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Evaluating the Synergistic Potency of Essential Oils and Antibiotics Against *Klebsiella pneumoniae* BLSE Strains

Karim Bariz[1], Lamia Trabelsi[2], Souad Lahcene[1], Mounir M. Salem-Bekhit[3], Gehan M. Elossaily[4*], Hayat Ali Alzahrani[5], Omar O. Alharbi[6], Mohamed Abbaci[7], Hocine Abbaci[8], Yacine Benguerba[9], Karim Houali[1]

[1] Laboratory of Analytical Biochemistry and Biotechnology (LABAB), Faculty of Biological Sciences and Agronomic Sciences. Mouloud Mammeri University, Tizi-Ouzou, Algeria
[2] National Institute of Marine Sciences and Technologies. Monastir, Tunisia
[3] Kayyali Chair for Pharmaceutical Industries, Department of Pharmaceutics, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia
[4] Department of Basic Medical Sciences, College of Medicine, AlMaarefa University, P.O. Box 71666, Riyadh 13713 Riyadh, Saudi Arabia
[5] Department of Medical Laboratory Technology, Faculty of Applied Medical Sciences, Northern Border University, Arar, Saudi Arabia
[6] Pharmaceutical Care Department, National Guard Health Affairs, Riyadh 11426, Saudi Arabia
[7] CRAPC Bou Ismail, Tipaza, Algérie
[8] Laboratoire de Maitrise des Energies Renouvelables, Faculté des Sciences de la nature et de la Vie, Université A/Mira Bejaia, Algérie
[9] Laboratoire de Biopharmacie Et Pharmacotechnie (LPBT), Ferhat Abbas Setif 1 University, Setif, Algeria

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ABSTRACT

Klebsiella pneumoniae producing extended-spectrum β-lactamases (ESBL) continues to pose huge therapeutic challenges in the treatment of infections, primarily urinary infections, due to its multidrug resistance to antibiotics. Therefore, there is a need for research on this topic to investigate ways to reduce the spread of antibiotic resistance, identify novel therapeutic approaches to treat these infections and gain a better understanding of the mechanisms of resistance. In this context, this study aimed to analyze the chemical composition of essential oils (EOs) of *Thymus algeriensis*, *Syzygium aromaticum*, and *Eucalyptus globulus* and assess their activity against *K. pneumoniae ESBL* strains, as well as the interaction type between these EOs and antibiotics used for the treatment of *K. pneumoniae* ESBL infections. The composition of the EOs was determined by gas chromatography-mass spectrometry (GC-MS). The activity of EOs was tested using the disc diffusion and liquid microdilution methods. The type of interaction between EOs and antibiotics was studied using the agar disk diffusion and chessboard methods. The analysis of the EO of *T. algeriensis* showed that the main compounds were thymol (23.14%), linalool (18.44%), and p-cymene (16.17%). The main constituents of EO of *E. globulus* were eucalyptol (54.29%), α-pinene (17.32%), aromadendrene (7.02%), and pinocarveol (6.32%). As for the EO of *S. aromaticum*, the major constituents were eugenol (80.46%) and eugenol acetate (16.23%). Results of the activity tests showed that all three EOs were active against the tested strains, with inhibition diameters ranging from 7.39±0.44mm to 32.4±1.05mm and minimum inhibitory concentrations (MICs) varying from 2 to 441.5±5.66 mg/ml. A synergistic interaction was obtained between amoxicillin-clavulanic acid and *T. algeriensis* EO against two strains of *K. pneumoniae* ESBL. These results demonstrate the potential of our EOs to inhibit multi-resistant pathogenic ESBL strains, as well as their synergistic interaction with antibiotics used in therapy, which could be an alternative to the use of antibiotics alone in treatment to fight against these multi-resistant pathogenic bacteria.

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Introduction

Since the discovery of penicillin in 1928, antibiotics have greatly contributed to the reduction of mortality from infectious diseases. However, the excessive, frequent, and uncontrolled use of antibiotics in human and animal health has led to the emergence and spread of bacterial resistance over time. Antibiotic resistance is now a major public health issue (1). *Klebsiella pneumoniae*, a Gram-negative bacillus of the *Enterobacteriaceae* family, is known to be highly virulent and multidrug-resistant. It is an opportunistic pathogen that causes respiratory tract infections, such as pneumonia, sepsis, and urinary tract infections (2). In 2017, this species was placed on the World Health Organization's Priority 1 (Critical) list, as an urgent research target for the development of new antibiotics. The evolution of *K. pneumoniae* strains, through point mutations and the horizontal transfer of mobile elements, together with the misuse of ß-lactam and other antibiotic families, has led to extended-spectrum ß-lactamases (ESBLs) and carbapenemases being produced and rapidly disseminated. Studies have shown that enzymatic mechanisms of antibiotic re-

