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Antimicrobial synergism and antibiofilm activities of *Pelargonium graveolens*, *Rosemary officinalis*, and *Mentha piperita* essential oils against extreme drug-resistant *Acinetobacter baumannii* clinical isolates

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Abstract: *Rosemary officinalis* L., *Pelargonium graveolens* L., and *Mentha piperita* L., essential oils are used by complementary medicine specialists simultaneously with traditional antibiotics for treatment purposes. The chemical composition of essential oils was analyzed by the gas chromatography-mass spectrometry method. *In vitro* antibacterial and antibiofilm activities of the essential oils were tested against extreme drug-resistant (XDR) colistin-resistant and colistin susceptible *Acinetobacter baumannii* clinical strains. The synergistic activities between essential oils and colistin antibiotics were investigated by the checkerboard method. The highest antibacterial effect was detected in mint essential oil (2.5–5 µl/ml), followed by pelargonium essential oil (5–20 µl/ml) and rosemary essential oil (5–20 µl/ml). The combination of rosemary essential oil or pelargonium essential oil with colistin showed strong synergistic activity in most of the bacterial strains tested (fractional inhibitory concentration index ≤ 0.5; synergy). As a result of the combination of mint essential oil and colistin, an indifferent effect was observed in only two bacterial strains, and other strains could not be evaluated. No antagonistic effects were observed in any of the tested essential oils. As a result of the effectiveness of the combination, the minimum inhibitory concentration (MIC) values of colistin in XDR-*A. baumannii* clinical isolates decreased 2–32 fold. Additionally, the sub-MIC concentration of essential oils exhibited an inhibitory effect (48–90%) against the biofilm layer of tested *A. baumannii* strains.

Keywords: *Acinetobacter baumannii*; essential oil; *Mentha piperita*; *Pelargonium graveolens*; *Rosemary officinalis*; synergistic activity.

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1 Introduction

Acinetobacter baumannii is a gram-negative, non-fermentative, aerobic, opportunistic pathogenic bacteria associated with nosocomial infections. It is often responsible for serious infections such as ventilator-related pneumonia, sepsis, meningitis, urinary tract, and wound infections. It can survive in hospital environments for a long time and adheres to surfaces, and forms a biofilm layer. The treatment of infections is getting more difficult day by day due to the intrinsic resistance of this bacteria to many antibiotics [1–3]. Conventional antibiotics, after a while which is frequently used in hospitals, exerted selective pressure on tolerant microorganisms, resulting in an increased frequency of multidrug-resistant (MDR) microorganisms, for a while [4].

The high virulence of the *A. baumannii,* which is naturally resistant to many antibiotics, is parallel with the multidrug resistance. With the worldwide prevalence of MDR *Acinetobacter* spp. bacterial infections, the use of colistin antibiotics has become apparent in treatment options [5]. However, due to the frequent use of this antibiotic in recent years, resistance to colistin has been reported worldwide [6, 7].

Nowadays, the need to develop natural, effective, and less toxic antimicrobial agents in the treatment of infections caused by MDR microorganisms is increasing. These natural compounds can be used alone or in combination with up-to-date antibiotics to increase the effectiveness of treatment. It is preferred for this purpose in essential oils from medical and aromatic plants [8, 9].

The antimicrobial properties of essential oils (EOs) and their compounds are widely researched in both industrial and academic fields [10, 11]. It has been reported that the combination of antibiotics used in the treatment of MDR bacteria and phytochemicals derived from plants exhibits synergistic activity, recently [9, 12].

In this study, it was aimed to evaluate the antibacterial and antibiofilm activities of rosemary (*Rosemary officinalis* L., EOR), pelargonium (*Pelargonium graveolens* L., EOP), and mint (*Mentha piperita* L., EOM) essential oils and the synergistic effects of these oils with colistin antibiotic against the extreme drug-resistant (XDR) colistin-resistant and colistin-susceptible *A. baumannii* clinical strains.

2 Materials and methods

2.1 Microbial strain

In this study, 10 XDR *A. baumannii* clinical isolates (five colistin-resistant and five colistin-susceptible) were used, which were isolated from different clinical samples (wound, blood, and urine); at the Clinical Microbiology Laboratory of Sivas Cumhuriyet University Research and Application Hospital.

