

Original Research Article

Susceptibility of Extended Spectrum Beta Lactamase Producing *Klebsiella Pneumoniae* Clinical Isolates To Antibiotics and Essential Oils

ABSTRACT

Aims: To determine the susceptibility of extended-spectrum beta-lactamase (ESBL) producing *Klebsiella pneumoniae* clinical isolates to antibiotics and essential oils - *O. compactum*, *O. marjoram* and *Thymus serpyllum*, to define the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and synergistic effect between essential oils and antibiotics.

Study design: This research included 30 isolates of *Klebsiella pneumoniae* obtained from clinical material provided from the University Clinical Center Tuzla.

Place and Duration of Study: Department of Biology, Faculty of science, University of Tuzla, between September 2019 and September 2020.

Methodology: The phenotypic disk diffusion method was used to determine sensitivity profile of isolates to antibiotics and essential oils and a confirmatory CLSI test with clavulanic acid for ESBL conformation. The MIC and MBC for essential oils were determined by microdilution test, and checkerboard method was used for determination of interactions between antibiotics and tested oils.

Results: *O. compactum* and *O. majorana* essential oils showed the same antimicrobial activity with 80.0% effect on ESBL *K. pneumoniae* isolates, *Thymus serpyllum* EO showed antimicrobial activity of 60.0%. The lowest MIC value had the *O. compactum* essential oil (MIC 6 mg/ml-10.5 mg/ml), followed by the *T. serpyllum* (MIC 17.2 mg/ml-43 mg/ml), while the *O. majorana* essential oil showed MIC values in range from 11 mg/ml to 39 mg/ml.

Conclusion: The results of the study showed the exceptional sensitivity of ESBL *K. pneumoniae* clinical isolates to the essential oils from *Origanum* and *Thymus* genera, which highly suggests their potential application in the struggle against these pathogens in the future.

Keywords: Klebsiella pneumoniae, essential oils, susceptibility

1. INTRODUCTION

In this era of antibiotic resistance, *Klebsiella pneumoniae* represents one of the most concerning pathogens involved in antibiotic resistance and as such, together with other highly important multiple drug resistant (MDR) pathogens, it has been classified as an ESKAPE organisms (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella*

pneumoniae, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*) [1]. Treatment of these organisms is a deep scientific concern. Recently, a significant increase in the incidents of ESBL-related infections has been observed throughout the globe [2].

Bacterial resistance has its own characteristics and will continue to worsen if not addressed properly. A growing number of studies are based on the research of the effect of unconventional antimicrobial agents which are widely present in nature and can be used to save the mankind concerning bacterial resistance [3]. Nowadays, the field of essential oils has been widely researched and it is one of the leading trends related to the use of "natural antibiotics". Plant essential oils have been used for hundreds of years as natural medicines to combat a multitude of pathogens, including bacteria, fungi, and viruses [4].

Essential oils, also known as volatile oils, are products of the secondary metabolism of aromatic plants. Essential oils are lipophilic and complex chemical compounds with high terpenic and phenolic contents [5]. Several essential oils confer antimicrobial activity by damaging the cell wall and membrane, leading to cell lysis, leakage of cell contents, and inhibition of proton motive force [6]. It is reported that they affect bacterial biofilms specifically by interfering with quorum sensing, inhibiting the peptidoglycan synthesis or reducing cell adherence [7]. In addition, there is no evidence of the development of bacterial resistance to them.

The aim of this study was to investigate antimicrobial activity of three essential oils, *Origanum compactum*, *Origanum majorana* and *Thymus serpyllum*, against *K. pneumoniae* clinical isolates that produce extended-spectrum beta-lactamases.

2. MATERIAL AND METHODS

2.1 Bacterial isolates and essential oils

The study included thirty ESBL isolates of *K. pneumoniae*, isolated from different human specimens (urine, sputum, gastric lavage, aspirate, throat swab) in the period from 2018. to 2019., at the University Clinical Centre in Tuzla. Isolation and identification of *K. pneumoniae* were performed by standard microbiological methods [8].

Further analyses and testing were conducted in the laboratory for microbiology, at the Faculty of Natural Sciences and Mathematics in Tuzla.