* Corresponding author. Email: jabdelmenam@mcst.edu.sa

sistance are the most common in *K. pneumoniae* (3). Recently, resistance to fosfomycin, carbapenems, tigecycline, and newer combinations such as meropenem-tazobactam, ceftazidime-avibactam, and ceftolozane-tazobactam have been reported around the world (4,5). The emergence of antibiotic-resistant strains and their spread across the globe necessitates research into the development of new molecules with natural antibacterial activity (6). Essential oils (EOs) have long been known for their antimicrobial properties and can act on the defense systems of cells, such as efflux pumps, quorum sensing, biofilm formation, and ATPase pump inhibition (7). The main components of EOs with antimicrobial activity are terpenes, alcohols, acids, esters, epoxides, aldehydes, ketones, amines, and sulfides such as terpineol, thujanol, myrcenol, neral, thujone, camphor and carvone (8,9). Several studies have demonstrated the effectiveness of EOs against multidrug-resistant *K. pneumoniae* strains, including ESBL and CTX-M positive strains (10,11). Combining antibiotics with EOs represents a novel exploration in the search for bioactive molecules with anti-microbial effects. The synergistic effect between these molecules could be a viable alternative to using antibiotics alone and help to overcome the problem of antibiotic resistance in therapy (12). *Eucalyptus globulus* EO possesses antimicrobial properties against various pathogenic bacteria such as *Mycobacterium tuberculosis*, *Staphylococcus*, and gram-negative bacteria like *K. pneumoniae*. The activity is mainly attributed to components like 1,8-cineole, p-cymene, limonene, β-pinene, and α-terpinol (13). Moreover, *Syzygium aromaticum* is rich in aromatic constituents, and its EO exhibit antioxidant, antifungal and antimicrobial activities due to the majority compound eugenol (14). Species from the Lamiaceae family are known for their therapeutic effects, and this includes *Thymus algeriensis*, a thyme species found in North Africa (Algeria, Libya, Morocco and Tunisia). Its EOs have various medicinal properties such as antimicrobial, antioxidant, anticancer, anti-ulcer, anti-diabetic, insecticidal, and anti-inflammatory activities (15). Our study aimed to first determine the chemical composition of the EOs of three plants two of them growing wild in Tizi-Ouzou region, namely *T. algeriensis* and *E. globulus*. For *S. aromaticum*, we used cloves directly purchased from an herbalist. We then conducted a study of their antibacterial activities and a second study of the association of these three EOs with antibiotics against clinical strains of *K. pneumoniae* ESBL and CTX-M positive. The main scope of this study was to explore the potential synergistic effects of combining antibiotics with the three EOs on these clinical strains. By assessing the efficacy of this combination, we hoped to discover if one of these EOs could enhance the efficacy of antibiotics against this strain of bacteria.

Materials and Methods

Plants and extraction of essential oils

Two plants were collected from the Tizi-Ouzou region in the locality of Aghrib (36° 48'08'' North 4°19'22'' East) for *T. algeriensis*, and from Yakouren (36°44'05'' North 4°26'19''East) for *E. globulus*. *S. aromaticum* was procured from a local herbalist in Tizi-Ouzou. The identification of the plants was done by Dr. Hocine Abbaci from the University of Bejaia. To extract the EOs, dried leaves were subjected to steam entrainment for 3 hours, after which the EO was recovered by decantation and dehydrated with anhydrous sodium sulfate. All pure EOs were stored at +4°C until use. The EOs yields were 1.8%, 2.5%, and 5.2% for *E. globulus*, *T. algeriensis*, and *S. aromaticum*, respectively.

Gas Chromatography-Mass Spectrometry (GC–MS) Analysis

The chemical composition of EOs was carried out at the Center for Physico-Chemical Analyses (CRAPC) in Tipaza, Algeria. GC-MS analyses were performed using a Hewlett Packard Agilent 6890 plus device equipped with an HP-5MS column (30 m, 0.25 mm, 0.25 µm) and a Hewlett Packard Agilent 5973 mass detector operating in ICT Scan mode (30 to 550). Helium was used as a carrier gas at a flow rate of 0.5 ml/min. The oven temperature program was as follows: 60°C for 8min, 2°C/min up to 250°C., and isothermal for 10min. The detector temperatures were 280°C. The constituents identification was based on comparing their relative retention indices (RI) and mass spectra with those of reference constituents in the NIST05 library or with mass spectra in the literature (16). The relative amount of the individual components of the total oil was expressed as a percentage of the peak area relative to the total peak area.

Tested Bacterial Isolates

Seven bacterial strains were utilized: three reference strains - *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Klebsiella pneumoniae* ATCC 700603 ESBL-positive (SHV-18) and four clinical strains of multidrug-resistant ESBL and CTX-M positive, *K. pneumoniae* 3520*, K. pneumoniae* 1216, *K. pneumoniae* 5111 and *K. pneumoniae* 3511. These strains were isolated from urine and pus samples at the Tizi-Ouzou University Hospital Microbiology-Parasitology Laboratory. Identification with MALDI-TOF and the search for resistance genes were conducted at the Laboratory of Molecular Microbiology, CHU ULB- ERASME, Belgium. The strains were then stored at -18 °C, revived on their selective media, and grown on heart-brain broth.

