i>	1.78e+09 ± 1.97e+08	1.12e+09 ± 1.15e+08	-37.08	0.0075

Data are showed as mean ± SD; p values were calculated by applying one-way ANOVA with Bonferroni correction for multiple comparisons.

A comparison between control growth and PAE with TEO of each strain at 24h was evaluated (Table 6). Significant differences were observed for *A. baumannii*, *S. aureus*, *E. faecalis*, and *E. coli*, which showed reduced growth rates after treatment with TEO compared to control at 24h. Moreover, substantial inhibition rates were observed for *K. pneumoniae* and *P. aeruginosa*, but no significant differences were shown.

4. Conclusions

Results showed a wide power of TEO and a great synergistic activity by the combination with KAN and COL against multidrug-resistant bacteria. The action was more evident on Gram-negative than on Gram-positive bacteria. The strongest reduction of growth provided by TEO was observed on *A. baumannii* and *K. pneumoniae*. The combination of TEO + KAN showed high synergistic activity against *A. baumannii*. In the same way, the combination COL + TEO showed great activity, overall on *P. aeruginosa* and *A. baumannii*, with an important reduction of growth. Finally, the triple combination KAN+COL+TEO caused a total inhibition of growth for *E. coli*, *P. aeruginosa*, and *A. baumannii* with low doses of all three molecules.

The antimicrobial properties of the complex biological mixture are, in part, ascribed to their lipophilic character, which allows their accumulation into cell membranes causing a break or an interference with biochemical processes necessary for the survival of the microorganism. Our results were consistent with the hypothesis formulated by other authors, according to any EO components that have a critical part to play in antibacterial activity for the synergistic effect [57, 58]. Therefore, it is possible to conclude that the particular composition of this TEO strongly influences the antimicrobial activity inasmuch as its variability depends on many factors such as environmental conditions, harvest time, the genotype of the plant, and extraction methods as described by some authors [59-61].

Moreover, the synergistic or antagonistic relationship between antimicrobials may result from competition for primary targets. On the other hand, a synergistic multi-target effect may arise, involving ion channels, enzymes substrates, metabolites, receptors, proteins,

transport proteins groups, DNA/RNA, ribosomes, and other complex chemical and physics mechanisms. Other possible explanations that we take into account may consider the interaction among different biochemical compounds that may cause changes in the structural shape of the molecules resulting in a decreased inhibitory activity. There are several aspects regarding the exact mechanism of the synergistic or antagonistic action between two compounds that could be considered to explain the conclusive effect [62]. These aspects are difficult to clarify without further molecular investigations since, in the current study, the interaction of TEO with two drugs was assessed simultaneously.

Indeed, in order to assess the impact of TEO in association with antimicrobials in our research, we characterized the chemical composition of TEO by GC-MS. Carvacrol was identified as the major component amounting to 73% of the mixture. This phenolic compound is always present in several species of plant belonging to *genus Labiatae*, and as reported in scientific literature and important reports, its presence is the primary cause of strong antibacterial action [63]. For our research is possible that antibacterial activity and the synergistic effect may be attributed to the high percentage of this molecule. Several authors demonstrated that phenolic components contained in EOs interact with model membranes and that their antibacterial effect may be ascribed to damage sustained by the microbial lipid fraction. In particular, phenols bind to the amine and hydroxylamine groups of the bacterial membrane, causing an altered permeability and resulting in the death of microorganisms [2, 9, 10]. Furthermore, despite being the main component of this TEO, we must assume that the minor components play a very important role. In fact, the control combinations with pure carvacrol only were not synergistic with respect to the microorganisms tested in the present work.

It is an important highlight as the colistin acts in a similar way of carvacrol. It is a polycation with both hydrophilic and lipophilic domains, and it interacts with the external bacterial membrane, altering its structure mainly through interaction with the lipopolysaccharide. Hence, the combination of EO with polymyxin would help the aminoglycoside drug to enter and act on the 30s subunit of the ribosomes, significantly increasing antibacterial activity. There are many reviews and articles about the synergism among active molecules of EO and antibiotics [64-69], but no one of previous studies evaluated the interaction of TEO with two drugs concurrently and very few studies have exploited the three-dimensional checkerboard assay [70] and a comparison with the more practical method described by El-Azizi [45].

The results shown indicate that although the activity of this TEO is not significantly different compared to the activity shown by other essential oils of thyme, the action of TEO obtained from *Coridothymus capitatus* in combination with aminoglycoside and polymyxin has significantly reduced the minimum inhibitory concentrations of all three molecules. This reduction also could involve the reduction of possible side effects (high doses of colistin cause nephrotoxicity and neurotoxicity [71], while high doses of kanamycin A can contribute to loss of hearing, also [72]), even if there are controversial thoughts about this argument [73]. Furthermore, natural substances have the disadvantage of having poor water solubility and not having optimal biological stability. These disadvantages can be overcome by the possible incorporation into lipid nanoparticles [74]. Indeed, there are scientific evidence in which “drug delivery vehicles” approach has demonstrated the improvement of efficacy, stability, and bioavailability both of natural compounds and antibiotic drugs [75-77].

To sum up, these combinations could be used to increase antibiotics susceptibility, and the active mixture could be incorporated into formulations for the treatment of MDR bacteria. Future investigations will focus on nano safe formulations for the development of new antimicrobial drug delivery systems.

