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ANTIBACTERIAL EFFECT OF NATURAL OILS – AN OPPORTUNITY TO SOLVE THE PROBLEM OF ANTIBIOTIC RESISTANCE ON THE EXAMPLE OF *PSEUDOMONAS* SPP.

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Abstract: Presently, the overuse of antibiotics is a great problem all over the world. The reason for this phenomenon is both primary and secondary resistance. Primary resistance is a congenital feature of microbes and does not depend on its contact with a drug. It is chromosomally coded and cannot be transmitted to other species of bacteria. Secondary resistance, on the other hand, develops as a result of contact with the antibiotic substance. Genes located in plasmids are responsible for the formation of this type of resistance. One plasmid often contains resistance genes for several different antibiotics. Plasmids can transfer gene-encoded resistance from one bacterial cell to another by conjugation and transduction. As a result of the overuse of antibiotics in humans and animals, a growing number of infections – such as pneumonia, salmonellosis, tuberculosis, and gonorrhea – are becoming more troublesome to treat. Antibiotic resistance leads also to longer hospital stays, higher medical costs and finally increased mortality. Now people are finally becoming aware of the consequences of the overuse of antibiotics. Thus, interest in natural bacteriostatic materials, such as plant essential oils, has observably grown. A number of scientific studies have confirmed the antimicrobial activity of plant-derived essential oils against pathogenic bacteria, including *Pseudomonas aeruginosa*. A very important advantage of plant oils is the fact that they are active in low, sub-lethal concentrations, without provoking the acquisition resistance mechanisms in bacteria. The aim of this review was to explain the mechanisms of antibiotic resistance formation on the example of *Pseudomonas aeruginosa* and to demonstrate that it is worth looking for alternative treatment methods which can lead to limiting the use of antibiotics. Finally, this work tries to explain how the oils work.

1.Introduction. 2. The characteristics of *Pseudomonas* genus. 2.1. *Pseudomonas aeruginosa.* 3. The mechanisms of antibiotic resistance in *Pseudomonas* spp. 3.1. Intrinsic resistance. 3.2. Adaptive resistance. 3.3. Plasmid resistance. 4. The most common resistances of clinical *P. aeruginosa* strains to antibiotics. 4.1. Resistance to aminoglycosides. 4.2. Resistance to fluoroquinolones. 4.3. Resistance to cephalosporins. 5. Essential oils from plants as a natural alternative for antibiotics. 5.1. Antibacterial activity of plant EOs against *Pseudomonas* spp. 5.2. How EOs work on the bacteria cell. 6. Summary

PRZECIWDROBNOUSTROJOWA AKTYWNOŚĆ OLEJKÓW ETERYCZNYCH SZANSĄ ROZWIĄZANIA PROBLEMU ANTYBIOTYKOODPORNOŚCI NA PRZYKŁADZIE BAKTERII *PSEUDOMONAS* SPP.

Streszczenie: Nadużywanie antybiotyków stanowi ogromny problem na całym świecie, powodując wzrost antybiotykoodporności u patogennych bakterii. Powodem tego zjawiska jest zarówno oporność pierwotna, jak i wtórna. Oporność pierwotna jest cechą wrodzoną drobnoustrojów i nie zależy od jego kontaktu z lekiem. Kodowana jest chromosomalnie i nie może być przekazywana innym gatunkom bakterii. Oporność wtórna natomiast pojawia się w wyniku kontaktu z substancją antybiotykową. Za powstawanie tego typu oporności odpowiadają geny zlokalizowane w plazmidach. Jeden plazmid zawiera często geny oporności na kilka różnych antybiotyków. Plazmidy mogą przenosić geny kodujące oporność z jednej komórki bakteryjnej na inną na drodze koniugacji i transdukcji. W wyniku nadużywania antybiotyków u ludzi i zwierząt coraz większa liczba infekcji – takich jak zapalenie płuc, salmonelloza, gruźlica i rzeżączka – staje się coraz trudniejsza w leczeniu. Odporność na antybiotyki prowadzi również do dłuższych pobytów w szpitalu, wyższych kosztów leczenia i ostatecznie do zwiększenia śmiertelności. Obecnie ludzie zaczynają być wreszcie świadomi konsekwencji nadużywania silnych środków bakteriobójczych. Dlatego poszukuje się rozwiązań alternatywnych. Przykładem jest wykorzystanie bakteriostatycznej aktywności lejków roślinnych pochodzenia roślinnego. Wiele badań naukowych potwierdziło działanie przeciwdrobnoustrojowe olejków eterycznych pochodzenia roślinnego wobec bakterii chorobotwórczych, w tym *Pseudomonas aeruginosa*. Bardzo ważną zaletą olejów roślinnych jest fakt, że są aktywne w niskich, sub-letalnych stężeniach, bez powodowania mechanizmów oporności u bakterii. Celem niniejszej pracy było wyjaśnienie mechanizmów powstawania oporności na antybiotyki na przykładzie bakterii *Pseudomonas aeruginosa* oraz wskazanie konieczności poszukiwania alternatywnych metod terapii, które mogłyby przynajmniej częściowo przyczynić się do ograniczenia spożywania antybiotyków. Podjęto także próbę wyjaśnienia mechanizmów oddziaływania olejów na komórki bakterii.

1.Wstęp. 2. Charakterystyka bakterii z rodzaju *Pseudomonas.* 2.1. *Pseudomonas aeruginosa.* 3. Mechanizmy antybiotykooporności u *Pseudomonas* spp. 3.1. Oporność wewnętrzna 3.2. Oporność adaptacyjna. 3.3. Oporność plazmidowa. 4. Najczęstsza oporność klinicznych szczepów *P. aeruginosa* na antybiotyki. 4.1. Oporność na aminoglikozydy. 4.2. Oporność na fluorochinolony. 4.3. Oporność na cefalosporyny. 5. Roślinne olejki eteryczne – alternatywa dla antybiotyków. 5.1. Przeciwdrobnoustrojowa aktywność olejków eterycznych względem *Pseudomonas* spp. 5.2. Mechanizm oddziaływania olejków eterycznych na komórki bakterii. 6. Podsumowanie

Key words: antibacterial activity, antibiotic resistance, plants essential oils, *Pseudomonas* spp.
Słowa kluczowe: aktywność przeciwdrobnoustrojowa, oporność na antybiotyki, roślinne olejki eteryczne, *Pseudomonas* spp.