Antibacterial activity of essential oils

A disc diffusion method was used to study the antibacterial activity of our EOs. Muller-Hinton Agar was seeded with a suspension of the target bacterium at 10^8 CFU/mL. Whatman No.1 paper discs with a 6mm diameter were placed on the bacterial mat and loaded with 10µl of pure EO. The boxes were then pre-diffused in the agar by storing them at 4 °C for 3 hours. After 24 hours of incubation at 37°C, the inhibition diameters were measured. Each test was repeated three times. The inhibition diameters were categorized as follows: Ø < 8mm as non-sensitive (-); 8mm < Ø< 14mm as sensitive (+); 14mm <Ø < 20mm as very sensitive (++) and finally Ø > 20 mm as extremely sensitive (+++). A Chloramphenicol disc (30µg) was utilized as a positive control (17).

Determination of minimal inhibitory concentration

The minimum inhibitory concentration (MIC) of EOs against ESBL strains of *K. pneumoniae* was determined using the 96-well microplate microdilution method in broth according to the Clinical and Laboratory Standards Institute (CLSI). Semi-logarithmic dilutions of *T. algeriensis*,

E. globulus, and *S. aromaticum* EOs (433.5-0.84mg/ml), (441-0.125mg/ml) and (512-1mg/ml), respectively, were prepared in Muller-Hinton broth with tween 80 at a final concentration of 1% (v/v). Each well was then filled with 50µl of EO dilution and 50µl of the bacterial suspension at a concentration of 10^6 CFU/ml. A growth control was then performed with Muller-Hinton broth alone and with tween 80 at 1% to exclude the antibacterial effect of tween 80. After being incubated for 18h at 37°C, 30 µl of resazurin at 0.015% was added to each well and incubated at 37°C for 3 hours. The MICs of the EOs were determined by noting the first blue, unchanged well after 3 hours of incubation with resazurin, and expressed in mg/ml.

Screening the Synergistic Effect of the EO with Antibiotic Discs

Three antibiotics Amoxicillin-clavulanic acid (20/10µg) (AMC); Ceftazidime (30µg) (CAZ) and Levofloxacin (5µg)(LEV) (Liofilchem brand) were tested alone and in combination with three EOs on Mueller Hinton agar seeded with 10^8 CFU/ml. 10µl of each EO was impregnated onto the antibiotic discs. After 24 hours of incubation at 37°C, the diameters of the inhibition zones around the discs of the EOs alone, antibiotics alone, and discs combined antibiotic- EOs were measured. The results were then analyzed for antagonism and synergy by comparing the diameters of inhibition in combination (EO + antibiotic) with the sum of the diameters of the inhibition zones of the two agents tested separately (EO and antibiotic) (18).

Checkerboard method

The synergistic interaction between *T. algeriensis* EO and amoxicillin-clavulanic acid against strains *K. pneumoniae* 3511, *K. pneumoniae* 1216, and *K. pneumoniae* ATCC 700603 ESBL-positive (SHV-18) was confirmed by this study. Dilutions were prepared for both amoxicillin-clavulanic acid (256 -2µg / ml) and EO of *T. algeriensis* (433.5-0.84 mg/ml). Each microplate well contained 25µl of the EO, 25 µl of amoxicillin-clavulanic acid, and 50 µl of the bacterial suspension (10^6 CFU/ml). The microplates were then incubated at 37°C for 24 hours, and 30 µl of resazurin was added to each well. The MICs of amoxicillin-clavulanic acid and EO combined or not were also determined (11). The interaction between amoxicillin-clavulanic acid and EO was measured using the fractional inhibitory concentration index (FICI).

$$FIC = \frac{MIC\ of\ essential\ oil\ or\ antibiotic\ in\ combination}{MIC\ of\ essential\ oil\ or\ antibiotic\ alone} \quad [1]$$

$$FICI = FIC\ essential\ oil + FIC\ antibiotic \quad [2]$$

Results were interpreted as synergy (FICI < 0.5), addition (0.5 < FICI ≤ 1.0), indifference (1.1 < FICI ≤ 4), and antagonism (FICI > 4).

Statistical Analysis

The data from synergetic tests between antibiotics and EOs were analyzed using the Mann-Whitney's HSD (honestly significant difference) test at $p < 0.05$ to investigate differences between single molecules and combination groups. Three independent experiments were performed to carry out all antimicrobial analyses, and the results were presented as the mean ± standard deviation and were subjected to the one-way ANOVA followed by Tukey's HSD (honestly significant difference) test at $p < 0.05$. All statistical analyses were performed using SPSS Statistics (Version 25.0).

Results

EOs Chemical Compositions

The composition of the EOs of *T. algeriensis*, *E. globulus*, and *S. aromaticum* examined in this study is given in Table 1. Results show that *T. algeriensis* EO contains the majority components thymol (23.14%), along with linalool (18.44%), p-Cymene (16.17%), Carvacrol (4.98%), α.-Pinene (4.57%), and Geraniol (4.22%) at lower proportions. Other components such as β-caryophyllene, α-thujene, camphene, β-myrcene, p-mentha-1,3-diene, and thymol methyl ether are present at very low levels.

