„CAROL DAVILA" UNIVERSITY OF MEDICINE AND PHARMACY,
BUCHAREST
DOCTORAL SCHOOL
PHARMACY

SELECTION OF NATURAL PHARMACEUTICAL SUBSTANCES CANDIDATES FOR NEW INNOVATIVE ANTI-INFECTIVE MEDICINES, WITH ANTIBACTERIAL AND ANTIBIOFILM EFFECT

PhD THESIS SUMMARY

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2024

Content

List of scientifical papers published

Articles published in ISI Q1 listed journals:

- **Răzvan Neagu**, Violeta Popovici, Lucia-Elena Ionescu, Viorel Ordeanu, Andrei Biță, Diana Mihaela Popescu, Emma Adriana Ozon și Cerasela Elena Gîrd: Phytochemical Screening and Antibacterial Activity of Commercially Available Essential Oils Combinations with Conventional Antibiotics against Gram-Positive and Gram-Negative Bacteria. Antibiotics 2024, 13, 478;

- **Răzvan Neagu**, Violeta Popovici, Lucia Elena Ionescu, Viorel Ordeanu, Diana Mihaela Popescu, Emma Adriana Ozon și Cerasela Elena Gîrd: Antibacterial and Antibiofilm Effects of Different Samples of Five Commercially Available Essential Oils. Antibiotics 2023, 12, 1191;

1. Introduction

Justification of choosing the theme

Medical therapy has been based since ancient times on medicines, using their empirically observed pharmacological effect and their psychological (placebo) effect.

Anti-infective medicines are used to treat diseases caused by bacteria, viruses, fungi, protozoa and animal parasites, being considered the most important group of medicines. Anti-infectives for systemic use are classified in the international nomenclature of medicines (Anatomical, Therapeutic, Clinical) with the ATC code J01. This is explained by the fact that infectious diseases have World Health Organization (WHO) coding group 1, being the most common and most serious diseases, often epidemic or pandemic in nature, causing the most cases of illness and death worldwide. Before the era of antibiotics over 40% of the population died from microbial infections.

The pharmacology classifies antimicrobial chemotherapeutics according to effect as follows: antibacterial, antiviral and antiparasitic. In the current list of Essential Medicines, WHO 23/2023, antimicrobials represent the most numerous group, because the infectious etiology is also very diverse and implicitly also the microbial sensitivity to anti-infective medicines. The lack of effectiveness is also compounded by bacterial resistance to antibiotics. One of the resistance and virulence mechanisms of pathogenic bacteria is the formation of biofilm, which gives them a special resistance to the immune defense and, most importantly, to anti-infective medicines, including antibiotics.

The history of anti-infective medicines is mixed with the history of medicine and pharmacy, in which phytotherapy played the main role, and the symbolic color of pharmacy remained the green of plants. Many plants, known as "medicinal" but also others, have antimicrobial or immunostimulant properties, because the plant kingdom also has to defend itself against the invasion of microbes, which are ubiquitous. There are thousands of known medicinal plants, hundreds of species are monographed in the oldest and newest Pharmacopoeias of the world, and thousands more plant species are under study, representing the largest reservoir of pharmacologically active substances, including for anti-infective medicines.

As a result, it is imperative to discover innovative anti-infective medicines, as a substitute, complementary or adjunctive treatment of antibiotic therapy, as antibacterial and/or antibiofilm.

I. The general part

1. Essential oils and interactions with antibiotics

1.1.Bacterial resistance to antibiotics – general aspects

Infectious diseases are diseases caused by pathogenic or conditionally pathogenic microorganisms that parasitize other beings. Pathogenic microbes have been and continue to be the leading cause of human morbidity being responsible for approximately 15 million deaths annually [1]. Anti-infective medicines contain antimicrobial substances, mainly chemotherapeutic: antibacterial, antiviral, antifungal and antiparasitic.