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1. Introduction

The increase in consumption of antibiotics has provoked the formation of resistance mechanisms in bacteria as a result of chromosomal changes or the exchange of genetic material via plasmids and transposons. This problem involves many pathogenic bacteria, including *Pseudomonas* spp. For instance, *P. aeruginosa* (responsible for severe nosocomial infections) is a clinically significant pathogen with confirmed resistance to multiple classes of antibiotics, such as fluoroquinolones, β-lactam antibiotics, and aminoglycosides [9, 53, 78, 81, 85]. The overuse of antibiotics in treatment and in industry will only further increase resistance, and will thus render it impossible to treat infections caused by drug resistant bacteria. Therefore, it is necessary to reduce the consumption of antibiotics and look for alternative substances with antimicrobial activity, which are not antibiotics, like essential oils from plants.

Essential oils (EOs) are in fact not oils, but they are very poorly soluble in water, and this makes them similar to oils. Plant-derived EOs can be obtained from various plant parts, including seeds, flower, buds, twigs, leaves, woods, fruits, and roots. They are usually prepared by fragrance extraction techniques such as distillation (including steam distillation), cold pressing, or extraction (maceration) [24, 89, 92]. Generally, EOs are complex mixtures of hundreds of individual aroma compounds [2]. All of these bioactive components have their own significant activity and that is why EOs acquire antimicrobial [73], antioxidant [20], antimycotic [13], antiviral [84], antiparasitic [94], antitoxicogenic and insecticidal [2] properties.

The majority of EOs are classified as GRAS (Generally Recognized As Safe). Moreover, due to their fragrance and flavour as well as antimicrobial properties, they can be used in food products [9–10, 48, 81, 88]. Although the food industry primarily uses EOs as flavourings, they represent a rich source of natural antimicrobials for foods preservation, which is currently being widely studied [4, 30, 32, 40]. The interest in EOs and their application in food preservation has been significantly amplified in recent years by the increasingly negative consumer perception of synthetic preservatives [40]. It has been demonstrated that EOs are able to inhibit the growth of some food spoilage bacteria such as *Clostridium* spp. (among others *C. botulinum*, *C. perfringens*) [46], *Bacillus* spp. (*B. stearothermophilus, B. cereus*) [29], *Lactobacillus* spp. (*L. acidophilus*) [65] and also food-related *Pseudomonas* spp. (*P. fluorescens*, *P. aeruginosa*, *P. lundensis*, *P. fragi*, *P. putida*, *P. orientalis*) [6, 49–50, 70–71].

Despite pioneering work that has elucidated the mode of antimicrobial action of a few EOs constituents in model food systems or in real food [among others 4, 30, 32], any detailed knowledge about how most of the compounds work is still lacking [40]. This knowledge is necessary to predict how EO`s work on different microorganisms cells.

The purpose of this review is to provide an overview of current knowledge about the mechanism of antibiotic resistance in bacteria on the example of the *Pseudomonas* species, antimicrobial activity as well as EOs mode of action and their constituents.

2. The characteristics of *Pseudomonas* genus

The genus *Pseudomonas* was first described by Migula in 1894 in just two concise sentences: "Cells with polar organs of motility. Formation of spores occurs in some species, but it is rare" [61]. Additional information about *Pseudomonas* was provided by Migula only in his later works. In 1895, he presented the *Pseudomonas pyocyanea* species which was renamed *P. aeruginosa* [48, 60–61].

Bacteria from the *Pseudomonas* genera were eagerly researched due to their widespread occurrence in the natural environment and capacity to grow in very simple media [72]. As a result of the study, it was demonstrated that *Pseudomonas* plays a key role in the process of mineralizing organic matter in nature. Moreover, Dooren de Jong's (1926) research introduced the methodology for the phenotypic characterization of strains, which has had a significant impact on taxonomic research on the genus for nearly 40 years [51].

The *Pseudomonas* genus represents a diverse group of bacteria, characterized by an enormous metabolic capacity which is manifested, *inter alia*, by the ability to adapt to diverse and challenging environments, the capability to synthetize a variety of low-molecular-weight biopolymers (among others, polyhydroxyalkanoates and gellan) [62, 86] and also by the ability to degrade recalcitrant compounds (among others, atrazine, chlorobiphenyl, m-toluate, polychlorinated biphenyl, nitrobenzene, 1,4-dichlorobenzene, 1,2,4-trichlorobenzene, naphthalene) [7, 15, 17, 19, 35, 42, 63, 69, 97]. Moreover, it is one of the most ecologically significant groups of bacteria – members of that genus are even present in terrestrial and marine environments and also in association with animals and plants. Such a free spread of bacteria from this species in the natural environment certainly results from its genomic diversity and genetic adaptability [42].

The cells of bacteria belonging to *Pseudomonas* genera are rod-shaped (less than 1 μm in diameter and not more than 4–5 μm in length) (Fig. 1), Gram-negative, polarly flagellated (in some species lateral flagella may also be produced) and they do not produce spores. Fimbriae pili can be observed in the cells of many species

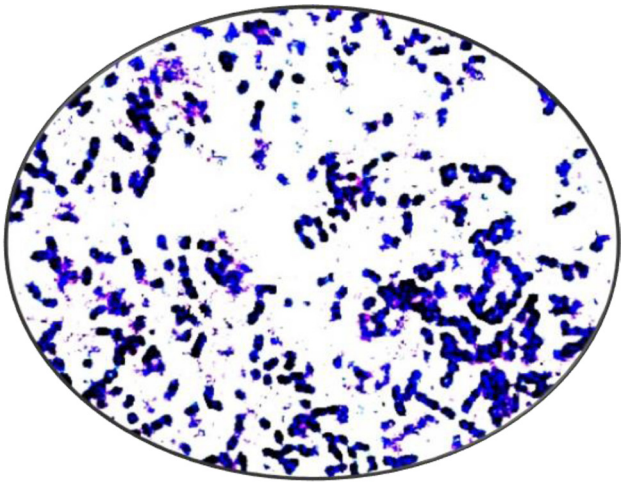

Fig. 1. ***Pseudomonas orientalis.***
The cells were coloured by crystal violet (1000x, Axiovert).

and probably all species are able to perform cell-to-cell contact, phage adsorption, attachment to cell surface and twitching motility. *Pseudomonas* strains are absolute aerobes. For many strains, nitrate can act as an electron acceptor under aerobic conditions [72].

The *Pseudomonas* genus includes many pathogenic species. However, of these *P. aeruginosa* is the most commonly associated with antibiotic resistance.

2.1. *Pseudomonas aeruginosa*

P. aeruginosa, like most Gram-negative pathogens, shows an increasing resistance to the majority of standard antibiotics used. A particularly dangerous phenomenon is the accumulation of resistance after the exposure of these bacteria to antibiotics and cross-resistance between them. This is responsible for multi-drug resistance, *inter alia*, in *P. aeruginosa*. This situation is particularly worrying because it creates a state reminiscent of the pre-antibiotic era [8].