For this study, three different essential oils were tested: *O. compactum*, *O. majorana* and *T. serpyllum* produced by Pranarom (B-7822 Ghislenghien, Belgique), indicating that the major components (>90%) of the essential oils *O. compactum* and *T. serpyllum* are carvacrol and thymol, while the major component of the essential oil of *O. majorana* is terpinen-4-ol. The reference strain of *K. pneumoniae* ATCC (American Type Culture Collection) 2342 was used as a control strain.

2.2 Methods

Antibiotic susceptibility testing was performed by the Kirby-Bauer disk diffusion method [9]. The following commercially available antibiotic discs were used: amoxicillin (30µg), cefalexin (30 µg), gentamicin (10 µg), amikacin (30 µg), imipenem (10 µg), piperacillin (75µg), ampicillin (10 µg), meropenem (10 µg), ciprofloxacin (10 µg), ceftazidim (30 µg), cefotaksim

(30 µg), ceftriaxone (30 µg), cefepime (30 µg) and aztreonam (30 µg) (Mast Group LTD, UK). The results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [10].

K. pneumoniae isolates resistant to cephalosporins of the third and fourth generations were further analyzed using the phenotypic methods to confirm the production of extended-spectrum beta-lactamases. The confirmation of ESBL production was performed by combined disc method [11].

The antibacterial effect of the essential oils of *O. compactum*, *O. majorana* and *T. serpyllum* was tested for ESBL *K. pneumoniae* isolates using the diffusion method according to Clinical laboratory standards institute (CLSI) guidelines [12]. Bacterial suspension inoculated on Mueller-Hinton agar was tested with 10, 20 and 50µL of each essential oil. After incubation period (35°C/24h), interpretation of the results was performed by measuring the diameters of the inhibition zones in millimetres [13].

MIC was determined by the microdilution method using microtiter plates with 96 wells in accordance with instructions provided by the CLSI [12]. For this test, a double dilution series of essential oil or antibiotic dissolved in Dimethyl Sulfoxide (DMSO), were applied on plates, in volume of 10 µL, and 90 µL of bacterial strain inoculated in Mueller-Hinton broth (MHB) were added. The final volume in each well was 100 µL, the final density of the bacterial cells was 10⁶ Colony-forming units (CFU)/ml. Concentrations of the tested essential oil ranged from 86 mg/ml to 6 mg/ml and initial antibiotic concentrations was 10 mg/ml. After incubation the bacterial growth was detected by adding 20 µL of 0.5% triphenyl tetrazolium chloride (TTC). The MIC is defined as the lowest concentration of the examined essential oils at which there is no visible bacterial growth. The minimum bactericidal concentration (MBC) matched the lowest concentration of the tested antimicrobial agent producing negative subcultures after the incubation period at appropriate temperature during 24-hour period.

2.3 FIC analysis

The synergistic effect between two antimicrobial substances was determined by the *checkerboard* method [14]. based on the previously determined MIC values. The obtained MIC values were used for the determination of the FIC (Fractional inhibitory concentration) index and for interpretation of the type of interaction between the antimicrobial agents according to the following formula:

FIC of antimicrobial agent A = MIC of agent A in combination / MIC of agent A alone

FIC of antimicrobial agent B = MIC of agent B in combination / MIC of agent B alone

(FIC index) = FIC of agent A + FIC of agent B.

3. RESULTS AND DISCUSSION

A total of 30 *K. pneumoniae* clinical isolates were tested for antibiotic susceptibility. The test results are summarized and shown in Table 1.

Table 1. Cumulative results of *K. pneumoniae* clinical isolates susceptibility to antibiotics

Class of antibiotics	Antibiotic	Sensitive		Intermediate		Resistant	
		n	%	n	%	n	%
Aminoglycosides	AK	12	40,0	0	0,0	18	60,0
	GM	4	13,3	0	0,0	26	86,0
Carbapenems	MEM	3	10,0	0	0,0	27	90,0
	IMP	20	66,6	0	0,0	10	33,3
Beta lactams	AX	0	0,0	0	0,0	30	100
	PIP	0	0,0	0	0,0	30	100
	AMP	0	0,0	0	0,0	30	100
Cephalosporins	I CEF	12	40	0	0,0	18	60,0
	III CTX	2	6,0	0	0,0	28	93,3
	IV FEP	5	16,6	0	0,0	25	83,3
	III CAZ	2	6,0	0	0,0	28	93,3
	III CRO	11	36,6	0	0,0	19	63,3
Fluoroquinolones	CIP	4	13,3	2	6,0	24	80,0

