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Objective: Determine the susceptibility of beta-lactamase producing clinical isolates of Pseudomonas aeruginosa to antibiotics and essential oils, define the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for tested oils.

Material and Methods: This study included 120 P. aeruginosa isolates from clinical material. A disc diffusion method was used to for determination of antibiotic and essential oil susceptibility profile. For the phenotypic detection of beta-lactamase producing isolates, a disk diffusion method was used according to the CLSI guidelines with clavulanic acid. MIC was determined by microdilution test in broth. MBC was recorded after determination of MIC, it was corresponded to the lowest concentration of the essential oil yielding negative subcultures after incubation at appropriate temperature for 24 h.

Results: Of the three tested oils, Origanum compactum oil had the strongest antimicrobial effect (MIC 6.4 mg/ml - 9.3 mg / ml) on P. aeruginosa isolates followed by Thymus serpylum oil (MIC 13 mg / ml to 78 mg / ml) and Origanum majorana oil (MIC 21,5 mg/ml do 43 mg/ml).

Conclusion: Beta-lactamase producing P. aeruginosa isolates resistant to third and forth generation of cephalosporin antibiotics, showed susceptibility against tested essential oils.

INTRODUCTION

Pseudomonas aeruginosa (P. aeruginosa) is an important opportunistic clinical pathogen, causing a variety of healthcare-associated infections, such as pneumonia, sepsis, wounds, and urinary tract infections [1]. Infections caused by P. aeruginosa are often difficult to treat because of its intrinsic and acquired resistance to many commonly prescribed antimicrobial agents, eventually leading to the emergence of multidrug-resistant P. aeruginosa (MDRPA) strain Strateva [2]. P. aeruginosa has been shown to possess a high level of intrinsic resistance to most antibiotics through restricted outer membrane permeability, efflux systems that pump antibiotics out of the cell and production of antibiotic-inactivating enzymes such as beta-lactamases [3]. A great number of P. aeruginosa strains generate various classes of extended spectrum beta-lactamases (ESBLs) which allow the bacterium to tolerate against extended–spectrum cephalosporins, such as cefepime and ceftazidime and they have been reported with a developing frequency [4]. The resistance of certain clinical pathogens has reached an alarming level and shows a significant impact on the clinical treatment outcome [5]. The problem of resistance is not limited to the hospital environment. Bacterial resistance has its characteristics and will continue to deteriorate unless adequate action is taken. An increasing number of studies are based on research into the effects of unconventional antimicrobial agents, those that are widely present in nature. One of the actions to mitigate the drug-resistance problem includes the development of new antimicrobials and in this sense essential oils are being investigated for potential antibacterial activities. Many plant oils or extracts have been reported to have antimicrobial properties and this is attributed to their ability to synthesize aromatic substances, most of which are phenols or oxygen-substituted derivatives [6]. Plant essential oils have been used for hundreds of years as natural medicines to combat a multitude of pathogens, including bacteria, fungi, and viruses. Essential oils are lipophilic and complex chemical mixes with high terpenic and phenolic contents. Antibacterial activities of essential oils and their components have been widely studied over the past few years [7]. Several essential oils confer antimicrobial activity by damaging the cell wall and membrane, leading to cell lyses, leakage of cell contents, and inhibition of proton motive force [8]. In addition, there is evidence that they effectively kill bacteria without promoting the acquisition of resistance.

Due to their lipophilic character, they are able to pass through cell membranes, disrupt the different phospholipids, polysaccharides and fatty acid layers, and finally permeabilize cells causing a loss of integrity. Clove, thyme or oregano essential oils among others are also able to affect bacterial biofilms specifically by interfering with quorum sensing, inhibiting the peptidoglycan synthesis or reducing cell adherence [9]. Finally, many essential oils are relatively easy to obtain, have low mammalian toxicity, and degrade quickly in water and soil, making them relatively environmentally friendly [10].