P. aeruginosa is a common cause of health care-associated infections, including pneumonia and infections of the bloodstream, urinary tract, and surgical-site infections. Generally, more than 6,000 (13%) of the 51,000 health care-associated *P. aeruginosa* infections occurring in the United States each year are multi-drug resistant. Thus, infections caused by *P. aeruginosa* are responsible for 400 deaths per year [52]. In Europe, it is estimated that *P. aeruginosa* is responsible for 10% of all hospital-acquired infections, but this number is constantly on the rise [3, 8, 80]. The factors influencing the growing resistance in *P. aeruginosa* include the low permeability of its cell wall, the genetic capacity to express a wide repertoire of resistance mechanisms, the constitutive expression of various efflux pumps, and the production of antibiotic-inactivating enzymes. Moreover, the evidence that it can acquire additional resistance genes from other organisms via plasmids, transposons and bacteriophages is presented [48–49, 59]. Because of this complex multidrug resistance, *P. aeruginosa* is used as a model organism in research on resistance or susceptibility to synthetic or natural antibacterials [3, 5, 36, 47–49, 54, 81].

3. The mechanism of antibiotic resistance in *Pseudomonas* spp.

Antibiotics are among the most effective drugs used for human therapy. However, the synthesis of large numbers of antibiotics over the past four decades has caused complacency about the threat of bacterial resistance. Antibiotics are extensively used not only in human treatment but also for animal farming and agricultural purposes. Unfortunately, they may be released into the natural environment and pollute it. The most evident consequence of antibiotic release into the natural environments is the phenomenon of resistant bacteria [9, 58]. Antibiotic-resistant bacteria that are very difficult or even impossible to eradicate are becoming increasingly common and are posing a global health crisis [58], because the release of residues containing human microbiota into an environment containing bacteria with resistive elements significantly increases the possibility that human-linked bacteria may acquire novel resistance determinants (the contact of human microbiota with other types of microbiota in different ecosystems will increase the possibility of genetic variation and the possible emergence of novel mechanisms of resistance) [58].

Generally, the resistance of bacteria to antibiotics can be determined by genes encoded in the chromosome or/and some mobile elements such as plasmids, transposons and integrons. However, new resistance mechanisms are constantly being described, and new genes and vectors of transmission are identified on a regular basis [8, 28, 58]. Bacteria may be naturally resistant to some groups of antibiotics or they can acquire resistance due to different genetic events such as mutations or the transfer of genetic information through direct contact with cells [23, 58].

3.1. Intrinsic resistance

Microorganisms in every environment on earth are exposed to antimicrobials and therefore some species (such as *P. aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*) possess an intrinsically low susceptibility to antibiotics. Free-living opportunistic pathogens often have large genomes that allow the colonization of diverse environments through metabolic versatility that helps to degrade and resist the toxicity

of compounds present in these ecosystems. This can include large numbers of biodegradative enzymes which cooperate in the modification and utilization of antibiotics as a food resource. Additionally, efflux pumps – originally involved in signal trafficking or resistance to toxic compounds produced by plants or rhizosphere-associated microbiota – can be also used for effluxing antibiotics [41, 57, 77].

P. aeruginosa has been classified within the group of six highly antibiotic-resistant bacteria that are the primary causative agents of nosocomial (hospital-acquired) infections [66]. An understanding of how this intrinsic resistance works was not easy to come by and was tackled by Murray *et al.* [66]. Primarily, they assumed that this resistance is related to gene expression in the presence of nonlethal levels of an antimicrobial with the idea that genes which are differentially regulated by low levels of an antimicrobial will provide an immediate insight into factors important for intrinsic resistance. However, it turned out that most of the genes identified in these studies have proven not to be significant in terms of intrinsic resistance. Another idea of theirs was based on screening transposon mutant libraries for increased or decreased susceptibility to sub-MIC antimicrobials These studies revealed novel resistance determinants, although it is necessary to continue them in order to test more antibiotics and confirm all the results obtained [66].

3.2. Adaptive immunity

The exposure of susceptible *P. aeruginosa* to an aminoglycoside results in rapid drug concentration-dependent killing followed by a phase of bacterial refractoriness characterized by slow drug concentration-independent killing. This adaptive resistance, which is distinct from the post-antibiotic effect and which disappears when the organism is no longer incubated with the aminoglycoside, has been observed *in vitro*, in animal models of infection, and in patients with cystic fibrosis. This adaptive resistance is certainly not a result of mutation events. Reduced intracellular accumulation of aminoglycosides (which is concomitant to adaptive resistance) was first interpreted as the consequence of a lower drug uptake across the bacterial envelopes. Supporting this assumption, pleiotropic changes in the protein profiles of the cytoplasmic membrane were detected in drug-exposed bacteria. The membrane potential – the driving force for drug entry – appears to be marginally diminished in adaptively resistant bacteria. This finding agrees well with the observation that surviving bacteria grow normally during the post-exposure refractory phase [37]. According to Lambert [49], aminoglycosides are not able to cross the outer membrane via porin channels. They promote their own uptake by binding to the lipopolysaccharide on the outer face of the membrane. Aminoglycosides