Bacterial strains were identified by Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF-MS) (Bruker Biotyper Daltonik, Germany) system from colonies that were planted on sheep blood agar and grown after overnight incubation at 35 ± 2 °C. *In vitro* susceptibilities of bacterial strains to colistin, antibiotics were determined by the liquid microdilution method.

The results were evaluated according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards (≤2 μg/ml; susceptible and >2 μg/ml; resistant). *In vitro* susceptibilities of bacterial strains to other antibiotics were determined using the BD Phoenix automated system (Becton Dickinson, USA). XDR *A. baumannii* was defined as nonsusceptible to ≥1 agent in all but ≤2 categories [13].

2.2 Essential oils and antibiotic

Rosemary (EOR) and mint (EOM) essential oils were purchased from "Misbahce" trading company (Ayvalık/Balıkesir, TR); pelargonium (EOP) essential oil was supplied by the "COSMER Kimya" trading company (Pendik/Istanbul, TR); used in this study. Colistin sulfate antibiotic was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Stock colistin antibiotic solution was prepared by dissolving 12.8 mg of the powder in 1 ml sterile distilled water.

2.3 Gas chromatography/mass spectrometry (GC-MS) analysis

The components of essential oils were determined using Thermo Scientific™ TRACE™ 1310 Gas Chromatograph systems. DB-5 (5% phenyl) methylpolysiloxane column (30 m × 0.25 mm, film thickness 0.25 μm) was used in the analysis. Helium was used as the carrier gas with a flow rate of 5.1 ml/min. One microliter of the sample diluted 1/40 in methanol was injected for analysis.

The oven temperature was programmed as follows 70 °C holds for 15 min, then 2 °C/min to 180 °C hold for 5 min, and finally 5 °C/min to 230 °C and hold for 15 min. Essential oil components were determined by comparing the mass spectra obtained with the library data. Quantitative data were obtained by calculating the relative percentage of the total peak area.

2.4 Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs)

The microdilution method was used in 96 well microtiter plates (MTPs) to determine minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) [14]. First of all, the stock solutions of EOs were prepared by dissolving in dimethyl sulfoxide (DMSO) as 100 μl/ml. The final concentration of DMSO was determined to be 1%. Two-fold serial dilutions of EOs and colistin antibiotics were prepared using cation-adjusted Mueller–Hinton broth (CAMHB). The concentration range of essential oils and colistin antibiotics was 40–0.07 μl/ml, and 0.25–128 μg/ml, respectively. Twelfth wells were used for growth control and sterility control. The working concentration of DMSO was tried for positive control. The concentrations of bacterial isolates were adjusted to a concentration of McFarland 0.5 in sterile saline using overnight culture. The final concentration of bacteria in wells is diluted to be 5×10^5 colony-forming units (cfu/ml). The prepared microplates were incubated at 37 °C for 18–24 h.

MIC was determined as the lowest concentration causing no visible growth in the MTP well [15]. To determine the MBC value, 10 μl was taken from the wells where no growth was observed in the microplate where the MIC value was studied, and inoculated into Mueller Hinton agar (MHA) medium. The minimum value was defined as MBC, where no growth was observed after 24 h incubation at 37 °C, indicating that the original inoculum was killed by 99.9% [16].

2.5 Synergistic activity

The microdilution broth checkerboard method was derived from the standard procedure developed by the Clinical and Laboratory Standards Institute (CLSI-2018) to determine the *in vitro* antimicrobial efficacy of the association with colistin together with the EOR, EOM, and EOP against XDR *A. baumanni* bacterial strains. The assays were carried out according to the protocol described by [17]. The combined effectiveness of colistin antibiotics and EOs for each isolate were tested in 96 wells microplate, briefly.

The ranges of colistin concentrations tested were 0.003–8 and 0.25–128 μg/ml for colistin-susceptible and colistin-resistant strains, respectively. The concentrations of EOP, EOM, and EOR were prepared in the range of 0.31–20 μl/ml in the CAMHB. The bacteria were inoculated into the wells so that the final bacterial concentration in the wells was 5×10^5 bacteria/ml. The prepared microplates were incubated at 37 °C for 24 h and then evaluated.