The EO of *E. globulus* is composed primarily of eucalyptol (54.29%), with α-pinene (17.32%) as a second major component. aromadendrene (7.02%), and pinocarveol (6.32%) are present at relatively lower concentrations. Lastly, the EO of *S. aromaticum* is dominated by eugenol (80.46%), followed by eugenol acetate (16.23%). Other components such as β-Caryophyllene, α-Terpinene, and isoeugenol are present at very low levels.

Antibacterial activity

Table 2 shows the results of studying the antibacterial activity of our three EOs. The greatest IZD was observed in *T. algeriensis* EO, with values ranging from 13.81±0.56mm to 32.4±1.05mm and MICs ranging from 3.38±0.2 to 433.5±2.53mg/ml. Of the *K. pneumoniae* ESBL (KP3520) strains, three (KP 3511, KP 1216, and KP5111) were found to be extremely sensitive to *T. algeriensis* EO. *S. aromaticum* EO yielded IZDs ranging from 8.17±0.22mm to 17.67±0.29mm and MICs ranging from 2mg/ml to 32mg/ml, with only one strain of *K. pneumoniae* ESBL (*K. pneumoniae* 5111) being very sensitive to this EO. Finally, *E. globulus* EO IZDs ranged from 7.39±0.44mm to 18.33±0.15mm, with corresponding MICs ranging from 13.78±0.62 to 441.5±5.66mg/ml. The profile of *K. pneumoniae* ESBL strains vis-à-vis this EO was described as sensitive.

Screening the Synergistic Effect of the EO with Antibiotic Discs

Tables 3, 4, and 5 in this study demonstrate two types of interactions between the three antibiotics and three EOs: antagonism and synergy. Specifically, a synergy-like interaction was observed between amoxicillin-clavulanic acid and *T. algeriensis* EO on two pathogenic strains (KP 1216 and KP 3511) and the reference strain *K. pneumoniae* ATCC 700603 ESBL-positive. Compared to the amoxicillin-clavulanic acid disc alone, the IZD of the combined disc increased from 2 to 4mm, indicating a synergistic effect. The Mann-Whitney test showed a significant difference ($p < 0.05$) in the antibacterial activity of the combined disk (amoxicillin-clavulanic acid-*T. algeriensis* EOs) and the single disks of amoxicillin-clavulanic acid and *T. algeriensis* EOs , which were tested, against the *K. pneumoniae* 3511, *K. pneumoniae* 1216 and *K. pneumoniae* ATCC 700603 strains.

Checkerboard method

The checkerboard method was used in this study to

Table 1. Chemical composition of essential oils of *T. algeriensis*, *E. globulus*, and *S. aromaticum.*

Thymus algeriensis		*Eucalyptus globulus*		*Syzygium aromaticum*	
Constituents	%	Constituents	%	Constituents	%
α-Thujene	1.39	3-Carene	0.1	Eucalyptol	0.09
α.-Pinene	4,57	α-Pinene	17.32	Chavicol	0.07
2,4(10)-Thujadien	0.08	β-Pinene	0.2	Eugénol	80.46
Camphene	0.14	Benzaldehyde	0.05	Isoeugenol	0.14
Sabinyl acetate	0.08	Eucalyptol	54.29	β-Caryophyllene	1.31
Sabinene	0.08	β-ocimene	0,16	α-Caryophyllene	0.18
2-β.-pinene	0.91	Dehydro-p-cymene	0.24	Eugenol acetate	16.23
1-Octen-3-ol	0.78	D-Fenchyl alcohol	0.22	2-oxide Eugenol	0.05
β-Myrcene	2,33	Pinocarveol	6.32	α-Terpinene	0.26
3-Octanol	0.21	(+)-Pinocarvone	2.95	δ-cadinene	0.04
p-Mentha-1,3-diene	1.87	Borneol	0.45	3-methyl- Adamantane	0.13
p-Cymene	16.17	Cyclohexanol	0.1	Dansyl-dl-methionine	0.04
p-Mentha-1,4-diene	0,70	Pseudocarveol	0.83	Gurjunene	0.05
β-ocimene	0.32	Camphene	0.46	Trans-2-(2-Pentenyl) furan	0.03
Terpinolen	0.16	(+)-Carvone	0.09		
Linalool	18.44	Isobornyl formate	0.08		
cis-Ocimene	0.16	2-Pyridylmethanol	0.19		
trans-Pinocarveol	0.18	β-Gurjunene	0.35		
Borneol	0.25	(+)-Aromadendrene	7,02		
4-Carvomenthenol	0.39	(+)-β-Selinene	0.22		
α-Ocimene	0.28	(+)-Ledene	1.03		
Thymol methyl ether	2.1	(+)-Valencene	0.21		
α-Citral	0.11	α-pinene oxyde	0.05		
Geraniol	4.22	Humulene	0.11		
Thymol	23.14				
Carvacrol	4.98				
β-Caryophyllene	2.63				

Table 2. Inhibition zone diameters (IZDs) (mm) and MICs for essential oils of *T. algeriensis*, *E. globulus*, and *S. aromaticum* against tested bacteria strains. The results of the ANOVA test and Tukey test of multiple comparisons showed a statistically significant difference ($p < 0.05$) in the antibacterial activity of the three EOs and chloramphenicol for each bacterial strain tested.