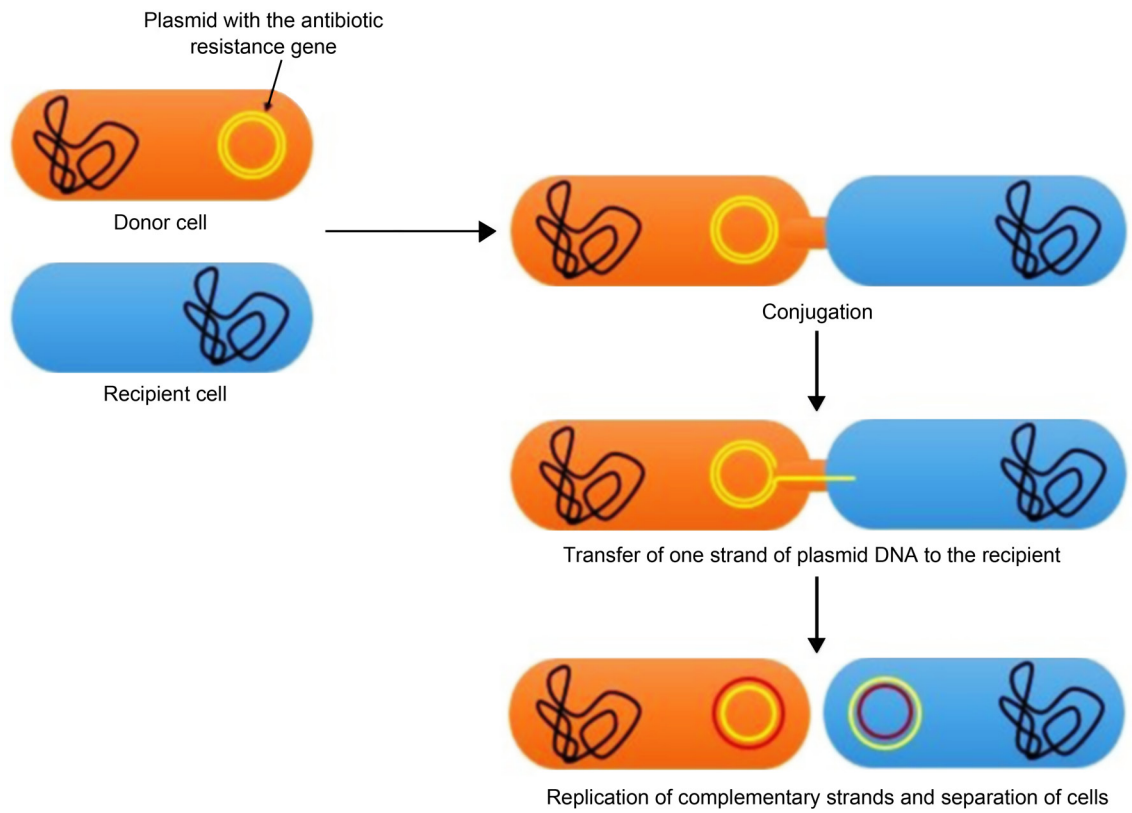

Fig. 2. Resistance arising as a result of spontaneous mutation in chromosomal DNA.

destroys the permeability barrier of the outer membrane and allows the antibiotics to penetrate through the wall to the cytoplasmic membrane. Aminoglycosides are then actively transported into the cells, where they interfere with protein synthesis at the ribosomes. Lambert [49] stated that resistance to aminoglycosides in laboratory strains of *P. aeruginosa* can be observed due to the overexpression of an outer membrane protein, OprH, which protects the lipopolysaccharides from binding the antibiotics but he underlined that this form of resistance has not been encountered widely in clinical isolates.

In clinical practice, colistin and polymyxin B are most commonly used to treat *P. aeruginosa* infections. Thus, an increase in the number of polymyxins-resistant clinical isolates is being reported. The polymyxins are antimicrobial cyclic oligopeptides that interact with the phospholipids of bacterial cell membranes and thereby lead to increased cell wall permeability and, as a result, the death of the bacteria. In *P. aeruginosa*, polymyxin resistance is strictly associated with covalent addition of 4-amino-L-arabinose to phosphate groups within the lipid A and core oligosaccharide moieties of lipopolysaccharides. Genes in the *arnBCADTEFpmrE* operon encode enzymes responsible for synthesis and transfer of L-Ara4N to lipid A. This amino-sugar modification interferes with charge interactions between phosphate groups within lipopolysaccharides and amino groups within the cyclic polymixin oligopeptide. In Gram-negative bacteria (including *P. aeruginosa*) the PmrAB and PhoPQ two-component regulatory systems stimulate transcription of the *arnBCADTEF-pmrE* operon in response to antimicrobial peptide exposure or divalent cation depletion. The sensor kinase PmrB activates the transcriptional response regulator PmrA through a phosphotransfer relay. Activation of PmrA can also occur as a consequence of *pmrB* mutation, previously observed as a cause of polymixin resistance in a laboratory strain as well as in clinical isolates [64].

3.3. Plasmid resistance

Plasmid is most commonly a fragment of double-stranded circular DNA present in bacterial cells, physically separated from chromosomal DNA, which can replicate independently of the chromosome and has the ability to move to other cells. Some of the genes carried by the plasmid have a beneficial role for the host cells. One such example is enabling the host cell to survive in an environment that would be lethal or restrictive for growth. Some of these genes encode traits for antibiotic resistance as well as resistance to heavy metals [23, 55]. These resistance plasmids can be transferred from cell to cell through the mechanisms of transformation, conjugation and transduction. Conjugation is a very rare phenomenon, probably because it involves strict requirements. The donor cell must contain a conjugative element (it can be transposon or plasmid), and donor as well as recipient cells must establish physical contact that is sufficiently stable to allow the transfer of DNA. Both cells must be metabolically active to allow DNA synthesis (Fig. 3). The transduction process requires a metabolically active donor cell in which transducing phage particles are produced during viral reproduction. The recipient can be spatially and temporarily separated from the donor, because the genetic

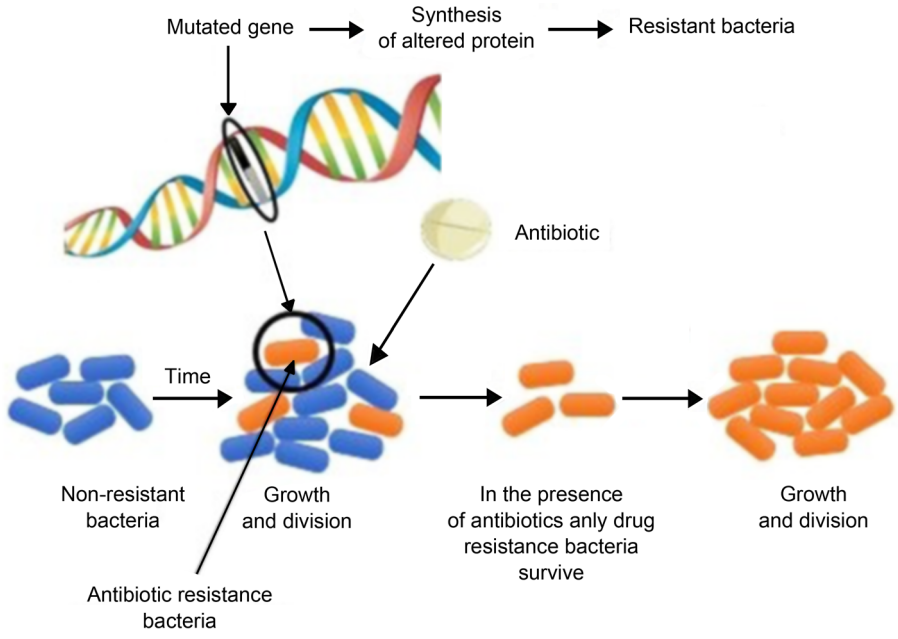

Fig. 3. Transfer of plasmids containing resistance genes from cell to cell.