Fractional inhibitory concentration index (FICi) values were calculated according to the single and combined MIC values of both agents. FICi values for two combined antimicrobial agents were calculated as follows:

$$\sum \text{FICi: FICA} + \text{FICB} = \frac{(\text{MIC of EO A in combination})}{(\text{MIC of EO A alone})} + \frac{(\text{MIC of Col in combination})}{(\text{MIC of Col B alone})}$$

The FICi was interpreted as follows; synergistic effect: FICi ≤ 0.5, additive effect: 0.5 < FICi < 1, indifferent effect: 1 ≤ FICi < 4, and antagonist effect: FICi ≥ 4 [18].

Isobolograms were plotted using GraphPad Prism, ver. 8.0 software (GraphPad Software, Inc. California, CA) to present the FICindex of the combinations [19].

2.6 Effect of the EOs on the biofilm formation

The effects of EOs on biofilm formation of *A. baumannii* were detected by MTP assay [20]. To determine the minimum biofilm inhibitory concentration (MBIC); EOR, EOM, and EOP prepared in MIC and sub-MIC (MIC/2, MIC/4, and MIC/8) concentrations with tryptic soy broth (TSB) containing 2% of glucose and bacterial inoculum (0.5 McFarland (10^8 cfu/ml)) were incubated in a U-bottomed 96 wells MTP. The final volume was 200 µl per well on the MTP. Only medium and bacteria were inoculated into the wells for negative control testing. The microplates were incubated at 37 °C for 48 h in a shaking oven. The contents of the wells were then drained and the microplates were gently washed three times with sterile distilled water to remove planktonic cells. The bacteria attached to the surface of the plates dried at room temperature were stained with 0.1% of crystal violet for 30 min. After this period, the paint on the plaques was washed three times with distilled water and removed. Pure ethanol was added to the dried microplates. After 20 min, the optical density (OD) of the microplate content was measured at 570 nm by the UV–Vis spectrophotometer (Bio-Tek, Winooski, USA). Percent inhibition of biofilm was calculated using the equation described by Onsare and Arora (2015) [21].

$$\%\ \text{Inhibition} = 100 - \left(\frac{\text{OD570 sample}}{\text{OD570 control}} \times 100\right)$$

The minimum biofilm eradication concentration (MBEC) test was studied using a method similar to the MBIC method. Unlike the MBIC test, *A. baumannii* isolates were allowed to form biofilms in a TSB medium for 48 h at 37 °C at the beginning of the test. Then, MIC and sub-MIC (MIC/2, MIC/4, and MIC/8) concentrations of EOs were inoculated into all wells. Microplates were incubated at 37 °C for 24 h. Microplates were stained using the crystal violet staining method described above. All assays were carried out in triplicate.

2.7 Statistical analysis

The statistical analysis of the research was done by IBM-SPSS 25.0 (IBM Co., Armonk, NY, ABD) program. The variance analysis of the data was performed using the one-way analysis of variance method. "Student's *t*-test" was applied for comparison between the two groups. Results were collected from three independent experiments tested in triplicate. Treated samples were analyzed in comparison with the negative control. $p < 0.05$ were considered statistically significant.

3 Results

3.1 Chemical composition

Chemical composition analyzes of the EOs, used in the study are shown in Table 1. According to GC-MS analysis results, the most common components in the chemical composition of EOP are; citronellol, geraniol, and citronellyl formate (in order; 28.3, 14.5, and 10.3%); the most common components in the chemical composition of EOM are; menthol, menthone, and menthyl acetate (in order; 32.9, 23.1, and 7.8%), and the most common components in the chemical composition of EOR are; camphor, 1,8-cineole, α-pinene, and camphene (in order; 23.4, 18.7, 21.6, and 8.2%).

3.2 Antimicrobial susceptibility

The results from the antibacterial assay show that EOM possessed antimicrobial activities with MIC values ranging from 2.5 to 10.0 µl/ml and MBC values from 2.5 to 10.0 µl/ml against all the tested microorganisms. EOR and exhibited antibacterial activity with MIC and MBC values ranging from 5.0 to 20.0 µl/ml, and EOP displayed a potential antibacterial activity with MIC values ranging from 5.0 to 20.0 µl/ml and MBC values from 10.0 to 20.0 µl/ml. The colistin antibiotic was active in the range of concentration 0.125–16 µg/ml. The MIC and MBC values of EOs against *A. baumannii* isolates are illustrated in Table 2.