Currently, the therapy of infectious diseases caused by bacteria is a challenge for modern medicine, due to the expansion of the phenomenon of bacterial resistance to antibiotics, and the World Health Organization (WHO) launched the warning, as early as 2017, that we risk entering the post-antibiotics era [2]. The spread of antibiotic resistance is the result of their inappropriate and excessive use [3].

At the moment, many of the bacteria are resistant to several classes of antibiotics, not just one antibiotic (polypharmacoresistant bacteria) [4]. Worldwide, about 50% of infections acquired in hospitals are caused by polypharmacoresistant bacteria. The polypharmacoresistant bacteria that cause the most problems are vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus* (MRSA) strains, methicillin- and vancomycin-resistant *S. aureus* strains, extended-spectrum beta-lactamase-producing bacteria: some strains of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Helicobacter pylori*, *Klebsiella pneumoniae* and *Escherichia coli*. There are also other important polypharmacoresistant pathogens: *Mycobacterium tuberculosis*, penicillin-resistant *Streptococcus pneumoniae*, *Legionella pneumophila*, *Salmonella spp.* and *Shigella spp.* [5]. In Romania, according to statistics, 40% of infections are caused by antibiotic-resistant pathogens and they cause 1470 deaths per year [6].

Antibiotics act on bacteria through bactericidal (kills bacteria) or bacteriostatic (inhibit bacterial multiplication) mechanisms. Bacterial antibiotic resistance is the ability of microorganisms to survive and multiply in the presence of an antibiotic. Resistance can be natural or acquired [7].

Also, one of the resistance mechanisms used by bacteria is biofilm formation, which is also a virulence mechanism [8]. Unicellular microorganisms can organize themselves into quasi-multicellular structures, passing from the planktonic life form to the biofilm form. Under

the protection of the biofilm, the microbial cells become resistant to the action of antibiotics and the immune system, which makes it difficult to treat biofilm infections. Up to 80% of chronic bacterial infections are associated with biofilm formation. Because biofilm infections contribute significantly to patient mortality and healthcare costs are high, new strategies to treat these infections are needed [9].

1.2.Combating antibiotic resistance and the concept of synergism of action

An innovative approach is represented by the use of combinations and synergistic associations resulting from the use of 2 or more antibiotics, or between an antibiotic and a substance that has the ability to potentiate the pharmacological effect of the antibiotic or with an antibacterial effect through other mechanisms that act complementary, which potentiates the effect and prevents the establishment of bacterial resistance to that antibiotic. When the effect of the combination is greater than the sum of the individual effects of the components, then it is synergism of action. A combination that has an additive effect is when its effect is equal to the sum of the individual effects. If the effect of the combination is less than the sum of the individual effects of the components, then it is antagonism. Indifference is present when there are no interactions between the agents of the combination [10].

Numerous data from the specialized literature indicate that essential oils have the ability to modulate the activity of antibiotics on pathogenic bacteria.

The direct benefits of using a synergistic combination are: broadening the spectrum of antibacterial action, the possibility of reducing doses with the decrease of antibiotic toxicity, as well as decreasing the possibility of developing resistance [11].

An example of synergistic association is the use of agents capable of inhibiting the enzymes responsible for the degradation of antibiotics by inhibiting the active *sites* of these enzymes. Beta-lactamase inhibitors, such as clavulanic acid, sulbactam and tazobactam, in combination with amoxicillin, are well-known examples of this approach. The frequent and inappropriate use of clavulanic acid has led to the development of resistance to combinations of beta-lactam antibiotics and beta-lactamase inhibitors [12].

In the current context, the interest in the scientific substantiation of phytopharmaceutical formulations both as an alternative and as a complementary component of synthetic medicines therapy is a topic of scientific interest.

II. Personal contributions

2. Determination of the antibacterial and antibiofilm efficacy of 5 essential oils marketed in Romania

2.1. Working hypothesis and specific objectives

The present study, **Neagu et al.** [13], aims to explore the antibacterial and antibiofilm effects of five essential oils marketed in our country. Four of them (eucalyptus oil, rosemary oil, clove oil and peppermint oil) are registered by the EMA as HMP for human and veterinary use [14], with regularly updated monographs [15]. The results were compared with the best-known data from the specialized literature. In addition, a complex statistical analysis was performed to support the obtained results.