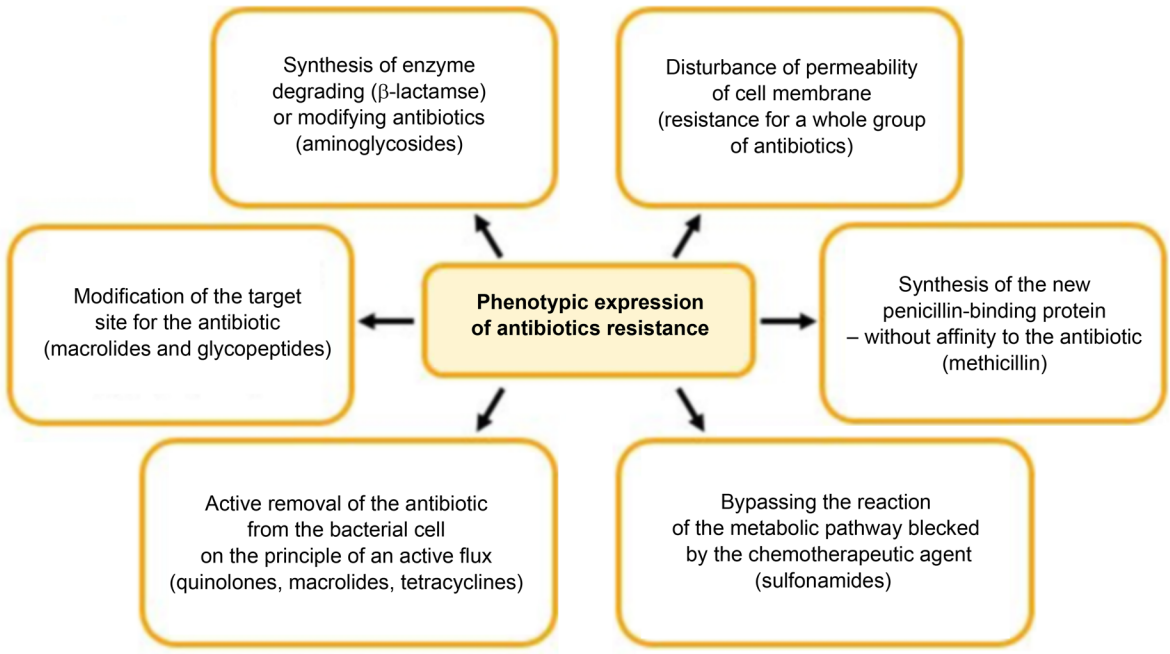

Fig. 4. Phenotypic expression of antibiotic resistance. Based on Doughari et al. [20].

information in the transducing particle can persist. In the transduction process the resistance gene can be transferred by phages because they are often resistant to many physical and chemical agents and can survive in the environment. The transformation process does not require a living donor cell, because the release of DNA during death and cell lysis is sufficient to provide free DNA. The persistence and dissemination of DNA in the environment determine how far in time and space the recipient cell can be separated from the donor. The recipient must be physiologically active to be able to adopt DNA. A close genetic relationship between donor and recipient cells is not necessary for the transfer of genes by transformation [14, 55, 93].

Studies of multiple antibiotic resistance determined by plasmids and transposons in Gram-negative bacteria (including *Pseudomonas* spp.) lead to the discovery of integrons. Integrons are genetic elements that represent an excision – an integration system involved in the capture, mobilization, and expression of gene cassettes conferring resistance to β-lactams, aminoglycosides, chloramphenicol, trimethoprim, sulfonamides, spectinomycin, streptomycin, and other antibiotics. All known integrons possess three elements essential for capturing exogenous genes: the integrase (*intI*) gene, a primary recombination site (*attI*), and a strong promoter. Gene cassettes are captured with the use of *attI* which allows recombination between the attI of the integron and a 59-nt element (also known as *attC* site) of a cassette. Unlike other excision-integration systems, integrons have multiple tandem gene cassettes inserted in one *attI* site and *intI* adjacent to *attI* rather than located in a gene cassette [43].

4. The most common resistance of clinical *P. aeruginosa* strains to antibiotics

P. aeruginosa, nosocomial pathogen, among other illnesses, are a cause of pneumonia, cystic fibrosis, meningitis, abscess, soft tissue infections, urinary tract infections, catheter associated infections, corneal infections and conjunctival erythema [15]. *P. aeruginosa* strains possess a diversity of resistance mechanisms which may lead to multi-drug or even pan-drug resistance. Standard antibiotic regimes against *P. aeruginosa* (penicillin, cephalosporins, aminoglycosides, fluoroquinolones, and carbapenems) are becoming increasingly ineffective due to the rise in drug resistance [15, 21, 23].

The mechanism of antibiotic resistance in *P. aeruginosa* is multi-factorial and includes the expression of multiple antibiotic modifying enzymes (e.g, aminoglycoside modifying enzymes, β-lactamases including extended-spectrum -lactamases and metallo-lactamases), antibiotic efflux pumps (MexAB-OprM, MexEF-OprN, MexCD-OprJ, and MexXY-OprM) as well as the acquisition of chromosomally or plasmid encoded antibiotic resistance genes. Also chromosomal mutations caused by alterations in DNA gyrase and topoisomerase IV gene and lower membrane permeability in *P. aeruginosa* can contribute to antibiotic resistance [15].

4.1. Resistance to aminoglycosides

Aminoglycosides are commonly used in the treatment of infections caused by *P. aeruginosa* such as tobramycin, gentamicin, amikacin [79]. The mecha-

nism of bacterial resistance to aminoglycoside antibiotics is very complicated and can run in three directions. In the first mode, the antibiotic can be subjected to enzymatic modification, the structure of the cellular receptor may change and the membrane permeability and transport of the antibiotic into the interior of the cell are disturbed [80, 83]. The second mechanism of bacterial resistance to aminoglycosides (characterized especially for Gram-negative bacteria, including *P. aeruginosa*) is based on the disruption of the energy-dependent antibiotic transport system to the cell receptor (ribosome 30S subunit), as a result of which the antibiotic does not reach the target site of action [80].

The third mechanism is based on a chromosomal mutation and leads to a change in the S12 protein of the ribosome 30S subunit and the loss of antibiotic binding ability [23]. Bolard *et al.* [98] documented that resistance of clinical strains of *P. aeruginosa* to aminoglycosides resulted from the production of transferable aminoglycoside-modifying enzymes, of 16S rRNA methylases, and mutational derepression of intrinsic multidrug efflux [98].

4.2. Resistance to fluoroquinolones

Fluoroquinolones have a broad range of antibacterial activity which is useful in the treatment of different diseases (e.g., urinary tract infections, gastrointestinal infections, respiratory tract infections, sexually transmitted diseases, bone and joint infections, and infections of the skin and soft tissue) [80]. The fluoroquinolones resistance genes are located in bacterial chromosome and mutations are the main mechanism of this resistance. The resistance to these antibiotics is predominantly caused by mutations in DNA topoisomerase II (gyrase) and topoisomerase IV enzymes [79]. These genes are responsible for the introduction and/or removal of supercoils in and the catenation/decatenation of DNA. Thus, they play an essential role in the replication, transcription, recombination, and repair of DNA. Gyrase is the preferred target of fluoroquinolones in Gram-negative bacteria; therefore, the resistance mutation first occurred in this enzyme. In some highly resistant isolates, additional mutations in topoisomerase IV can be observed. Both gyrase and topoisomerase are each comprised of two subunits, with fluoroquinolones resistance mutations occurring typically in the region assigned as "quinolone resistance determining region" (QRDR) of GyrA and/or ParC. Such mutations commonly occurred in fluoroquinolones-resistant *P. aeruginosa* [79].