3.3 Measurement of synergistic effect

Synergistic actions of EOM, EOP, and EOR were detected by the checkerboard assay and calculating of the FICi, against planktonic growth isolates. The results of the combination with colistin antibiotic together with EOM, EOP, and EOR against *A. baumannii* (Ac-b) strains are shown in Table 3. No antagonistic effect was observed in any of the isolates studied.

For EOM, an indifferent effect was seen in two susceptible isolates of colistin, and for EOP, an indifferent effect was seen in two colistin-resistant isolates. For EOR, a

Table 1: Chemical composition of essential oils of EOP, EOM, and EOR.

No.	Compound	Relative percentage (%)		
		EOP	EOM	EOR
1	Citronellol	28.3%	–	–
2	Geraniol	14.5%	–	–
3	Citronellyl formate	10.3%	–	–
4	Geranyl formate	3.8%	–	–
5	Geranyl butyrate	2.3%	–	–
6	γ-Eudesmol	8.5%	–	–
7	Geranyl tiglate	1.8%	–	–
8	β-Cadinene	2.9%	–	–
9	Citronellyl acetate	0.5%	–	–
10	Phenetyl tiglate	1.7%	–	–
11	Rose oxide B	1.4%	–	–
12	Citral	0.9%	–	–
13	Neral	0.4%	–	–
14	Other	1.8%	–	–
15	Sabinene	–	0.4%	–
16	Myrcene	–	0.2%	–
17	D-Limonene	–	2.5%	–
18	Eucalyptol	–	5.5%	–
19	Menthofuran	–	2.3%	–
20	Menthyl acetate	–	7.8%	–
21	β-Ylangene	–	0.5%	–
22	Neo-menthol	–	4.3%	–
23	Terpinen-4-ol	–	0.5%	–
24	Menthol	–	32.9%	–
25	α-Terpineol	–	0.7%	–
26	Germacrene	–	0.7%	–
27	Piperiton	–	1.7%	–
28	α-Fenchene	–	–	1.3%
29	Camphene	–	–	8.2%
30	Limonene	–	–	2.6%
31	Carene	–	–	0.7%
32	β-Myrcene	–	–	2.6%
33	α-Terpinene	–	–	0.4%
34	Cymene	–	–	2.0%
35	1,8-Cineole	–	–	18.7%
36	Camphor	–	–	23.4%
37	β-Caryophyllene	–	–	1.6%
38	β-Fenchyl alcohol	–	–	2.9%
39	α-Terpinolene	–	–	0.6%
40	Borneol	–	–	3.1%
41	Unidentified	–	–	1.2%
42	γ-Terpinene	–	0.2%	0.6%
43	β-Pinene	–	1.1%	5.3%
44	Isomenthone	7.4%	4.8%	–
45	β-Bourbonene	1.8%	0.5%	–
46	Caryophyllene	2.1%	5.3%	–
47	Menthone	3.2%	23.1%	–
48	Linalool	4.6%	–	1.0%
49	α-Pinene	0.8%	0.8%	21.6%

EOP; Pelargonium essential oil, EOR; Rosemary essential oil, EOM; Mint essential oil.

synergistic effect was observed in seven isolates studied. As a result of the checkerboard analysis, the essential oil that has the least effect is EOM. A paired sample *t*-test showed no significant difference between ΣFICi results of colistin-resistant and colistin-sensitive isolates ($p > 0.05$).

It was found that colistin and EOP or EOR combinations decreased the colistin MIC value of XDR Ac-b bacterial isolates resistant to colistin by 2–32 fold (FICindex ≤ 0.5; synergy). It was determined that, the MIC/4 concentration of EOR with the MIC/8 concentration of colistin have a synergistic effect (FICi: 0.37) against the Ac-b-6 bacterial isolate; the MIC/8 concentration of EOP oil with the MIC/4 concentration of colistin have a synergistic effect (FICi: 0.37) against the Ac-b-4 bacterial isolate. Further details about these results are shown in Table 3.

In addition, test results were also represented by the isobologram generated by plotting the FICs of EOs and colistin (Figure 1).

3.4 Inhibition of biofilm formation

EOR, EOM, and EOP were found to have strong activity in biofilm inhibition formed by *A. baumannii* bacteria at sub-MIC (MIC/2) concentrations. The results obtained for the inhibition of biofilm formation are shown in Figure 2.