2.2. Materials and Method

2.2.1. Materials

All chemicals and reagents were of analytical grade. The bacterial strains tested for antibacterial and antibiofilm efficacy are representative for pathogenic bacterial species: Gram-positive (*S. aureus*) and Gram-negative (*E. coli* and *P. aeruginosa*) strains.

The antibacterial and antibiofilm effectiveness testing was carried out on five essential oils with 2-4 variants of each, marked with 1, 2, 3 and 4:

- *Origani aetheroleum* 1, 2 (oregano essential oil, OEO);
- *Eucalypti aetheroleum* 1, 2 (eucalyptus essential oil, EEO);
- *Rosmarini aetheroleum* 1, 2 (rosemary essential oil, REO);
- *Caryophylli aetheroleum* 1, 2, 3 (clove essential oil, CEO);
- *Menthae aetheroleum* 1, 2, 3, 4 (peppermint essential oil, PEO).

Broad-spectrum antibiotics were chosen as standard: Gentamicin, Streptomycin and Amoxicillin & Clavulanic Acid.

2.2.2. Method for determining antibacterial activity

The method used was adapted from the specialized literature [16,17]. It involved the cultivation of bacteria in 96-well plates with Muller-Hinton medium in contact with essential oil samples and incubating at 37^0C for 24 hours.

Sample preparation

The sample were O/W emulsions prepared with an essential oil concentration of 30% w/w; the emulsifier used was Poloxamer 407, 5% concentration in water, as previously mentioned [18]. Each emulsion was diluted with double distilled water to obtain the final concentration of each essential oil stock solution of 25 mg/ml.

Standard antibiotic solution preparation

All antibiotic solutions were prepared with double-distilled water, the final concentration of the stock solution being 0.5 mg/ml.

2.2.3. Antibiofilm activity determination method

The method was adapted from the literature [19,20] and detailed in our recently published article [21]. After incubation, bacterial biofilm production was evidenced by staining with 0.1% gentian violet after removing the culture medium, washing twice with sterile distilled water, and drying at room temperature under air flow. After removing the dye, the microplates were dried at 50^0C for 60 min. The dye incorporated into the bacterial cells that formed the biofilm was solubilized with 95% ethanol for 10 minutes under continuous stirring at 450 rpm.

2.2.4. Quantification and interpretation of antibacterial and antibiofilm activities

The antibacterial and antibiofilm effect of the essential oils was determined by reading the absorbances separately on each experimental variant using the EnSight Multimode Plate Reader. Depending on the measurement needs, the operator selected and modified the wavelengths (562 nm for the antibacterial effect and 570 nm for the antibiofilm activity). The final absorbance value is the arithmetic mean of the 100 readings per second / per well taken by the multimodal reader software [22]. The calculation formula is presented in the following equation:

$$(1) \qquad Efficacy\ \% = 100\ - \frac{\text{mean of Sample absorbance value}}{\text{Reference absorbance value}} \times 100$$

The results obtained were compared with those achieved by standard antibiotics.

2.3. Results and discussions

All the data recorded from the results are summarized in Figure 2.1, highlighting the place of the essential oils in relation to both antibacterial efficacy (*ABE*) and antibiofilm efficacy (*ABfE*) against all the bacteria tested at all three dilutions.