Macrolides	ATM	8	26,6	0	0,0	22	73,3
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*n – total number of isolates

*AK- amikacin, GM - gentamicin, AX - amoxicillin, MEM - meropenem, IMP - imipenem, CIP – ciprofloxacin, PIP - piperacillin, AMP - ampicillin, CEF - cefalexin, CTX - cefotaxim, FEP - cefepime, CAZ - ceftazidime, ATM - aztreonam, CRO - ceftriaxon.

Antibiotic susceptibility of *K. pneumoniae* clinical isolates showed 100% (30/30) resistance to beta lactam antibiotics amoxicillin, piperacillin and ampicillin. A high degree of resistance was recorded towards first-generation cephalosporins - 93.3% isolates were resistant to cefotaxime (28/30). Resistance was particularly expressed for third and fourth generation of cephalosporins, is 93.3% isolates were resistant to ceftazidime (28/30), 83.3% to cefepime (25/30), and 93.3% to cefotaxime (28/30). For aminoglycoside antibiotics amikacin and gentamicin, resistance of 60% (18/30) and 86% (26/30), respectively, was observed. Resistance to fluoroquinolones (ciprofloxacin) manifested 80% of isolates (24/30), and to macrolides (azithromycin) 73% (22/30). Sensitivity to carbapenems was diverse, 90.0% of isolates were resistant to meropenem (27/30), while among all tested antibiotics, the highest sensitivity isolates expressed to imipenem- 66.6% were sensitive to this antibiotic (20/30). The production of extended-spectrum beta-lactamases enzymes with combined disk method was confirmed in 30/30 tested *K. pneumoniae* clinical isolates.

Since imipenem showed the best antimicrobial effect on ESBL *K. pneumoniae* isolates, it was selected as a reference antibiotic for further tests in the microdilution method, Table 2.

Table 2. Imipenem MIC and MBC values for 10 beta lactamase positive *K. pneumoniae* isolates

Isolate	Imipenem (10 µg)	
	MIC (mg/ml)	MBC (mg/ml)
31	0,0075	0,909
33	0,0826	0,909
39	0,0075	0,909
41	0,0075	0,909
50	0,0000056	0,00068

52	0,0075	0,909
53	0,0000056	0,00068
54	0,0000056	0,00068
56	0,0075	0,909
59	0,0826	10
ATCC 2342	0,0000056	0,00068

The antibacterial activity of *O. compactum*, *O. marjorana* and *T. serpyllum* essential oils was tested on all 30 ESBL positive *K. pneumoniae* isolates, and the cumulative sensitivity results were shown in Table 3.

Table 3. Cumulative results sensitivity of ESBL isolates of *K. pneumoniae* to essential oils

Essential oil	Volume 10 µl		Volume 20 µl				Volume 50 µl					
	Sensitive		Resistant		Sensitive		Resistant		Sensitive		Resistant	
	n	%	n	%	n	%	n	%	n	%	n	%
<i>Origanum compactum</i>	0	0	30	100	23	76,6	7	23,3	24	80,0	6	20,0
<i>Origanum majorana</i>	0	0	30	100	20	66,6	10	33,3	24	80,0	6	20,0

Thymus

0 0 30 100 17 56,6 13 43,3 18 60,0 12 40,0

serpyllum

Essential oils of the *Origanum* genera had the greatest antimicrobial effect at 50 μ L of volume. 80.0% (24/30) of beta-lactamase-producing isolates of *K. pneumoniae* showed sensitivity to *O. compactum* and *O. majorana* oils. *T. serpyllum* essential oil also showed a high antimicrobial activity of 60.0% with effective results on 18/30 beta-lactamase producing isolates.

For further analysis, ten randomly selected ESBL *K. pneumoniae* isolates were tested to determine the minimum inhibitory and minimum bactericidal concentrations of the essential oils, Table 4.