EOR, EOM, and EOP at MIC/2 concentration caused 48–84, 55–92, and 52–90% inhibition of biofilm formation for all tested strains, respectively, compared to the control group without treatment. The MBIC values of EOR, EOM, and EOP vary between 10 and 20, 5 and 10, and 10 and 20 µl/ml for the tested strains, respectively (Table 4). The MBEC values for all tested strains were determined as >40 µl/ml for EOR, >20 µl/ml for EOM and EOP.

When the results of biofilm inhibition shown as "%" in Figure 2 were analyzed, it can be said that the average biofilm activity of all EOs was similar, and there was no statistically significant difference in terms of efficiency.

4 Discussion

The augmentation of antibiotic-resistant bacteria and the inadequacy of newly developed antibiotics in the treatment of infectious diseases make it necessary to develop alternative treatment strategies [22]. The combination of conventional antibiotics with natural bioactive agents is one of these treatment options. Essential oils and their components form part of the phytochemical group with such therapeutic

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of essential oils (µl/ml) against Ac-b clinical isolates.

Bacterial strains		EOM (µl/ml)		EOR (µl/ml)		EOP (µl/ml)		Colistin (µg/ml)	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
XDR colistin-resistant *A. baumannii* strains	Ac-b-1	10	10	20	20	20	20	16	32
	Ac-b-2	2.5	2.5	5	10	5	10	16	32
	Ac-b-3	5	5	10	10	10	20	8	16
	Ac-b-4	5	5	10	10	10	10	8	16
	Ac-b-5	10	10	20	20	20	20	8	16
XDR colistin-susceptible *A. baumannii* strains	Ac-b-6	5	10	10	20	10	20	0.25	0.5
	Ac-b-7	5	5	10	10	10	20	0.25	0.25
	Ac-b-8	5	10	10	10	10	20	0.25	0.5
	Ac-b-9	10	10	20	20	20	20	0.125	0.25
	Ac-b-10	5	5	10	10	10	20	0.125	0.25

EOP; Pelargonium essential oil, EOR; Rosemary essential oil, EOM; Mint essential oil.

Table 3: The *in vitro* synergistic activity of EOs in combination with colistin against *Acinetobacter baumannii* strains.

		EOM (µl/ml)				EOP (µl/ml)				EOR (µl/ml)			
	Strains	$MICC_{col}$	$MICC_{eo}$	FIC_{index}	Eff	$MICC_{col}$	$MICC_{eo}$	FIC_{index}	Eff	$MICC_{col}$	$MICC_{eo}$	FIC_{index}	Eff
Colistin (µg/ml)	Ac-b-1	NP	NP	NP	–	NP	NP	NP	–	NP	NP	NP	–
	Ac-b-2	NP	NP	NP	–	8.0	2.5	1.0	**ind**	NP	NP	NP	–
	Ac-b-3	NP	NP	NP	–	0.5	0.62	**0.12**	**syn**	0.25	2.5	**0.28**	**syn**
	Ac-b-4	NP	NP	NP	–	2.0	1.25	**0.37**	**syn**	0.25	2.5	**0.28**	**syn**
	Ac-b-5	NP	NP	NP	–	8.0	5.0	1.25	**ind**	NP	NP	NP	–
	Ac-b-6	0.12	2.5	1.0	**ind**	0.06	0.31	**0.27**	**syn**	0.03	2.5	**0.37**	**syn**
	Ac-b-7	NP	NP	NP	–	0.12	2.5	0.75	**add**	0.03	2.5	**0.37**	**syn**
	Ac-b-8	NP	NP	NP	–	0.06	1.25	**0.37**	**syn**	0.03	1.25	**0.28**	**syn**
	Ac-b-9	0.12	5.0	1.5	**ind**	0.03	2.5	**0.37**	**syn**	0.01	2.5	**0.2**	**syn**
	Ac-b-10	NP	NP	NP	–	0.03	2.5	**0.5**	**syn**	0.03	0.31	**0.28**	**syn**

Ac-b: *Acinetobacter baumannii*, MICCcol and MICCeo: Minimum inhibitory concentration of colistin and essential oil in combination. FICindex: Fractional inhibitory concentration index. Synergistic effect (in bold) FICindex ≤ 0.5. Additive effect 0.5 < FICindex < 1. Indifferent effect 1 ≤ FICi < 4. Antagonistic effect FICindex ≥ 4 [18]. NP: Not Performed.

activities. The potential synergy of essential oils with antibiotics has been frequently investigated to alleviate the antimicrobial resistance burden [11, 23].