The objective of the present study was the determination of the antimicrobial activity of three different plant Origanum compactum (O. compactum), Origanum majorana (O. majorana), and Thymus serpyllum (T. serpyllum) essential oils on drug resistant clinical isolates of P. aeruginosa.

MATERIAL AND METHODS

Bacterial strains and essential oils

One hundred and twenty bacterial isolates of P. aeruginosa, isolated from a variety of human material (urine, sputum, gastric lavage, aspirate, throat swab) in period between 2017-2018 period, identified with standard methods by CSLI guidelines, and stored at -20°C at the Microbiology department in University Clinical Centre Tuzla. The reference strain P. aeruginosa ATCC 27853 was used as a control strain. Isolates were cultivated on Cetrimide Agar (MHA, Merck, Germany). Bacterial culture was enriched in the Mueller Hinton Broth (MHB, Merck, Germany) at 35°C for 24 h before the antimicrobial susceptibility and essential oils antimicrobial activity tests.

Three different essential oils are used in this study: O. compactum, O. majorana and T. serpyllum oil. The essential oils were purchased from and certified by Pranarôm International (B-7822 Ghislenghien, Belgique). Gas chromatography coupled with mass spectrometry (GC-MS) reports, detail the major constituents, were joined with each essential oils.

Antibacterial susceptibility testing

Antibacterial susceptibility studies were carried out by Kirby and Bauer disk diffusion technique using commercially available antibiotic discs [11]. Bacterial culture in peptone water (Himedia, Mumbai, India), containing 0.5 McFarland turbidity (1 x 10^8 cfu/ mL), was swabbed in Mueller Hinton agar (MHA, Mumbai, India) plate. Antibiotic discs were placed on it by maintaining about 24 mm distance with each other. Inhibition zone diameter was measured after overnight incubation at 35°C and results were interpreted according to EUCAST guidelines [12]. Antibiotic discs used in this test: [gentamicin (10 µg), amikacin (30 µg), imipenem (10 µg), piperacillin (75 µg), meropenem (10 µg), ciprofloxacin (10 µg), ceftazidime (30 µg), cefepim (30 µg) and aztreonam (30 µg)].

Phenotypic determination of beta-lactamase producing isolates

P. aeruginosa isolates resistant to third generation of cepfalosporines, were phenotypically analyzed for beta-lactamase production. Detection test was performed to confirm the beta-lactamase positive isolates by combined disk method [13].Bacterial suspensions were prepared and spread evenly on Muller Hinton agar plates (suspension concentrations equal to 0.5 McFarland). Ceftazidime and cefepime disks (30 µg each) were applied on the plate 20 mm apart (centre-to-centre) from amoxicillin/clavulanic acid (30/10 µg) and ceftazidime/clavulanic acid (30/10 µg). Deformation of the inhibition zone around cephalosporin disks towards the central disk of amoxicillin with clavulanic acid (synergism) ceftazidime/clavulanic acid (30/10 µg) indicates that the relevant isolate was beta-lactamase producing strain.

Agar diffusion method for essential oils

Antibacterial activity of three essential oils O. compactum, O. majorana and T. serpyllum, was primarily tested against beta lactamase positive strains of P. aeruginosa using agar diffusion method according to CLSI guidelines with some modifications [14,15]. Bacteria were cultured on Luria-Bertani medium for 24h and on Cetrimide agar (Merck, Germany) overnight at 35 °C to obtain individual colonies. Then, the colonies were suspended in 0.9% sterile salin to achieve turbidity equal to 0,5 McFarland standard (1,5 x 10^8 CFU/ml). After that, Mueller Hinton agar plates (HiMedia, India) were inoculated with bacterial suspension. For each essential oil antimicrobial effect was tested at 10 µl, 20 µl and 50 µl volume. Plates were incubated at 35 °C for 24 h.