	Thymus algeriensis		*Eucalyptus globulus*		*Syzygium aromaticum*		*Chloramphenicol*
	IZD	**MIC**	**IZD**	**MIC**	**IZD**	**MIC**	**IZD**
K. pneumoniae ATCC 700603	13.81±0.56	27.1±0.56	7.39±0.44	110.25±3.37	11.83±0.78	2±0.51	24±0,00
E. coli ATCC 25922	25.86±0.95	27.1±0.8	18.33±0.15	13.78±0.62	17.67±0.29	2±0.58	25±0,00
P. aeruginosa ATCC 27853	13.27±0.75	433.5±2.53	10.37±0.13	441.5±5.66	8.17±0.22	4±0.67	22±0,00
K. pneumoniae 3520	19.17±0.56	13.45±0.31	9.17±0.44	27.56±0.80	12.33±0.67	16±0.11	26±0,00
K. pneumoniae 3511	24±1.05	13.45±0.71	11.45±1.2	55.12±0.77	12.34±0.48	32±0.55	26±0,00
K. pneumoniae 1216	21±0.55	27.1±0.45	13.80±0.9	27.56±0.73	13.5±1.1	4±0.41	27±0,00
K. pneumoniae 5111	32.4±1.05	3.38±0.2	11±0.66	27.56±0.29	16.5±0.38	2±0.19	28±0,00

confirm the synergy observed on a solid medium between the disc of Amoxicillin-Clavulanic Acid and the EO of *T. algeriensis*. The results, depicted in Table 6, indicate an Inhibitory Fraction below 0.5 for the three strains tested: *K. pneumoniae* 3511, *K. pneumoniae* 1216, and *K. pneumoniae* ATCC 700603, demonstrating a synergistic effect between the EO and Amoxicillin-Clavulanic Acid. The MICs for Amoxicillin-Clavulanic Acid decreased by 25% in *K. pneumoniae* 1216 and 12.5% in *K. pneumoniae* 3511 and *K. pneumoniae* ATCC 700603, respectively. The Mann-Whitney test showed significant differences ($p < 0.05$) in the antibacterial activity expressed as MIC of the combination of amoxicillin-clavulanic acid-EO of *T. algeriensis* and the MIC of amoxicillin-clavulanic acid tested alone against the strains *K. pneumoniae* 3511, *K. pneumoniae* 1216 and *K. pneumoniae* ATCC 700603. The decrease in the MIC of amoxicillin-clavulanic acid in combination with EO of *T. algeriensis* is statistically significant.

Discussion

Plants are subjected to biotic and abiotic stresses that influence their physiology and growth. In order to resist

Table 3. Inhibition zone diameters (IZDs) (mm) of *E. globulus* essential oil and antibiotics, alone and in combination, against tested bacteria strains.

.	Antibiotics	IZD with antibiotics	IZD essential oil	Sum of the IZD with the Antibiotics and the Essential Oil	IZD association with Antibiotics and Essential Oil	Effect
K. pneumoniae ATCC 700603	AMC	14±0,00	9±0,13	23	13.5±0,55	A
	CAZ	06±0,00		15	11±0,43	A
	LEV	20±0,00		29	21±0,88	A
E. coli ATCC 25922	AMC	19±0,00	18±0,35	37	19.5±0,87	A
	CAZ	22±0,00		40	22.5±0,22	A
	LEV	32±0,00		50	40±0,40	A
P. aeruginosa ATCC 27853	AMC	06±0,00	06±0,58	12	06±0,11	A
	CAZ	20±0,00		26	20±0,97	A
	LEV	24±0,00		30	25±0,50	A
K. pneumoniae 3520	AMC	12±0,00	11±0,99	23	13±0,12	A
	CAZ	06±0,00		17	9.5±0,43	A
	LEV	23±0,00		33	22±0,96	A
K. pneumoniae 3511	AMC	13±0,00	08±0,62	21	11.5±0,66	A
	CAZ	06±0,00		14	08±0,88	A
	LEV	06±0,00		14	08±0,48	A
K. pneumoniae 5111	AMC	12±0,00	12±0,95	24	14±0,77	A
	CAZ	06±0,00		18	10.5±0,94	A
	LEV	19±0,00		31	19±0,23	A
K. pneumoniae 1216	AMC	10±0,00	13±0,53	23	14.5±0,41	A
	CAZ	06±0,00		19	10±0,45	A
	LEV	21±0,00		34	21±1,3	A

A: antagonistic effect, S: Synergistic effect.