In this study, the antibacterial, antibiofilm, and synergistic activity (with colistin antibiotics) of EOR, EOP, and EOM against clinical isolates of XDR-*A. baumannii* bacteria were investigated. The highest antibacterial effect was detected in EOM (2.5–10 µl/ml), followed by EOR and EOP (5–20 µl/ml).

The antimicrobial activity of essential oil may depend on one or both of its main components. However, in this activity, the interaction between the basic components, as well as the components that are present in small amounts is also important [11].

Menthol and menthone, which are among the monoterpenes that are in high amounts in mint, have been reported to disrupt the structure of proteins embedded in the membrane, inhibit the process of cellular respiration, and also cause cell death by disrupting the transport of ions across cell membranes [24]. It was determined that the total amount of menthol and menthone in the EOM content we used in our study was of 56%. It is reported that as the amount of menthol and menthone in the content of EOM increases, the antimicrobial activity increases [25].

It is reported that the antibacterial activity of EOR is caused by the components 1,8-cineole, camphor, and α-pinene [26, 27]. In our study, it was determined that EOR contains high levels of camphor (23.4%), α-pinene (21.6%), and 1,8 cineole (18.7%).

Mekonnen et al. showed that EOR with a high amount of α-pinene has a medium antibacterial activity with varying effects in different bacterial strains [28].

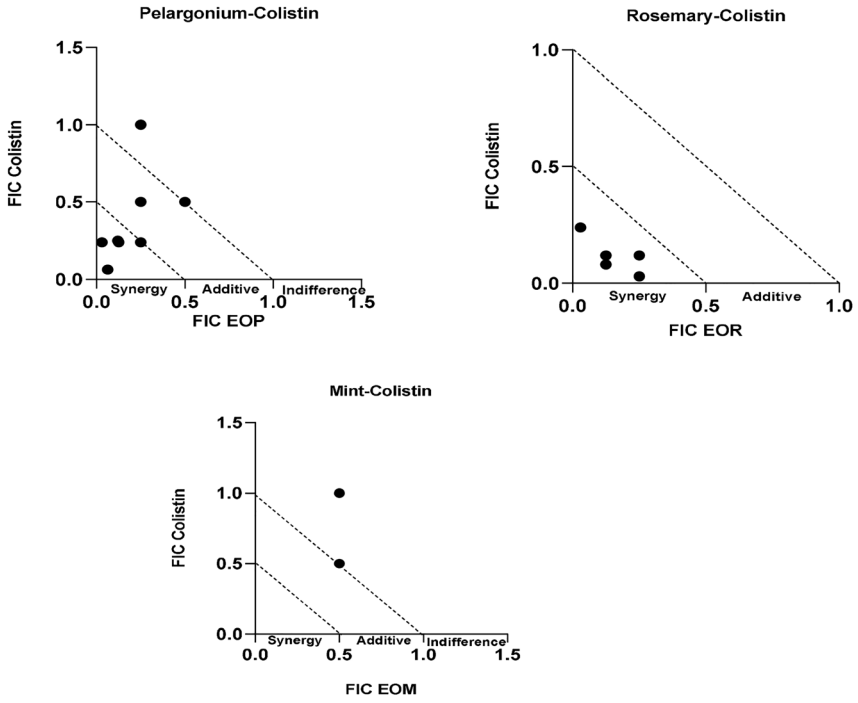

Figure 1: Isobolograms revealing the synergy (FICindex ≤ 0.5), additive (0.5 < FICindex < 1), and indifferent (1 ≤ FICindex < 4) effects of colistin and essential oils against Ac-b clinical isolates.