Reporting of results was carried out by measuring the diameters of the inhibitory zones in millimetres, with a transparent ruler and caliper. The zones of inhibition were interpreted by Pirvu et al [16]. The criteria for evaluating the antimicrobial value of plant extracts predict that the inhibition zone of less than 10 mm indicates the insensitivity ie resistance of the bacteria to essential oil, 10 to 15 mm indicates poor antimicrobial activity of the oil, 16 to 20 mm moderate antimicrobial activity of the extract, and the zone of inhibition of 20 mm indicates the pronounced antimicrobial activity of a given extract.

Determination of the Minimum Inhibitory (MIC) and Minimum Bactericidal Concentration (MBC) of essential oils

The minimum inhibitory concentration (MIC) for O. compactum essential oil was assessed by 96 well broth micro-dilution method in Muller Hinton Broth (MHB) as per the guidelines given by Clinical and Laboratory Standards Institute [17]. The suspensions of overnight fresh bacterial cultures were adjusted at 0.5 McFarland turbidity. The essential oil was dissolved in dimethylsulfoxide (DMSO) to render the proper dissolution with MHB. Then, a series of double

dilutions was made and 10 µL at 90 µL inoculated MHB, was introduced in a microtiter plates with 96 wells. The final volume in each well was 100 µl, final density of bacterial cells was 106 CFU/ml, and concentrations of the examined oil were in the range of 86 mg/ml to 6,4 mg/ml. Microtiter plates were incubated for 24 hours at 35 °C. Bacterial growth was detected by adding 20 µl of 0.5% aqueous triphenyl-tetrazolium chloride solution (TTC). MIC is defined as the lowest concentration of investigated essential oils in which there is no visible growth bacteria, red colour colonies at the bottom of the recess microtiter plates after adding TTC. The minimum bactericidal concentration (MBC) corresponded to the lowest concentration of the essential oil yielding negative subcultures after incubation at appropriate temperature for 24 h.

RESULTS AND DISCUSSION

One hundred and twenty clinical isolates of P. aeruginosa were tested for antibiotic susceptibility. The test results are summarized and shown in Table 1.

Table 1. Antibiotic resistance profile of P. aeruginosa

Antibiotic	Sensitive		Intermediate		Resistant	
	n	%	n	%	n	%
Amikacin- AK	89	74,1	3	2,5	28	23,3
Gentamicin- GEN	67	55,8	0	0	53	44,1
Meropenem - MER	59	49,1	7	5,8	61	50,8
Imipenem - IMP	60	50,0	4	3,3	56	46,6
Ciprofloxacin - CIP	90	75,0	0	0	30	25,0
Piperacilin - PIP	25	20,8	0	0	95	79,1
Ceftazidim - CAZ	82	68,3	0	0	28	33,6
Cefepime - FEP	78	65,0	0	0	26	31,2
Aztreonam - ATM	30	25,0	2	1,6	88	73,3

The results of antimicrobial tests (Kirby Bauer) showed that ciprofloxacin (75%) and amikacin (74,1%) were the most efficient antipseudomonal agents. Strains that showed resistance to third and fourth generation of cephalosporins, were taken for further phenotypic analysis of beta-lactamase production. The antibiotic profile of these strains is shown in Table 2.

Table 2. Phenotypic detection of beta-lactamase producing P. aeruginosa isolates

Number of isolate	CTX (30µg)	FEP (30µg)	CAZ (30µg)	ATM (30µg)	CRO (30µg)	CAZ+KL (30/10µg)	CTX+KL (30/10µg)	AUG (30µl)
1	R	S	R	S	R	S	S	S
2	R	R	S	R	R	S	S	S
3	R	R	R	R	R	S	S	S
4	R	R	R	R	S	S	S	S
5	R	R	R	R	R	S	S	S
6	R	R	S	R	S	S	S	S
7	R	S	R	S	R	S	S	S
8	R	R	R	R	R	S	S	S
9	R	R	R	R	R	S	S	S

* CTX - Cefotaxime, FEP - Cefepime CAZ - Ceftazidime, , ATM - Aztreonam, CRO - Ceftriaxon, CAZ+KL – ceftazidime with clavulanic acid, CTX+KL – cefotaxime with clavulanic acid, AUG –amoxicillin with clavulanic acid.