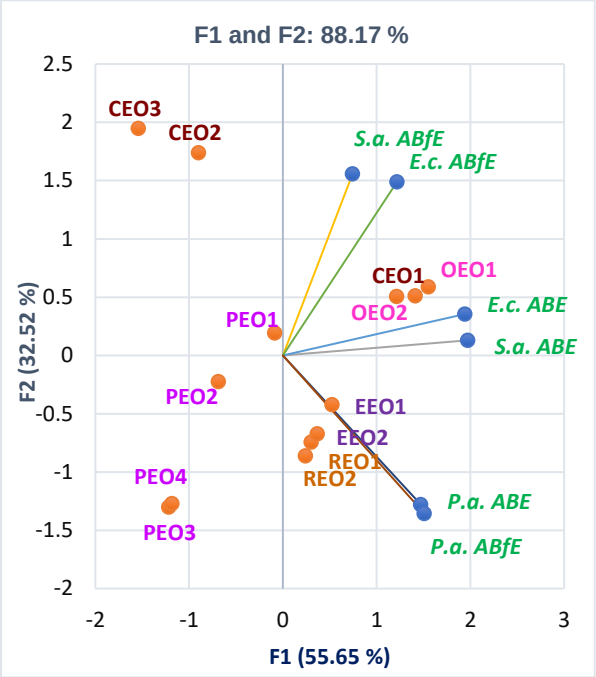


Figure 2.1. PCA-Biplot displays the antibacterial and antibiofilm efficacy of essential oils against Gram-positive and Gram-negative bacteria.

As an overview (see **Figure 2.1.**), samples from different manufacturers of the same essential oil showed similar activities; only clove and peppermint oils showed greater differences. Compared to the results of other studies, the small differences between EEO1-2 and REO1-2 samples do not seem to influence the antibacterial effects. There were appreciable differences in the chemical composition of the CEO1-3 samples and notable differences in the content of bioactive constituents in the PEO1-4 samples, resulting in large differences between the antibacterial and antibiofilm effects.

All essential oils showed antibacterial and antibiofilm activities at the first decimal dilution against all Gram-positive and Gram-negative bacteria tested and an MIC value > 25 mg/mL. *E. coli* showed the lowest susceptibility to all commercially available essential oils – for 15 essential oil samples no antibacterial and antibiofilm effects were detected at the following two dilutions.

Only essential oils with a high content of highly active metabolites showed non-significant differences at all decimal dilutions. Essential oils with many bioactive compounds in moderate content showed a substantial decrease in antibacterial potential.

3. Determination of antibacterial and antibiofilm efficacy of combinations of commercially available essential oils associated with antibiotics

3.1. Working hypothesis and specific objectives

The present study, **Neagu et al.** [23], aims to investigate the antibacterial and antibiofilm activity as well as the potential interactions for five essential oils that recorded the best antibacterial and antibiofilm efficacies in the previous testing stage - lavender essential oil (LEO), clove essential oil (CEO), oregano essential oil (OEO), eucalyptus essential oil (EEO) and peppermint essential oil (PEO) – combined in binary and triple combinations with antibiotic medicines - tetracycline (TET), neomycin (NEO) and bacitracin (BAC) tested against *S. aureus*, *E coli* and *P. aeruginosa*.

3.2. Materials and Method

3.2.1. Materials

All chemicals and reagents were of analytical grade. The bacterial strains on which the antibacterial and antibiofilm efficacy were tested, are representative of pathogenic bacterial species and were purchased from suppliers authorized to sell ATCC products: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853).

The 5 essential oils that were analyzed are: clove essential oil (CEO), eucalyptus essential oil (EEO), lavender essential oil (LEO), peppermint essential oil (PEO) and oregano essential oil (OEO) and the conventional antibiotics used are: neomycin, tetracycline hydrochloride and bacitracin.

3.2.2. GC-MS analysis

Materials and method

A gas chromatography-mass spectrometry (GC/MS) analysis was performed to identify and quantify the constituents of clove, eucalyptus, lavender, peppermint and oregano essential oils by perceptible elucidation by comparing their mass spectra with reference (NIST Library 2020). A Thermo Scientific Focus GC (Norristown, PA, USA) with an AI/AS 3000 autosampler coupled to a DSQ II mass detector and equipped with a TraceGOLD TG-624 column 60 × 0.25 × 1.4 (mm). The injection volume was 1 μL, at a flow rate of 1.3 mL/min and a split ratio of 1:50 using helium as the carrier gas. The initial oven temperature was set at

110°C and held for 5 minutes. After that, it was increased to 220°C at a rate of 2°C/min and held for 15 minutes. The temperature of the MS transfer line was maintained at 240°C. The ion source temperature was 230°C and the electron impact ionization was set at 70 eV. The spectra were analyzed in full scan mode in the mass range from 50 to 450 and the retention times (RT) of all constituents were recorded [24].