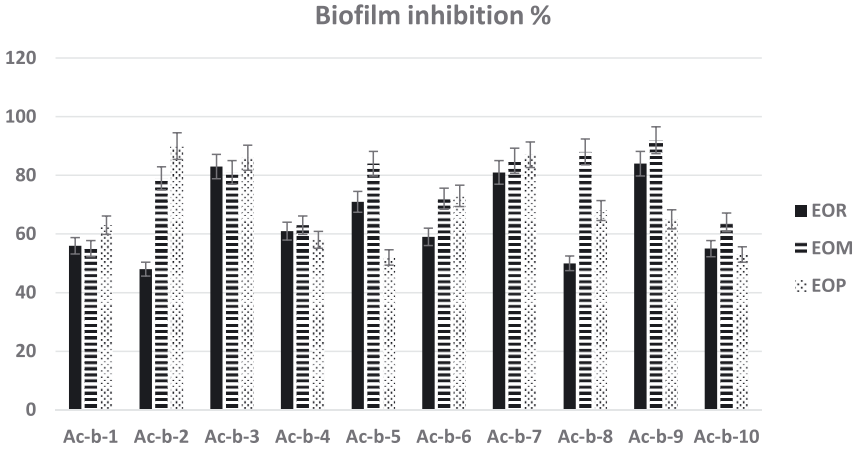

Figure 2: Antibiofilm activity against Ac-b clinical isolates of Rosemary essential oil (EOR), Mint essential oil (EOM), and Pelargonium essential oil (EOP) in MIC/2 concentration.

Table 4: The *in vitro* antibiofilm activity of EOs against clinical *Acinetobacter baumannii* isolates.

Bacterial strains		EOR		EOM		EOP	
		MBIC (µl/ml)	MBEC (µl/ml)	MBIC (µl/ml)	MBEC (µl/ml)	MBIC (µl/ml)	MBEC (µl/ml)
1	Ac-b-1	20	>40	10	>20	20	>40
2	Ac-b-2	20	>40	5	>20	10	>40
3	Ac-b-3	10	>40	5	>20	10	>40
4	Ac-b-4	20	>40	10	>20	20	>40
5	Ac-b-5	10	>40	5	>20	20	>40
6	Ac-b-6	20	>40	5	>20	20	>40
7	Ac-b-7	10	>40	5	>20	10	>40
8	Ac-b-8	20	>40	5	>20	20	>40
9	Ac-b-9	10	>40	5	>20	20	>40
10	Ac-b-10	20	>40	10	>20	20	>40

EOP; Pelargonium essential oil, EOR; Rosemary essential oil, EOM; Mint essential oil.

The quantitative composition of EOs depends on many factors, such as climate changes, growing conditions, harvest date, and distillation methods [29]. Jiang et al. indicated that EOR contains mainly 1,8-cineole (26.54%) and α-pinene (20.14%). Sienkiewicz et al. reported that EOR consists mainly of 1,8-cineole (46.4%), camphor (11.4%), and α-pinene (11.0%) components [30, 31]. Bajalan et al. evaluated the antimicrobial activities of EOR against gram-positive and gram-negative bacterial strains, and they reported that EOR has a remarkable antimicrobial activity [32]. Probuseenivasan et al. reported that EOR has strong antibacterial effects on several selected standard bacterial strains [33]. It has been shown that EOR interacts with the microbial cell membrane, causing changes, altering the transport of electrons, and causing disruption of the membrane structure and its functionality [34].

The geraniol which is a type of monoterpene, that is a high amount in EOP, has been reported that has antimicrobial activity against gram-negative and gram-positive bacteria. Monoterpenes cause perturbation of the lipid fraction in the plasma membrane of microorganisms, and the permeability of the membrane changes and the cell dies [35].

In our study, the combination of EOR and EOP with the colistin antibiotic showed significant synergistic activity on the bacterial strains tested. The synergistic effect (FICi from 0.25 to 0.37) was determined for seven bacterial strains tested in EOR. In EOP, a synergistic effect (FICi from 0.12 to 0.5) for six bacterial strains, an indifferent effect for two bacterial strains, and an additive (add) effect for one bacterial strain were determined. The combinations of EOP and EOR with colistin antibiotics reduced MIC values in XDR-*A. baumannii* clinical isolates by 2–32 fold (FICi ≤ 0.5; synergy). It was determined that the oil with the weakest synergistic effect was EOM. Indifferent effect (ind) was detected in only two of the bacterial strains that were tested, and other bacterial strains could not be evaluated. No antagonist effect was detected with the colistin antibiotic in any of the tested essential oils.

In this study, although a moderate level of antibacterial activity was found in the EOR and EOP, it can be said that EOs have strong synergistic activity. This result indicated that although the antibacterial activity of a natural compound alone is low, its antimicrobial activity may increase with the combined application. The antimicrobial synergy mechanisms of natural compounds have not been fully elucidated. But some authors suggest that phytochemicals disrupt the cell wall structure of the bacteria or facilitate the entry of antibiotics into the cell by increasing the permeability of the cytoplasmic membrane. And they also suggest that some of these chemicals are efflux pumps or penicillin-binding protein inhibitors [4].