An active efflux of the agents via antibiotic efflux pump is a typical phenomenon in *P. aeruginosa* where efflux-mediated resistance to quinolones (along with mutations in the genes of gyrase and topoisomerase) seems to predominate as a mechanism of resistance to these agents. The active efflux mechanism is due to the broad substrate specificity of the fluoroquinolones efflux systems, which are capable of accommodating a variety of clinically relevant antimicrobial agents in addition to them [80]. Four members of the Resistance Nodulation Division family of multi-drug efflux systems, MexAB-OprM, MexCD-OprJ, MexEF-oprN, and MexXY-OprM are described to accommodate fluoroquinolones. Expression of *mexAB-oprM*, controlled directly or indirectly by three repressors MexR, NalD and NalC and mutations in *mexR*, *nalC* and *nalD*, have been observed in fluoroquinolone-resistant clinical *P. aeruginosa* isolates [79].

4.3. Resistance to cephalosporins

Besides fluorquinolones, β-lactam antibiotics are most frequently applied in the treatment of bacterial infections (e.g, bacterial meningitis, otitis media, salmonellosis). During the past 30 years, emergence and dissemination of β-lactam resistance in some pathogenic strains such as *P. aeruginosa* has become a serious problem worldwide. Of particular concern is the increasing resistance to 3rd and 4th cephalosporins generation and carbapenems. Gram-negative bacteria pursue various molecular strategies for developing resistance to these antibiotics, most commonly the ability to hydrolytic cleavage of the β-lactam ring of cephalosporins by β-lactamases, antibiotic expulsion by chromosomally encoded efflux mechanisms and reduced drug uptake owing to the loss of outer membrane porin proteins [79]. In *P. aeruginosa* four molecular classes (A-D) of β-lactamases, including metal dependent (Zn^{2+} – equiring, class B) and metal-independent (active site serine, classes A, C, and D) β-lactamases have been observed (79). *P. aeruginosa* typically carries chromosomal genes for two of the β-lactamases. It acts as a class C cephalosporinase (AmpC) and a class D oxacillinase (PoxB) [79]. AmpC is a well-known β-lactamase while *PoxB* activity was only detected in lab mutants lacking AmpC. AmpC is induced by a number of β-lactam antibiotics; therefore, it contributes to the internal resistance to the number of β-lactam antibiotics. However, AmpC cannot be induced by monobactams, the anti-pseudomonal penicillin piperacillin and many of the newer cephalosporins such as cefotaxime, ceftriaxone, ceftazidime and then the resistance is dependent upon mutational derepression of *ampC* (in fact, mutational derepression of *ampC* is the most common mechanism of resistance to β-lactams in *P. aeruginosa*) [79].

The original β-lactamases were plasmid-encoded class A enzymes with limited-spectrum of activity that could only hydrolyze penicillin and an older narrow-spectrum of cephalosporins. However, there are

reports in the literature about the presence of acquired β-lactamases in *P. aeruginosa* with an extended-spectrum of β-lactamase (ESBL) enzymes (classes A and D) capable of hydrolyzing a broader range of β-lactams, including a broad spectrum of cephalosporins, monobactams and carbapenemases (classes A, B and D) that hydrolyze most β-lactams, including carbapenems (excluding aztreonam) [76, 79].

Because bacteria produce effective resistance mechanisms against antibiotics commonly available and in use, and in addition, this resistance can be passed between them, the focus should turn to a quest for alternative methods of inhibiting bacterial metabolic activity. If, however, in some cases chemical antibiotics could be replaced by natural substances, the phenomenon of antibiotic abuse could be overcome and the spread of the antibiotic resistance phenomenon may be stopped or at least slowed down. Plant oils are an example of such natural bacteriostatic substances. There are currently many *in vitro* studies on the mechanisms of their antimicrobial activity.

5. Essential oils from plants as a natural alternative for antibiotics

Essential oils are mixtures of many aromatic compounds. They include, among others, terpenes, terpenoids, phenols and tannins, which determine their biological functions, such as antimicrobial, antioxidant and anti-inflammatory activity. Natural oils are found in the cells of oil plants, namely, those containing more than 0.01% oil [26]. EOs are obtained from plants by steam distillation, cold pressing or extraction with organic solvents, as well as by a combination of these methods [11–12]. They have antimicrobial activity against many pathogenic microorganisms, including food pathogens and microbes responsible for the spoilage of food products. It is believed that due to differences in the cell wall structures of Gram-positive and Gram-negative bacteria, EOs have a weaker effect against Gram-negative bacteria, but oils containing thymol and carvacrol, for example, show comparable activity in both cases. Some EOs have a comparable or stronger antimicrobial effect than chemotherapeutics and preservatives. In addition, even long-term use of oils does not induce the immunization of microorganisms, as is the case with synthetic substances [12].

5.1. Antibacterial activity of plant EOs against *Pseudomonas* spp.

Many plant EOs are known to inhibit the growth of *Pseudomonas* strains [e.g., 3, 27, 31, 48, 56, 67, 68, 74, 75, 82, 87, 94, 96]. The antibacterial activity against some *Pseudomonas* species of oregano, rosmarinus, thymus [1], satureja [16, 74, 75] eucalyptus [74], basil, citrus, fennel, lemongrass [75] has been confirmed. Examples of the minimal inhibitory concentrations (MIC) of selected EOs with biostatic activity against bacteria from *Pseudomonas* genera are collected in Table I. A comparison of the value of MICs is impossible, because the authors used different units and in most cases the data needed to re-calculate such values are missing. However, the MIC values are generally very low. That ability of EOs to inhibit bacteria grow in low concentrations is the main advantage of using them. According to literature data, the exposure of cells to such concentrations of natural antimicrobial agents neither kills bacteria nor stops their growth and thus does not cause selective pressure or the development of resistance mechanisms in bacteria cells [74].

5.2. How EOs work on the bacteria cell

The mechanism of antimicrobial activity of EOs is closely related to the type and amount of antimicrobial-active compounds in their composition. The active ingredients of EOs are a diverse group of organic compounds with a low molecular weight [12, 34, 40, 45]. The composition of the essential oil of a given plant may vary depending on the part of the plant from which the oil was obtained (leaves, flowers, etc.), the location and conditions of cultivation, and methods of isolation [12, 33, 45]. Due to the variability of the composition of EOs, the mechanism of their action is still not fully understood [34, 40, 48].