Only nine isolates showed deformation of the inhibition zone around cephalosporin disks towards the central disk of amoxicillin with clavulanic acid (synergism), and were identified as beta-lactamase producing strains Table 2. Deformation of the inhibition zone around cephalosporin disks towards the central disk of amoxicillin with clavulanic acid (synergism) is shown in Figure 1.

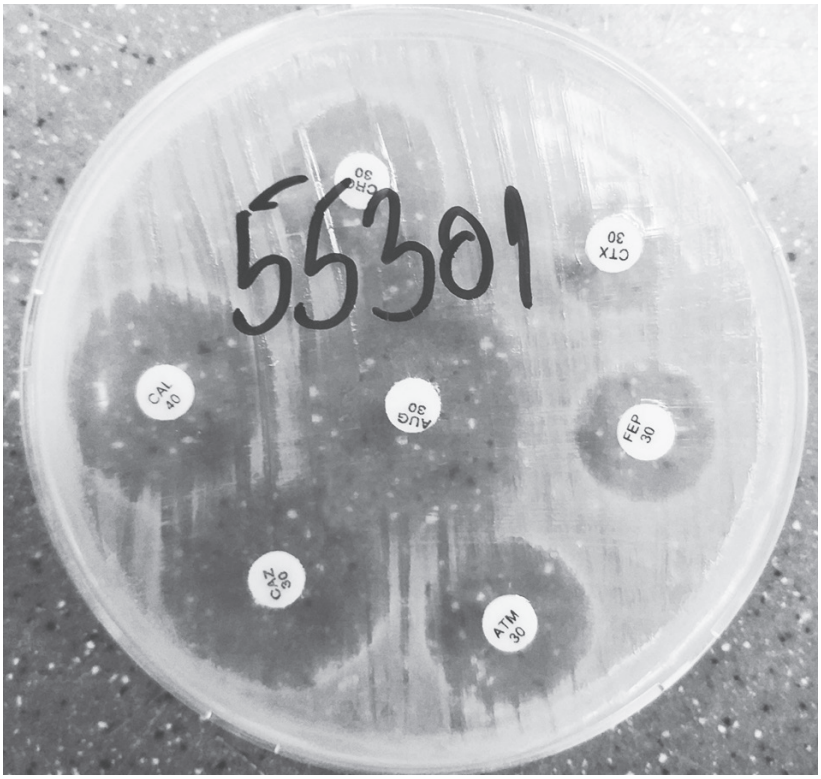

Figure 1. Deformation of the inhibition zone around cephalosporin disks towards the central disk of amoxicillin with clavulanic acid (synergism)

Due to the recorded high resistance toward the cephalosporins of third and fourth generation, 28 isolates were selected for further testing involving in vitro sensitivity to three essential oils (in a final volume of 20 and 50 µl) T. serpyllum, O. majorana and O. compactum (Table 3 and 4).

Table 3. Antimicrobial effect of essential oils on beta-lactamase producing P. aeruginosa isolates

Number of isolate	O.compactum oil	O.majorana oil	T.serpyllum oil	O.compactum oil	O.majorana oil	T.serpyllum oil
	Volume 20 µl			Volume 50 µl		
1	S	S	S	S	S	S
2	S	S	S	S	S	S
3	R	R	R	R	R	R
4	S	S	S	S	S	S
5	S	R	R	R	R	R
6	S	S	S	S	R	R
7	R	R	R	S	S	S
8	S	R	S	S	R	S
9	S	R	R	S	R	R

* S – sensitivitiy to essential oil, R - resistance to essential oil

Table 4. Antimicrobial effect of essential oils on third and fourth generation of cephalosporine resistant P. aeruginosa isolates