Table 4. Inhibition zone diameters (IZDs) (mm) of *T. algeriensis* essential oil and antibiotics, alone and in combination, against tested bacteria strains.

	Antibiotics	IZD with antibiotics	IZD essential oil	Sum of the IZD with the Antibiotics and the Essential Oil	IZD association with Antibiotics and Essential Oil	Effect
K. pneumoniae ATCC 700603	AMC	16.5±0,00	16±0,61	32	34±0,1	S
	CAZ	06±0,00		22	10±0,34	A
	LEV	22±0,00		38	23±0,62	A
E. coli ATCC 25922	AMC	20±0,00	25±0,97	45	20±0,70	A
	CAZ	22±0,00		47	12±0,51	A
	LEV	34±0,00		59	29.5±0,39	A
P. aeruginosa ATCC 27853	AMC	06±0,00	06±0,1	12	06±0,69	A
	CAZ	21±0,00		27	21±1,2	A
	LEV	24±0,00		30	24±1,44	A
K. pneumoniae 3520	AMC	11.5±0,00	19±0,92	30,5	19±0,98	A
	CAZ	06±0,00		25	12±0,88	A
	LEV	24±0,00		43	22.5±0,83	A
K. pneumoniae 3511	AMC	12±0,00	22±0,79	34	37±0,15	S
	CAZ	06±0,00		28	18±0,33	A
	LEV	07±0,00		29	24±0,57	A
K. pneumoniae 5111	AMC	10.5±0,00	32±0,36	42,5	29.5±1,45	A
	CAZ	06±0,00		38	12.5±0,64	A
	LEV	22±0,00		54	24±1,48	A
K. pneumoniae 1216	AMC	12±0,00	22±0,44	34	38±0,17	S
	CAZ	06±0,00		28	23±1,31	A
	LEV	22±0,00		44	22±1,66	A

A: antagonistic effect, S: Synergistic effect.

Table 5. Inhibition zone diameters (IZDs) (mm) of *S. aromaticum* essential oil and antibiotics, alone and in combination, against tested bacteria strains.

	Antibiotics	IZD with antibiotics	IZD essential oil	Sum of the IZD with the Antibiotics and the Essential Oil	IZD association with Antibiotics and Essential Oil	Effect
K. pneumoniae ATCC 700603	AMC	13±0,00	13±0,88	26	18±0,12	A
	CAZ	06±0,00		19	12±0,49	A
	LEV	21±0,00		34	19±0,88	A
E. coli ATCC 25922	AMC	10±0,00	17±0,9	27	17±0,99	A
	CAZ	19±0,00		36	14±1,1	A
	LEV	31±0,00		48	32±1,9	A
P. aeruginosa ATCC 27853	AMC	06±0,00	08±0,10	14	08±0,63	A
	CAZ	21±0,00		29	20±0,67	A
	LEV	23±0,00		31	18±0,58	A
K. pneumoniae 3520	AMC	10±0,00	12±0,23	22	13±0,43	A
	CAZ	06±0,00		18	12±0,64	A
	LEV	24±0,00		36	21±1,69	A
K. pneumoniae 3511	AMC	13±0,00	15±0,73	28	17±0,98	A
	CAZ	06±0,00		21	14±0,57	A
	LEV	07±0,00		22	15±0,6	A
K. pneumoniae 5111	AMC	11±0,00	14±0,66	25	17±0,95	A
	CAZ	06±0,00		20	12±0,81	A
	LEV	22±0,00		36	17±0,77	A
K. pneumoniae 1216	AMC	10±0,00	14±0,13	24	16±0,59	A
	CAZ	06±0,00		20	14±0,71	A
	LEV	24±0,00		38	20±1,8	A

A: antagonistic effect, S: Synergistic effect.

Table 6. Fractional inhibitory concentration and Fractional inhibitory concentration indices of *T. algeriensis* essential oil-amoxicillin-clavulanic acid against *K. pneumoniae* ATCC 700603 *K. pneumoniae*1216 and *K. pneumoniae*3511.

	MIC essential oil alone	MIC essential oil in combination	FIC essential oil	MIC AMC alone	MIC AMC in combination	FIC AMC	FICI	Interaction type
K. pneumoniae ATCC 700603	27.1±0.32	6.775±0.56	0.25	64±0.18	8±0.67	0.125	0.375	Synergy
K. pneumoniae 1216	27.1±0.77	3.38±0.19	0.124	256±0.39	64±0.91	0.25	0.375	Synergy
K. pneumoniae 3511	13.45±0.45	1.70±0.43	0.126	64±0.74	8±0.29	0.125	0.251	Synergy