Pseudomonas bacteria are particularly resistant to antibiotics and disinfectants because of the increased coherence of the cytoplasmic membrane, due to the higher content of lipoproteins [44, 95]. Ingredients of EOs are characterized by high lipophilicity, thanks to which they have the ability to penetrate the membrane and cell wall of microbial cells. These molecules, after penetrating the cell, disrupt its integrity [34, 45]. It should be noted that the components of EOs can eliminate bacterial plasmid antibiotic resistance by inhibiting the replication of a given plasmid, thanks to which they can also exhibit antimicrobial activity against strains resistant to previously used substances [45]. Unfortunately, several factors still limit the effectiveness of EOs on bacterial cells. It has been shown that the penetration of essential oil components into Gram-negative bacteria cells is a more complicated process than in the case of Gram-positive bacteria due to differences in the structure of their cell walls [10, 12, 34, 45]. Differences in cell structure between bacterial species and strains also determine the potency of the antimicrobial activity of a given essential oil. It has been proved that the

Table I
Minimum inhibitory concentrations of EOs against *Pseudomonas strains*

Origin of EO	Strain		Reference
	P. aeruginosa	*P. putida*	
	MIC		
Achillea millefolium	> 1000 µg/ml	N/A	85
Anethium graveolens	N/A	> 0.8% v/v	36–37
Aniba rosaeodora	> 2.0% v/v	N/A	84
Apium graveolens		> 0.8% v/v	36–37; 84
Artemisia dracunculus	N/A		36–37
Boswellia carterii	> 2.0% v/v	N/A	84
Cananga odorata			
Cedrus atlantica			
Chamæmelum nobile	N/A	> 0.8% v/v	36–37
Cinnamomum cassia		0.05% v/v	
Cinnamomum verum		0.1% v/v	
Cistus ladaniferus pineniferum			
Citrus aurantifolia	> 2.0% v/v	N/A	84
Citrus aurantium		> 0.8% v/v	36–37; 84
Citrus bergamia			
Citrus limetta	N/A		36–37; 84
Citrus limon	> 2.0% v/v		36–37; 84
Citrus x paradisi		N/A	84
Citrus reticulata	N/A	> 0.8% v/v	37
Citrus reticulata var. *madurensis*	> 2.0% v/v	N/A	84
Citrus sinensis	N/A	> 0.8% v/v	36–37
Commiphora myrrha	> 2.0% v/v	N/A	84
Coriandrum sativum		0.8% v/v	36–37
Corydothymus capitatus	N/A	0.025% v/v	
Cuminum cyminum		> 0.8% v/v	
Cucurbita pepo	> 2.0% v/v	N/A	84
Cupressus sempervirens		> 0.8% v/v	36–37; 84
Curcuma longa	N/A		36–37
Cymbopogon citratus	> 2.0% v/v	0.8% v/v	
Cymbopogon martinii		0.2% v/v	
Cymbopogon nardus		0.4% v/v	
Daucus carota		N/A	84
Eucalyptus dives	N/A	> 0.8% v/v	36–37
Eucalyptus polybractea	> 2.0% v/v	N/A	84
Eugenia caryophyllus	N/A	0.1% v/v	36–37
Foeniculum vulgare	> 2.0% v/v	N/A	84
Gaultheria procumbens			
Juniperus communis			
Laurus nobilis	N/A	> 0.8% v/v	36–37
Lavandula angustifolia	> 2.0% v/v	N/A	84
Lavandula latifolia	N/A	> 0.8% v/v	36–37
Macadamia integrifolia	> 2.0% v/v	N/A	54
Matricaria chamomilla	10.0 µg/ml		54
Melaleuca alternifolia	> 2.0% v/v	0.8% v/v	36–37; 84
Melaleuca cajuputi		> 0.8% v/v	

Table I
Continued

Origin of EO	Strain		Reference
	P. aeruginosa	*P. putida*	
	MIC		
Mentha piperita	3.0 µg/ml	N/A	54
Mentha x piperita	> 2.0% v/v		84
Mentha spicata			
Myristica fragrans	N/A	> 0.8% v/v	36–37
Myrtus communis cineoliferum			
Ocimum basilicum	> 2.0% v/v		36–37; 84
Origanum majorana		0.4% v/v	
Origanum vulgare		N/A	84
Pelargonium graveolens			
Pimpinella anisum			
Pimenta dioica	N/A	0.1% v/v	36–37
Pimenta racemosa	> 2.0% v/v	N/A	84
Pinus sylvestris		> 0.8% v/v	36–37; 84
Piper nigrum		N/A	84
Plantago major	> 1000 µg/ml		85
Pogostemon patchouli	> 2.0% v/v		84
Psidium guajava	> 1000 µg/ml		85
Punica granatum			
Rosmarinus officinalis	> 2.0% v/v	> 0.8% v/v	36–37; 84
Salvia lavandulifolia	N/A	0.8% v/v	36–37
Salvia officinalis	> 2.0% v/v	N/A	84
Salvia sclarea			
Santalum album			
Satureja hortensis	N/A	0.05% v/v	36–37
Syzygium aromaticum	> 2.0% v/v	N/A	84
Tea tree	4.0% v/v		87
Thymus vulgaris	> 2.0% v/v	0.05% v/v	36–37; 84
Vetiveria zizanioides		N/A	84
Zingiber officinale		> 0.8% v/v	36–37; 84

formation of exopolysaccharides occurring in *Pseudomonas* species causes their greater resistance to EOs than other Gram-negative bacteria [45].

The ingredients of EOs, due to their hydrophobicity, can affect the proportion of unsaturated fatty acids in the cell membrane and change their structure. The use of concentrations of oils lower than the MIC value in relation to bacteria can cause an increase in the amount of unsaturated fatty acids responsible for the fluidity of the cell membrane, which results in structural changes of the membrane [45]. Phenolic compounds contained in EOs also have a significant influence on their antibacterial activity. Treatment with thymol, carvacrol and eugenol may increase the content of saturated fatty acids while lowering the unsaturated fatty acid content in the bacterial cell membrane. This results in an increased stiffness of the cell membrane and eventually its degradation. Essential oil components may also affect the activity of enzymes responsible for the synthesis of fatty acids included in the outer layer of the cell [10, 12, 22].

Active substances in EOs can affect proteins in bacterial cells and affect cell division. These compounds also affect the expression of genes. They can affect the regulation and synthesis of, for example, enzymes or chaperone proteins, *inter alia* related to the thermal stress of the cell. The action of essential oil components in relation to cellular proteins completely disturbs the functioning of the bacterial cell [10, 12, 40, 45].