Number of isolate	O.compactum oil	O.majorana oil	T. serpyllum oil	O. compactum oil	O. majorana oil	T. serpyllum oil
	Volume 20 µl			Volume 50 µl		
10	S	S	S	S	S	S
11	S	R	R	S	R	R
12	S	R	R	S	R	R
13	S	R	R	S	R	R
14	S	R	R	S	R	R
15	R	R	R	R	R	R
16	R	R	R	R	R	R
17	S	R	R	S	R	R
18	S	R	R	S	R	R
19	R	R	R	R	R	R
20	S	S	S	S	S	S
21	S	R	R	S	S	S
22	R	R	R	S	S	S
23	S	S	R	S	S	R
24	R	R	R	R	R	R
25	R	R	R	S	R	R
26	S	R	R	S	R	R
27	R	R	R	R	R	R
28	S	R	R	S	R	R

* S – sensitivitiy to essential oil, R - resistance to essential oil

Sensitivity of P. aeruginosa isolates to different volumes of essential oils is presented in Table 5.

Table 5. Sensitivity of P. aeruginosa isolates to essential oils

Essential oil	Volume 20 µl				Volume 50 µl			
	Sensitive		Resistant		Sensitive		Resistant	
	n	%	n	%	n	%	n	%
Origanum compactum oil	19	67,8	9	32,1	21	75	7	25
Origanum majorana oil	7	25	21	75	9	32,1	19	67,8
Thymus serpyllum oil	6	21,4	22	78,5	8	32,1	19	67,8

A volume of 50 µl for O. compactum oil proved to be highly effective on most isolates; 21/28 isolates were sensitive (75%), while 7/28 isolates were resistant (25%), which is attributed to the high content of carvacrol and thymol, the main constituents of this essential oil [18]. A volume of 50 µl for O. majorana oil and T. serpyllum oil did not result in larger change in the number of sensitive isolates, sensitivity increased only with 2 isolates, for both essential oils (32.1%). From Table 3 and 4 it can be concluded that the antibacterial activity of these unconventional antimicrobial agents varied among the isolates and that the greatest antimicrobial effect nonetheless had the essential oil O. compactum. The results showed a dependence of antimicrobial activity on the amount of oil used in the test and, as expected, higher amounts of oil gave better results. Numerous scientific reports have highlighted an important antimicrobial activity of O. compactum essential oils. [15,19]. According Bouhdid et al., the essential oil from Origanum compactum has good antioxidant effect and a high antibacterial activity in vitro against standard reference strains of E. coli, P. mirabilis, S. aureus, L. monocytogenes, L. innocua, E. faecium and B. subtilis [19]. Species of the genus Pseudomonas (P. aeruginosa and P. fluorescens) have shown resistance to oil concentrations when used in low volumes, which is consistent with the results of our studies.

The minimum inhibitory and bactericidal concentrations of the essential oils tested for resistant P. aeruginosa strains are shown in Table 6.

Table 6. Minimum inhibitory and minimum bactericidal concentrations of essential oils

Number of isolate	Origanum compactum oil		Origanum majorana oil		Thymus serpyllum oil	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
1	7	8,4	21,5	43	39	78
2	9,3	14	28,6	86	26	78
3	R	R	R	R	R	R
4	8,4	10,5	43	86	13	26
5	R	R	R	R	R	R
6	8,4	10,5	R	R	R	R
7	8,4	10,5	R	R	R	R
8	6,4	8,4	R	R	R	R
9	8,4	10,5	R	R	R	R
23	8,4	10,5	43	86	R	R
ATCC 27853	7	8,4	14,3	21,5	13	26

*MIC - Minimum inhibitory concentrations ; MBC - minimum bactericidal concentrations; R - resistance to essential oil

MIC is defined as the lowest concentration of investigated essential oils in which there is no visible growth bacteria, red colour colonies at the bottom of the recess microtiter plates after adding TTC (Figure 2).