any change, plants adapt by producing specific secondary metabolites. Thus, the qualitative and quantitative composition of essential oils from the same plant will vary from one environment to another and even within the same biotope. Regarding *T. algeriensis*, our results differ significantly from those reported by other authors. Studies conducted in Algeria have shown that the EOs of this species are predominantly rich in camphor. For example, Khemkham et al (2022) (19). found that EO from the Djelfa region contained 11.9% camphor, 13.3% 1,8-cineole, and 5.3% α-pinene. Similarly, Adouane et al (2022) (20) reported that the EO from the Biskra region contained 37.29% camphor, 11.12% 1,8-cineole, 7.13% myrcene, and 5.54% borneol. A study conducted in the Laghouat region reported a predominance of carvacrol acetate (14.16%), limonene (11.49%), and α-pinene (9.26%) (21). Hazit et al (2009) (22) reported a thymol rate of 29.5% in the region of Blida (Algeria), while Labiad et al (2022) (23) reported a slightly similar content of 33.24% in Al Hoceima region (Morocco). Other authors have also identified thymol as the primary compound in EOs from both countries. Our results indicate that the EO of *T. algeriensis* from the Aghrib region (Tizi-Ouzou) belongs to the thymol chemotype, with thymol as the dominant compound. Compared to other studies, our findings show lower levels of eucalyptol (54.29%) in our EO extracted from *E. globulus*. In contrast, Boukhatem et al (2020) (24) and Assaggaf et al (2022) (25) reported higher eucalyptol content (94.03% and 90.14%, respectively) in the Lakhdaria region (Bouira, Algeria) and Larache region (Morocco). The minor compounds in this EO from Morocco include α-pinene (3.85%), β-pinene (0.62%), γ-terpinene (2.39%), α-phellandrene (0.96%), β-myrcene (0.58%), and camphene (0.48%). Selka et al (2022) (26) identified eugenol (69.14%) and β-caryophyllene (18.8%) as the primary compounds in the EO of *S. aromaticum* from Tlemcen (Algeria). In our study, we found that eugenol is the majority compound (80.46%), which is similar to the results reported by Boughendjioua (2018) (27) in the Skikda region (Algeria), where eugenol content was 80% and β-caryophyllene content was 2.9%. Several factors, including genetic and environmental factors of each plant species, can explain the qualitative and quantitative composition of EOs observed in this study. As Yang et al (2018) (28) noted, genetic, ontogenetic, morphogenetic, and environmental