Table 4. MIC and MBC of essential oils tested on 10 ESBL positive *K. pneumoniae* isolates.

Isolates	<i>O. compactum</i>		<i>O. majorana</i>		<i>T. serpyllum</i>	
	MIC	MBC	MIC	MBC	MIC	MBC
31	6.4	8,4	28,6	86	13	26
33	9,3	14	43	86	26	78
39	8,4	10,5	28,6	86	39	78
41	8,4	10,5	21,5	43	15,6	39
50	6	9,3	17,2	43	13	26
52	6.4	8,4	28,6	86	26	78
53	7	10,5	17,2	43	19,5	39
54	6,4	9,3	21,5	43	26	78

56	10,5	16,8	43	86	13	26
59	9,3	14	43	86	26	78
ATCC 2342	6	9,3	17,2	43	11,1	15,6

*Concentrations MIC and MBC are expressed in mg/ml

The minimum inhibitory concentration of *O. compactum* essential oil tested on 10 ESBL *K. pneumoniae* clinical isolates shows a range of values from 6 mg/ml to 10.5 mg/ml, for *O. marjorana* essential oil 17.2 mg/ml to 43 mg/ml, and for *T. serpyllum* essential oil, MIC values vary from 11.1 mg/ml to 39 mg/ml. For the reference strain *K. pneumoniae* ATCC 2342, the following results were obtained: *O. compactum* had the lowest MIC value of 6 mg/ml and MBK 9.3 mg/ml, for *O. marjorana* MIC value was 17.2 mg/ml, while for *T. serpyllum* the MIC value was 11.1 mg/ml.

The checkerboard method was performed on 2 ESBL *K. pneumoniae* isolates and ATCC 2342 reference strain. Due to the previous detection of imipenem as the antibiotic with the highest antibacterial activity on beta lactamase positive isolate, this strong conventional antimicrobial agent was selected for checkerboard method analysis, in combination with three essential oils *O. compactum*, *O. marjorana* and *T. serpyllum*. The obtained results are presented in Table 5.

Table 5. Synergistic effect of essential oils and imipenem tested on two ESBL *K. pneumoniae* isolates and a reference strain

Isolates and reference strain	<i>O. compactum</i>		<i>O. majorana</i>		<i>T. serpyllum</i>	
	FIC	Effect	FIC	Effect	FIC	Effect
	+		+		+	
	Imipenem		Imipenem		Imipenem	
ATCC 2342	1,61	Indifferent	2,89	Indifferent	1,11	Indifferent
31	1,54	Indifferent	1,26	Indifferent	2,18	Indifferent

FIC	Effect	FIC	Effect	FIC	Effect
1,03	Indifferent	1,41	Indifferent	1,23	Indifferent

The use of essential oils in treating various human diseases, especially infectious ones caused by multiple-resistant bacterial strains, can be an interesting alternative to synthetic medicine that can have side effects. Essential oils in combination with antibiotics can prevent the creation of the strains resistant to antibiotics. Due to the therapeutic problems associated with particularly resistant strains, essential oils can be useful in fighting infections caused by nosocomial pathogens.

The antibacterial characteristics of the essential oil from *Origanum* genera and their effect against pathogenic bacteria have been described before in many studies. Ibišević et al. (2019), tested antibacterial activity of the *O. compactum* essential oil on different Gram-positive and Gram-negative bacterial strains. This research included 75 clinical bacterial strains of *S. aureus*, *E. faecalis*, *E. coli*, and *K. pneumoniae*, and gained results confirmed its strong and significant antibacterial activity [15].

O. compactum, the essential oil with the highest antimicrobial activity against ESBL *K. pneumoniae* isolates, had the lowest MIC values (6 mg/ml to 10.5 mg/ml), then *T. serpyllum* (with MIC values from 11.1 mg/ml to 39 mg/ml), while the essential oil of *O. majorana* ranked the lowest in terms of antimicrobial activity (MIC 17.2 mg/ml to 43 mg/ml). Mohamed et al. (2018) also reported the positive effect of the *Origanum* and *Thymus* of the essential, especially *T. serpyllum* on *K. pneumoniae*, with MIC values of 9.4 mg/ml [16]. Orhan et al. (2011) examined the inhibitory effect of different types of essential oils, including the essential oils of oregano (*O. compactum* and *O. majorana*) on 10 beta-lactamase positive isolates of *K. pneumoniae* [17]. All the essential oils and their components used in that study showed incredible bacterial growth inhibition with MIC values ranging from 32 to 64 mg/ml, which is in accordance with our results.