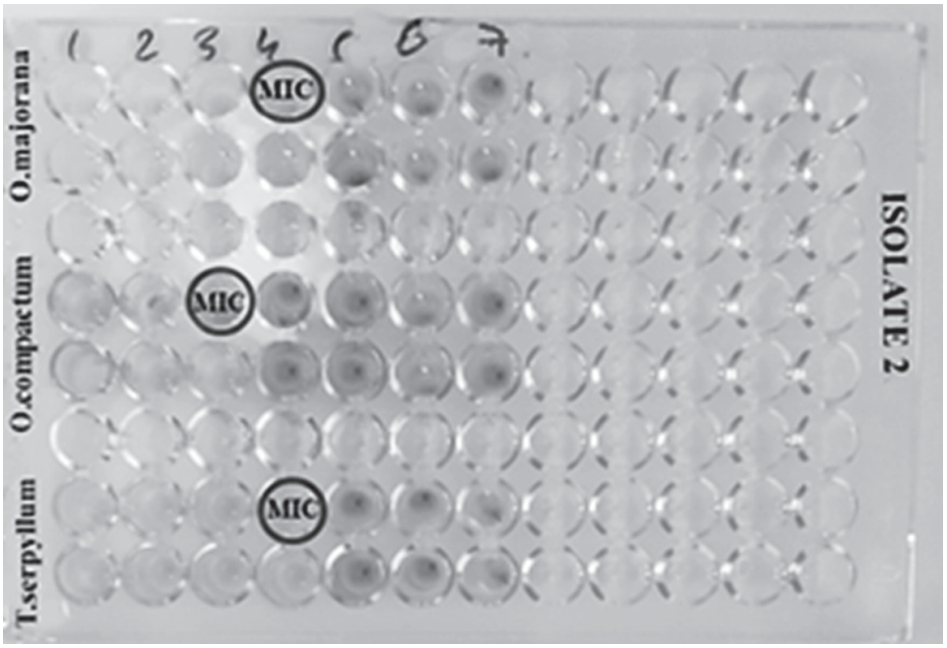

Figure 2. Dilution method for testing minimum inhibitory and bactericidal concentrations of essential oils

The minimum inhibitory concentration of O. compactum essential oil tested against 10 P. aeruginosa isolates, of which 9 was identified as beta-lactamase positive, shows a range from 6.4 mg / ml to 9.3 mg / ml. The MIC value O. majorana was ranged from 21.5 mg / ml to 43 mg / ml, while for T. serpyllum MIC values ranged from 13 mg / ml to 39 mg / ml. Results of this study showed that O. compactum oil undoubtedly poses the highest antimicrobial effectiveness against beta-lactamase producing P. aeruginosa isolates, followed by T. serpyllum oil, whiles O. majorana oil presented the weakest antimicrobial activity. The application of essential oils in the treatment of many human diseases, particularly infectious diseases caused by multidrug resistant bacterial strains, may be an interesting alternative for synthetic drugs that can have side effects. Essential oils used in combination with antibiotics might prevent antibiotic-resistant strain formation. Due to the therapeutic problems associated with particularly resistant strains, essential oils can be useful in fighting diseases caused by nosocomial pathogens.

CONCLUSION

In this research O. compactum oil undoubtedly showed the strongest antimicrobial activity against beta-lactamase producing P. aeruginosa clinical isolates with the lowest MIC values (6.4 mg/ml to 9.3 mg / ml), followed by T. serpyllum oil (MIC from 13 mg/ml to 78 mg / ml) with the last place in antimicrobial activity belonging to the essential oil of O. majorana. (21.5 mg/ml to 43 mg /ml). Such results may be related to dominant components in the essential oils, like carvacrol and thymol, as major constituents in O. compactum and T. serpyllum essential oil, relative to the essential oil O. majorana, in which the dominant component was terpinen- 4-ol. Since there are currently no new antipseudomonal antibiotics in use, attention should be focused on unconventional antimicrobial agents such as essential oils. Also, it should be considered that use of substances with many antimicrobial ingredients like essential oils, the opportunities for development of resistance are minimized.

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