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Conflicts of Interest

The authors declare no conflict of interest.

Supporting Information

The supplementary file is available about the designation of MDR bacteria (Table S1). Antimicrobial categories and agents used to define MDR following rules described by Magiorakos [41]: non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial categories, and the GC-MS analysis (Table S2). GC characterization of *Thymus capitatus* var. *coridothymus* essential oil. Res. Type TIC; Figure S1. Chromatogram of *Thymus capitatus* var. *coridothymus* essential oil.)

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Supplementary files

Materials and Methods

MIC and MBC assays

Bacteria used to belong to the collection of the Laboratory of Applied Microbiology (Department of Biomedical and Biotechnological Sciences, Università degli Studi di Catania, Italy). The strains were designated as multidrug resistant (MDR) by International standard definitions for acquired resistance. MDR was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories following the rules described by Magiorakos *et al* [41]. The antibiotics used to assess their level of resistance/sensitivity, according to guidelines of CLSI [42], were summarized in Table S1.

Table S1. Antimicrobial categories and agents used to define MDR following rules described by Magiorakos *et al* (2012): non-susceptible to ≥ 1 agent in ≥ 3 antimicrobial categories.

	Categories	Resistant to ..
<i>Acinetobacter baumannii</i>	Aminoglycosides	Gentamicin; Amikacin
	Antipseudomonal cephalosporins	Ceftazidime; Cefepime
	Antipseudomonal fluoroquinolones	Ciprofloxacin
	Polymyxins	Colistin
<i>Enterococcus faecalis</i>	Aminoglycosides	Gentamicin
	Streptomycin	Streptomycin (high level)
	Penicillins	Ampicillin
<i>Escherichia coli</i>	Fluoroquinolones	Ciprofloxacin
	Folate pathway inhibitors	Trimethoprim-sulphamethoxazole
	Monobactams	Aztreonam
	Penicillins	Ampicillin
	Phenicol	Chloramphenicol
	Polymyxins	Colistin
<i>Klebsiella pneumoniae</i>	Monobactams	Aztreonam
	Penicillins	Ampicillin
	Phenicol	Chloramphenicol
	Polymyxins	Colistin
<i>Pseudomonas aeruginosa</i>	Aminoglycosides	Gentamicin
	Antipseudomonal fluoroquinolones	Ciprofloxacin; Levofloxacin
	Phosphonic acids	Fosfomicin
	Polymyxins	Colistin
<i>Staphylococcus aureus*</i>	Aminoglycosides	Gentamicin
	Anti-staphylococcal β -lactams	Oxacillin
	Fluoroquinolones	Ciprofloxacin
	Lincosamides	Clindamycin
	Macrolides	Erythromycin

*an MRSA is always considered MDR by virtue of being an MRSA.

Results

GC-MS analysis

Table S2. GC characterization of *Thymus capitatus var. coridothymus* essential oil.

Res. Type TIC.		
RT (min)	Peak name	Area
6.522	β -Thujene	1.01E+09
6.77	α -Pinene	7.46E+08
7.34	Camphene	1.62E+08
8.328	β -Pinene	9.67E+07
8.733	β -Myrcene	8.54E+08
9.434	α -Phellandrene	2.05E+08
9.83	Terpinolene	1.06E+09
10.163	p-Cymene	5.91E+09
10.328	p-Mentha-1,3,8-triene	1.68E+07

RT (min)	Peak name	Area
11.549	γ -Terpinene	2.70E+09
13.465	β -Linalool	2.94E+08
16.645	Borneol	2.65E+08
17.09	L-Terpinen-4-ol	2.12E+08
22.175	Thymol	1.36E+08
22.551	Carvacrol	4.56E+10
27.319	β -Caryophyllene	3.15E+09

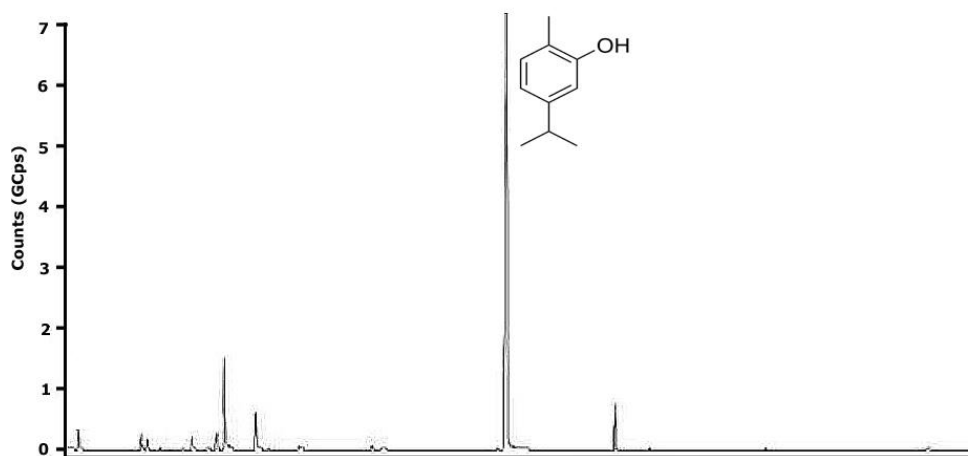


Figure S1. Chromatogram of *Thymus capitatus* var. *coridothymus* essential oil.