Results and discussions

All essential oil constituents were identified and quantified.

3.2.3. NMR and IR structural analysis of the tested antibiotics

Materials and method

Conventional antibiotics were structurally analyzed before entering the tests.

A Mercury 300 BB (Varian Medical Systems, Inc., Palo Alto, CA, USA) machine was used for the NMR analysis, operating at 300 MHz for ^{1}H spectra and 75,075 MHz for ^{13}C spectra. The solvent used for the solubilization of antibiotic powder samples was deuterated water (D_2O).

For the IR analysis, the Bruker Vertex 70 FT-IR device equipped with a reflection device (ATR) with a diamond crystal was used. The IR spectra were processed with OPUS 5.5 software (Bruker).

Results and discussions

NMR and IR spectra confirmed the chemical structures of neomycin, bacitracin and tetracycline hydrochloride.

3.2.4. Determination of antibacterial and antibiofilm activity through the microplate method

Materials and method

The method used was adapted from the literature [16,17] and successfully tested in the study presented in Chapter 2 [13].

Sample preparation

The samples were O/W emulsions prepared with an essential oil concentration of 30% w/w; the emulsifier used was Poloxamer 407, 5% concentration in water, as previously mentioned [13,25].

Each emulsion was diluted with double distilled water to obtain the final concentration of each essential oil stock solution of 25 mg/mL.

The binary combinations of essential oils were prepared in a 1:1 ratio.

The antibiotic medicines (neomycin, bacitracin, and tetracycline) were dissolved in double-distilled water with a final stock solution concentration of 5120 µg/mL.

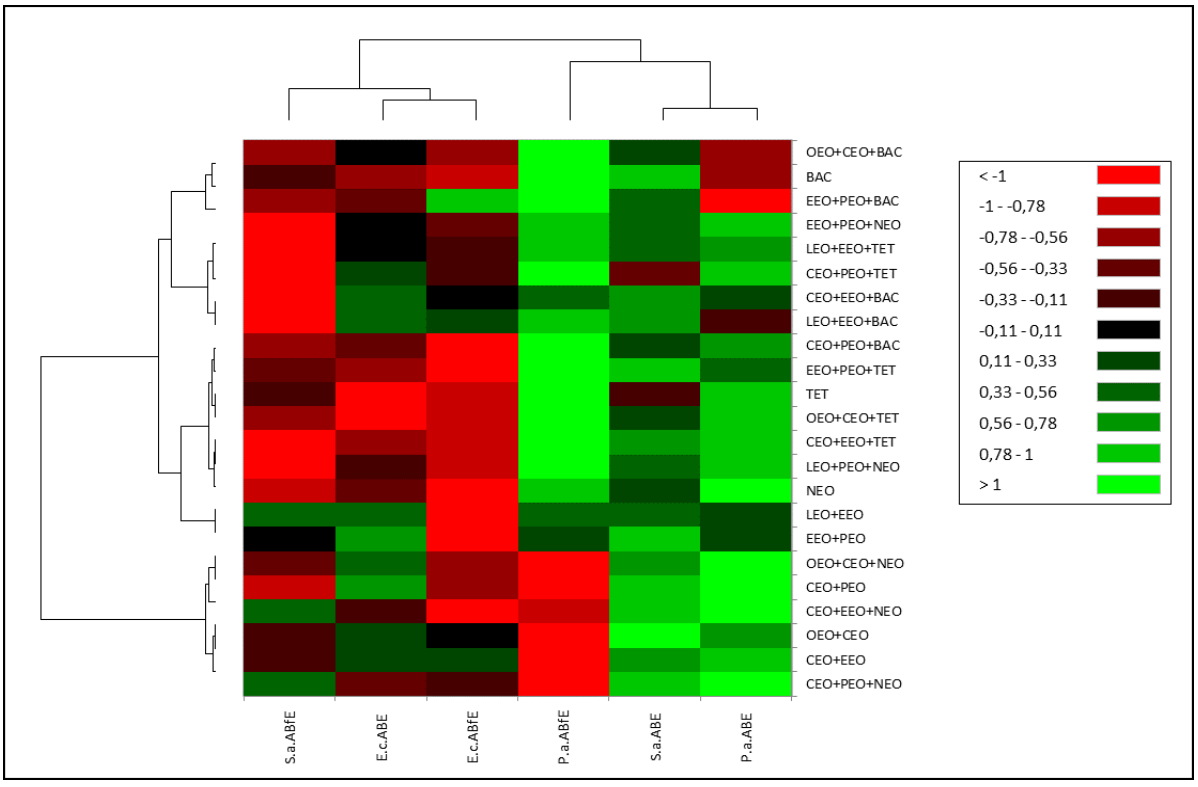
The triple combinations contained equal parts of each constituent (1:1:1).