Rosato et al. reported that the combination of EOM and gentamicin or ampicillin had a strong synergistic effect against many standard bacterial strains in their studies, but there is no apparent synergistic effect for *A. baumannii* standard bacterial strain [36].

Jarrar et al. investigated the efficacy of *Rosmarinus officinalis* L. ethanol extract and cefuroxime combination against methicillin-resistant *Staphylococcus aureus* bacteria and found synergistic activity in all studied bacterial strains. The authors noted that although the pure extract showed a low antimicrobial activity, it showed a strong synergistic effect with the antibiotic [37]. These results were consistent with our study. In one study, it was reported that the combination of EOP with ciprofloxacin antibiotics showed synergistic activity (FICi from 0.375 to 0.5) against uropathogenic standard bacterial strains (*Klebsiella pneumoniae* KT2, *Proteus mirabilis* PRT3, and *S. aureus* ST2.) [38]. In another study, it was found that the combination of geraniol (the main component in EOP), and clarithromycin or amoxicillin antibiotics showed a synergistic effect (FICi 19–0.32) against standard bacterial strains of *S. aureus* due to its strong anti-efflux activity [39].

The biofilm layer, which allows bacteria to be resistant to negative environmental conditions such as nutrient deficiency, and dryness, is quite complicated. The biofilm layer of bacteria is the main cause of persistent nosocomial infections, especially in immunocompromised patients. Frequent use of medical devices such as catheters and ventilators in intensive care units makes difficult the treatment of biofilm-related *A. baumannii* infections. Many antibiotics, including carbapenems and colistin, reduce the biofilm layer but do not completely remove the biofilm layer. Because concentrations above the MIC and MBC values should be applied to increase the effectiveness of antibiotics on the biofilm layer. For this reason, it has become very important to investigate new and natural antibiofilm agents in the fight against the biofilm layer [40–42].

The use of essential oils and by-products of their to deal with biofilm formation and development has become an important strategy. In our study, the effectiveness of the essential oils examined in MIC/2 concentration on the biofilm formation formed by clinical strains of the *A. baumannii* bacteria was found to be significantly high. It is reported that the nonlethal damage caused by essential oils on the bacterial cell wall negatively affects the "*adhesion to bacterial surfaces*" step, which is the first step of biofilm formation [43]. It is also thought that the addition of essential oils to abiotic surfaces can both contribute to

the elimination of planktonic cells and prevent bacteria from adhering to the surfaces [44]. *A. baumannii* is also frequently transmitted by abiotic surfaces. For this reason, pre-treatment of surfaces with plant extracts may be preferred as an application that reduces surface adherence and makes the environment unfavorable for biofilm formation. Chaieb et al. reported that many essential oils or natural agents inhibit bacterial biofilm formation due to their bactericidal effects and anti-adhesion potentials [45]. In another study, Lagha et al. reported that EOR has the highest biofilm activity on biofilm formation of *Escherichia coli* bacteria isolates among the essential oils they tested [46].

Because of their bactericidal impact on XDR-*A. baumannii* bacteria, EOM, EOR, and EOP have the potential to be used as a new antimicrobial agent. Mechanical ventilation, which is commonly used, especially in intensive care patient groups, causes the majority of patients to develop nosocomial pneumonia and increases the mortality rate. Thus, it may be possible to use such essential oils for medicinal purposes by inhaling them or to benefit from them while cleaning mechanical ventilators. Pre-treatment of such mechanical devices with plant extracts can be preferred as an application that reduces surface adsorption and makes the environment unfavorable for biofilm.

5 Conclusion

Our study is the first to report the combination of EOM, EOR, and EOP with colistin against XDR-*A. baumannii* bacterial strains. It was determined that the combination of EOR and EOP with colistin showed a high level of synergistic activity against colistin-resistant and colistin-sensitive *A. baumannii* bacterial strains in this study. It has also been found that this combination increases the effectiveness of colistin and reduces the MIC value by 2–32 fold. The use of essential oils in combination with antibiotics may be an innovative alternative to treatment protocols to reduce antimicrobial resistance and avoid the undesirable toxic effects of antimicrobial therapy. However, further *in vitro* and *in vivo* studies are required to assess the potential of this combination for therapeutic purposes.

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