In the study by Bedenić et al. (2015), which covers *in vitro* antibiotic susceptibility testing of *K. pneumoniae* clinical isolates that produce different types of beta-lactamase enzymes, it is noted that imipenem remains the medicine of choice for beta-lactamase positive isolates of *K. pneumoniae* because a relatively low rate of resistance to this antibiotic has been reported [18]. In our study, imipenem also showed the most effective activity and its significantly lower minimum inhibitory concentrations were determined in relation to the minimum inhibitory concentrations of all the tested essential oils. However, one of the major problems today is that many bacteria have become resistant to almost all of the available antibiotics. The strains of *K. pneumoniae* resistant to imipenem have already been described worldwide. The results of our study also show the rate of resistance to this antibiotic, which was 33.3% (10/30).

Examining the combinatory effects of essential oils and imipenem, in this research synergy was not established. On the contrary, the effect was indifferent in combinations with all three essential oils. In a study conducted by Rosato et al. it was reported that oregano oil in combination with gentamicin exhibited synergism against *B. cereus*, *B. subtilis* and one strain of *S. aureus*. In contrast, the combination with gentamicin against *E. coli*, *Acinetobacter baumannii* and another strain of *S. aureus* was less effective and more likely to be additive than synergistic [19]. A study investigating thyme oil by Van Vuuren et al. reported a synergistic effect in combination with ciprofloxacin against *S. aureus* and *K.*

pneumoniae. However, with a FIC between 0.5 and 1.0, this combination would not be classed as synergistic by other researchers [5].

The number of studies in which alternative plant agents are combined with conventional antibiotics in control of Gram-negative bacteria is still limited. Combined therapy is a relatively new concept. However, most studies involved with this matter have detected the presence of synergy *in vitro* but have not completely examined basic mechanisms of this common activity. Considering the fact that most components of essential oils have general harmful effects on cell membranes of bacteria and that most antibiotics have specific target molecules involved in synthetic processes in the cell, it is probable that synergy in most cases is a consequence of a multi-target effect.

It is known that gram-negative bacteria are more resistant to antibiotics than the gram-positive bacteria. The use of terpenes as a therapeutic alternative combined with antibiotics could amplify their competence of income to the cell. Moreover, antibiotics could permit an effective transport of the latter until reaching its bacterial cell target owing to the lipidic nature of the terpene. This type of synergism raises the question if the presence of beta-lactamases should be discouraging by a potential drastic effect of essential oils [20].

The use of essential oils in treating various human diseases caused by multiple-resistant bacterial strains can be an interesting alternative to synthetic medicine. Studies to date have indicated that these unconventional antimicrobial agents can be an interesting choice and alternative for reducing the use of antibiotics [21]. The results of our study are in accordance with this statement, indicating high susceptibility of *K. pneumoniae* beta-lactamase producing isolates to essential oils, namely 80% (24/30) to *O. compactum* and *O. majorana* and 60% (18/30) to *T. serpyllum*.

4. CONCLUSION

In this study, the susceptibility of 30 ESBL *K. pneumoniae* clinical isolates to three types of essential oils (*Thymus serpyllum*, *Origanum majorana* and *Origanum compactum*) was tested. The antibacterial activity of these unconventional antimicrobials varied among the isolates. The essential oils of *O.compactum* and *O.majorana* showed the greatest antimicrobial activity against the majority of isolates, with the susceptibility of 80% (24/30) of the tested isolates. The essential oil of *Thymus serpyllum* also showed a high antimicrobial activity of 60.0% affecting 18/30 ESBL positive isolates. Studies to date have indicated that unconventional antimicrobial agents can be an interesting choice and alternative for reducing the use of antibiotics. Since new antibiotics have not been developed in the fight against resistant enterobacteria, future research should be directed towards the use of unconventional antimicrobials such as essential oils, which can minimize resistance to antibiotics.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the

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