Determination of antibacterial and antibiofilm activity

The working protocol is identical to that described in Chapter 2.

Results and discussions

With the help of the data obtained from the tests, a Heatmap was drawn up with the help of which we can observe the differences regarding the antibacterial and antibiofilm activity of essential oils in binary combinations and triple combinations (essential oils + antibiotics) as can be seen in **Figure 3.1.**



Note: LEO - lavender essential oil; CEO – clove essential oil; OEO - essential oil of oregano; EEO - eucalyptus essential oil; PEO - peppermint essential oil; NEO - Neomycin; TET - Tetracycline; and BAC - Bacitracin. Values indicating synergism of action are marked in green, and the color intensity decreases from dark green to light green (indifference), and the color intensity increases from light red (low antagonism) to dark brown (high antagonism).

Figure 3.1. Representative heatmap of the antibacterial and antibiofilm effects, expressed as a percentage, for double and triple combinations.

As we can see on the heatmap, the best antibacterial effects were achieved on *S. aureus* and *P. aeruginosa*, and the best antibiofilm effect was achieved on *P. aeruginosa* biofilm.

Regarding the antibacterial activity on *S. aureus*, *E. coli* and *P. aeruginosa*, 6 triple combinations (essential oils + antibiotics) obtained a higher antibacterial efficacy value than those obtained by binary combinations and antibiotics tested individually.

Concerning the antibiofilm activity on *S. aureus*, *E. coli* and *P. aeruginosa*, 12 triple combinations (essential oils + antibiotics) obtained a higher antibiofilm efficacy value than those obtained by the binary combinations and antibiotics tested individually.

3.2.5. In vitro evaluation of antibacterial activity

Materials and method

An *in vitro* evaluation of the antibacterial activity of the tested solutions was performed using a semi-quantitative method (adapted Kirby-Bauer diffusimetric antibiogram [26]). The results - expressed as the diameter of the IZD inhibition zone (mm) - were measured after 24 hours of contact between the tested solutions and the pathogenic bacteria. The sample preparation protocol is identical to that described in 3.2.4.

Work technique

The minimum and maximum inhibition zones diameters (IZD) were measured in mm and the arithmetic mean was calculated using one decimal place. Sterile cellulose discs of Ø 6 mm diameter were impregnated with 10 µL of sample solutions and placed on Petri dishes with culture medium. Sterile borosilicate glass cylinders with a diameter of 6 mm Ø were placed on Petri dishes with culture medium and loaded with 100 µL of sample solutions.