factors can influence the biosynthesis and accumulation of secondary metabolites. Qaderi et al (2023) (29) similarly reported that environmental conditions such as CO_2 concentration, temperature, drought, and UV-B exposure can significantly impact the production and accumulation of secondary metabolites in plants. The variations in the composition of EOs highlight the complex interactions that various environmental factors can have on the plants, not to mention the role of bacterial and fungal endophytes or epiphytes that colonize these plants and produce secondary metabolites. Numerous studies from around the world have reported on the antibacterial properties of EOs. Mechaala et al. (2021) (30) found that *T. algeriensis* EO exhibited antibacterial effects against 3 strains of *K. pneumoniae* ESBL, with inhibition diameters ranging from 10.26 to 16.22mm and MICs ranging from 1.56 to 12.55mg/ml. Additionally, Vázquez-Ucha et al. (2020) (31) reported good activity of EOs from *Thymus zygis* and *S. aromaticum* against colistin-resistant strains of *K. pneumoniae* BMR. Kwiatkowski et al. (2022) (11) also found that compounds such as linalool, geraniol, eugenol, thymol, eucalyptol, and carvacrol exhibited bacteriostatic and bactericidal effects on strains of *K. pneumoniae* uropathogenic and carbapenemases positive. These results are consistent with our findings on the EOs of *E. globulus* (which contains primarily eucalyptol) and *S. aromaticum* (with eugenol as the major compound). Furthermore, Yang et al. (2021) (32) demonstrated the effect of linalool on pathogenic *K. pneumoniae* strains, which may also explain the action of *T. algeriensis* EO, whose second major constituent is linalool. The antibacterial activity of EOs evaluated in this investigation can be potentially attributed to the combined action of numerous components present in these oils, although in either small or average amounts. For instance, Feng et al (2022) (33) elucidated the inhibitory activity of pure geraniol against diverse strains of enterobacteria, including *E. coli* and *Salmonella typhimurium*. Sharma et al (2023) (34) also reported the suppressive effect of citral against *Pseudomonas aeruginosa* PAO1, and its capability to create a biofilm. Furthermore, Wang et al (2019) (35) studied the antibacterial activity of α-pinene against pathogenic enterobacteria such as *E. coli* and *Salmonella enterica*. Several studies have reported that the antibacterial effect of EOs can be attributed to their ability to alter the plasma membrane of bacteria, resulting in modifications of membrane permeability and the leakage of cytoplasmic proteins. This alteration interferes with the functioning of efflux pumps and cellular enzymes (36). For instance, Moo et al (2021) (37) found that eucalyptol or 1.8-cineole can increase the zeta potential and membrane permeability of *K. pneumoniae* KPC positive strains, resulting in a loss of intracellular proteins and nucleic acids, as well as an increase in oxidative stress and the peroxidation of membrane lipids. Linalool, the second major compound in the EO of thyme, also acts on *K. pneumoniae* KPC strains by inducing the loss of cytoplasmic and membrane proteins (30). Similarly, the hydroxyl group present on the C1 of the mono-terpene ring in thymol is responsible for its activity against pathogenic bacteria such as *S. aureus*, *P. aeruginosa*, and *E. coli* (38). Eugenol, a major constituent of the EO of *S. aromaticum*, has been reported to act on resistant strains of *K. pneumoniae* BMR and carbapenem by decreasing intracellular ATP concentration, intracellular pH, and hyperpolarizing the plasma membrane, thus increasing its permeability (39). Several studies have investigated the potential synergistic or additive effects between antibiotic molecules and EO compounds. For example, Kasrati et al (2016) (40) found a synergistic association between cefixime and thyme EO in inhibiting pathogenic *K. pneumoniae* strains. Yang et al (2021) (30) reported an additive effect between beta-lactam (meropenem) and linalool in inhibiting *K. pneumoniae* KPC strains. Oukil et al (2022) (41) reported a synergistic interaction between cefazolin and *T. algeriensis* EO against *K. pneumoniae* ATCC1603. Benameur et al (2018) (42) demonstrated a synergistic association between *T. vulgaris* EO and cefotaxime in inhibiting enterobacteria strains. The resistance of *K. pneumoniae* strains to combinations of β-lactamase inhibitors can be attributed to enzymatic mechanisms (ESBL), impermeability mechanisms (Ompk36 porin), or expression of efflux systems (acrAB), which reject antibiotics outside the cell (43). The study found a synergistic effect between amoxicillin and clavulanic acid, which could be explained by the constituents of *T. algeriensis* EO permeabilizing the plasma membrane or inactivating the BLSE enzymes of *K. pneumoniae* BLSE strains. This would result in *K. pneumoniae* strains becoming sensitive to the antibiotic once again. Thymol from *T. algeriensis* EO could destabilize the plasma membranes of the strains tested, allowing amoxicillin to penetrate the cell, as reported by Tian et al (2021) (44). Additionally, Farrag et al (2019) (45) obtained a synergistic effect between antibiotics (Cefoperazone, Piperacillin, Cefoperazone/sulbactam, Piperacillin/tazobactam) with thymol and gallic acid. EOs are believed to enhance the bioavailability of antibiotics within bacterial cells, leading to a decrease in MIC values. In addition, the synergistic effect observed may result from the inhibition of ESBL hydrolysis of amoxicillin-clavulanic acid. Shoeib et al (2022) (46) demonstrated the inhibition of CTX-M ESBL by methyl cinnamate, a major compound found in the EO of *Ocimum basilicum.* The mechanisms of action behind the synergistic or additive effects of combining EOs and antibiotics are still not fully understood. While membrane destabilization is the most commonly studied mechanism, it cannot explain all the observed effects. Therefore, it is premature to speculate about the future of these formulations, and further research is needed to evaluate their stability, in vivo efficacy, and toxicity. However, these combinations have the potential to address the issue of antibiotic resistance by allowing the use of lower doses of antibiotics that may have been abandoned due to side effects or resistance. EOs can also destabilize the resistance mechanisms of bacteria, making these combinations a promising approach to combat bacterial infections.

Conclusion

K. pneumoniae producing ESBL poses huge therapeutic challenges due to its multidrug resistance. Research is needed to reduce the spread of resistance, identify new ways to treat infections, and gain a better understanding of resistance mechanisms. In this context, we assessed the chemical composition of T. algeriensis, S. aromaticum, and E. globulus EOs and tested them against K. pneumoniae ESBL strains. GC-MS identified thymol, linalool, and

p- cymene as the main components of *T. algeriensis*; eucalyptol, α-pinene, aromadendrene, and pinocarveol in *E. globulus*; and eugenol and eugenol acetate in *S. aromaticum*. Results of the activity tests showed that all three EOs were active against the tested strains. The interaction tests showed a synergistic effect between amoxicillin-clavulanic acid and *T. algeriensis* EO on two *K. pneumoniae* ESBL strains. These findings suggest the potential of our EOs to inhibit multi-resistant pathogenic bacteria, as well as their synergistic interaction with antibiotics, which could be an alternative to antibiotic use alone.

Author Contributions

Conceptualization, B.K., H.A. and L.S.; methodology, M.S.B., and F.A.; validation, S.H.A., and W.M.; formal analysis, S.A., and N.A.; investigation, Y.G., and A.M.; resources, L.S. and B.K.; data curation, L.S. and M.S.B.; writing and original draft preparation, S.H.A., and A.M.; writing, review and editing, B.M., W.M., and K.H.; supervision, Y.G. All authors have read and agreed to the published version of the manuscript.

Author Contributions

Conceptualization, B.K., H.A. and L.S.; methodology, M.S.B., and F.A.; validation, S.H.A., and W.M.; formal analysis, S.A., and N.A.; investigation, Y.G., and A.M.; resources, L.S. and B.K.; data curation, L.S. and M.S.B.; writing and original draft preparation, S.H.A., and A.M.; writing, review and editing, B.M., W.M., and K.H.; supervision, Y.G. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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