The production of ATP in bacterial cells takes place in the cytosol by glycolysis. There is a correlation between intracellular and extracellular ATP concentration. The

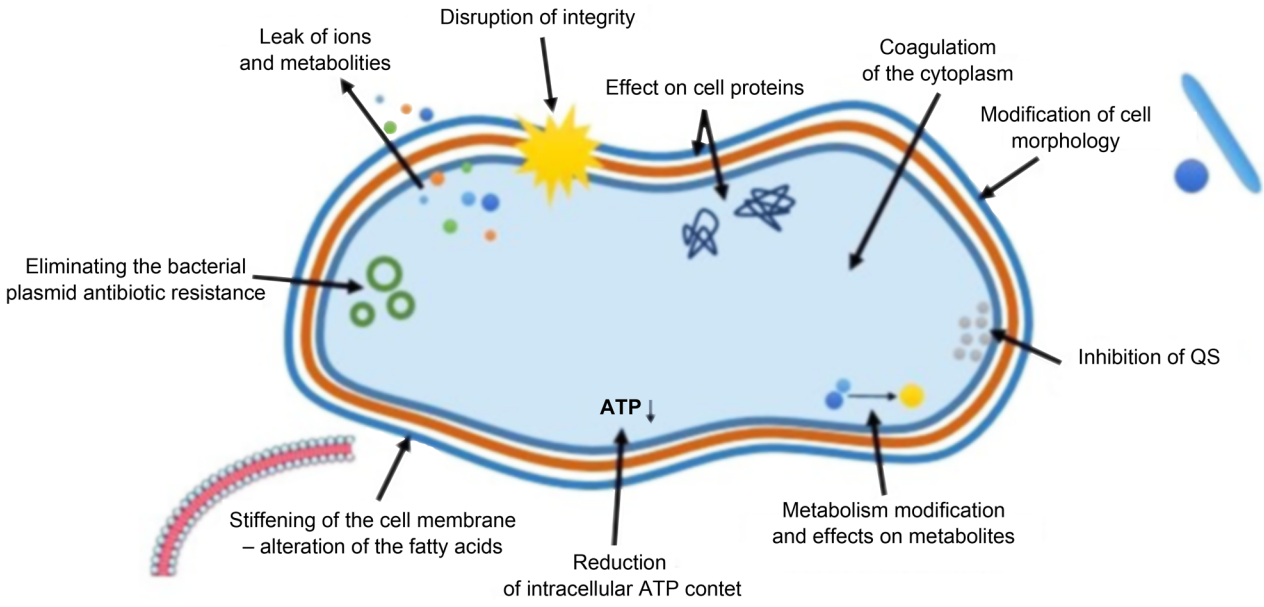

Fig. 5. The mode of antibacterial action of essential oils.

ingredients of EOs cause permeabilization of the cell membrane, thereby reducing the concentration of ATP inside the cell. Permeabilization of the cell membrane and leakage of ions (e.g. hydrogen and potassium) also causes excessive loss of inorganic phosphate, resulting in a reduction in the amount of intracellular ATP. The ATP content in the bacterial cell may also decrease due to the inhibitory activity of the oil components towards ATP-ase [10, 18, 40, 45, 48, 70]. EOs can affect bacterial cell metabolites [10, 40, 92, 95]. This effect may take place by interacting with metabolites already present in the cell or by altering metabolic pathways. This results in changes in the function of certain metabolites, as well as the synthesis of excessively small or large amounts of specific compounds. Changes in metabolic pathways may also result in the formation of new metabolites. This causes the occurrence of cellular stress and significant disturbances in the functioning of the cell [45].

EO components have the ability to modify the morphology of a bacterial cell. It has been shown that oblong-shaped cells are more sensitive to these compounds than coccoid ones. Changes in bacterial morphology are also different for Gram-positive and for Gram-negative bacteria. The action of EOs on bacteria can cause changes in the shape of cells, for example by extending or rounding them. It can be observed that bacterial cells treated with oils cease to be visibly separated from each other due to the degradation of their cell membranes. Damage to the protein-lipid membrane can lead to coagulation of the cytoplasm and a complete release of the cellular content. Changes of this type involve bacterial cell lysis [10].

The compounds included in the EOs are able to inhibit the phenomenon of Quorum Sensing (QS), which is an intercellular communication system occurring at a sufficiently high cell density. QS regulates a number of activities, such as biofilm formation, sporulation or virulence factors [19]. The expression of genes involved in QS results in the production of signalling molecules. The inhibition of the phenomenon by the components of oils takes place by inhibiting the synthesis, transport or secretion of signalling molecules. This leads to a reduction in the formation of bacterial biofilms, proteolytic activity and to the reduction of bacterial virulence [48].

EOs have multidirectional antibacterial activity, which is associated with the diversity of their composition. Each of the biologically active compounds present in the essential oil has a different mechanism of action. There is a synergy between the compounds, which makes the oils work more comprehensively than all their components separately. In general, the activity of each essential oil consists of a series of activities of individual substances or their complexes, which results, *inter alia*, in decreasing cellular respiration and lowering intracellular pH, and eventually may lead to the inhibition of bacterial cell growth or lysis [10, 12, 40, 45].

Although there are many scientific reports documenting (*in vitro*) the strong antibacterial activity of essential oils against Gram-negative and Gram-positive bacteria, there are no reports from in vivo experiments. We can only find the information that some plant oils/extracts are used in the folk medicine in Brazil to treat some bacterial infections [38]. Unfortunately, there is no data available on its form of application and we do not know the concentration of oils contained in natural medicines and how effective they are.

6. Summary

Bacterial adaptation to antibiotics through the last decades has been very successful, resulting in an increase in antibiotic resistance and posing considerable medical problems. Further overuse of antibiotics will lead to serious consequences – the number of antibiotic resistant strains of different species will increase [25]. At present, throughout the world in general (including Poland) *Enterobacteriaceae* producing the New Delhi-Metallo-beta-laktam-1 gene (NDM-1) (including *Klebsiella pneumoniae* strains assigned as New Delphi) is spreading at an alarming rate. NDM belongs to the group of metal-B-lactamases (MBL), which *Pseudomonas aeruginosa* can produce, after acquiring a gene conditioning them. Now, there is an increase in the number of isolated clinical strains that are currently producing MBL in the world. Those bacteria are resistant to all β-lactam antibiotics (apart from monobactams). They are a problem not only because of the limited possibilities to treat infections caused by them, but also due to the fact that they can convey the "super resistance gene" to other bacteria, often harmless, also transforming them into superbugs resistant to many commonly used drugs. The only way to prevent the growing problem of antibiotic resistance is to rationalize the consumption of antibiotics and use them for industrial purposes. Therefore, the task for modern scientists is to find substances with bacteriostatic and bactericidal activity (with novel targets and modes of action), against which bacteria have not developed defence mechanisms. One such trend is the use of essential oils as inhibitors of bacterial growth.

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