The Petri dishes were then incubated at 37°C and read after 24 hours. The minimum inhibitory concentration (MIC) was calculated from the arithmetic mean of the IZD. Negative controls were unimpregnated sterile cellulose discs or uncharged glass cylinders.

The indicative method for evaluating the Quantity/Effect correlation (Q/Ef index) specific to the screening of antimicrobial agents consists in the calculation of the MIC value:

$$Effect\ (MIC) = \frac{\text{Q (µg)}}{\text{V (µl)}}$$

Q – the quantity (µg) of sample solution applied.

V – the volume of the environment in which the test sample diffused and inhibited microbial multiplication.

The fractional inhibitory concentration index (FICI) was calculated for the double and triple combinations as follows:

$FICI_{AB} = (MIC_{A\ in\ combination} / MIC_{A\ alone}) + (MIC_{B\ in\ combination} / MIC_{B\ alone})$

$FICI_{ABC} = (MIC_{A\ in\ combination} / MIC_{A\ alone}) + (MIC_{B\ in\ combination} / MIC_{B\ alone}) + (MIC_{C\ in\ combination} / MIC_{C\ alone})$

Results and discussions

The calculation of MIC values from IZD values led to the assessment of the interaction between essential oils in binary combinations and essential oils and conventional antibiotics in triple combinations by analyzing each FICI index (see **Figure 3.2.**).

Our study reported a synergistic binary combination (OEO+CEO) against *P. aeruginosa*, another partial synergy (EEO+PEO) against *S. aureus* and another additive combination (CEO+PEO) against *E. coli*. Similar observations regarding these antibacterial effects are available for conventional antibiotics combined with essential oils. The previously mentioned binary combinations led to four partially synergistic triple combinations with antibiotic medicines (TET and NEO) that were revealed on *S. aureus* and *E. coli*, three containing TET.

Bacteria	*S. aureus*		*E. coli*		*P. aeruginosa*	
Sample	**SaFICI1**	**SaFICI2**	**EcFICI1**	**EcFICI2**	**PaFICI1**	**PaFICI2**
CEO+EEO	5.00	1.60	4.90	11.30	13.00	1.10
CEO+EEO+BAC	1.35		6.91	18.47		2.79
CEO+EEO+NEO	1.94	1.15	6.05	2.33	5.56	7.50
CEO+EEO+TET	3.52	8.52	1.97	2.46	2.93	3.54
CEO+PEO	6.00	2.20	1.00		1.80	
CEO+PEO+BAC	5.38		1.27			
CEO+PEO+NEO	9.33	2.52	1.11	2.16	1.29	
CEO+PEO+TET	3.09	0.74	0.92	1.59	1.49	2.62
EEO+PEO	0.80	1.40	3.70	7.00	9.80	4.20
EEO+PEO+BAC	1.25	3.54	8.14	10.51	18.35	3.22
EEO+PEO+NEO	1.66	0.53	3.78	3.93	2.56	3.68
EEO+PEO+TET	2.37	15.22	0.92	1.99	3.78	3.74
LEO+EEO	1.20	2.50	15.60	11.10	11.70	5.00
OEO+CEO	2.60	9.90	4.00	10.00	3.90	0.20
LEO+EEO+NEO	3.83	4.27	11.35	5.04	10.73	2.05
OEO+CEO+NEO	3.98	6.67	3.70			1.16
LEO+EEO+BAC	3.76	5.32	23.61	26.28		3.03
OEO+CEO+BAC	3.02	3.77	4.16	10.48		
LEO+EEO+TET	4.83	3.20	3.74	4.47	4.97	8.27
OEO+CEO+TET	4.87	1.80	4.42	4.96		14.23

Note: LEO - lavender essential oil; CEO – clove essential oil; OEO - oregano essential oil; EEO - eucalyptus essential oil; PEO - peppermint essential oil; NEO - Neomycin; TET - Tetracycline; and BAC - Bacitracin. FICI - Fractional Inhibitory Concentration Index. If IZD = 0 mm, the substance has no effect; there is no MIC. FICI ≤ 0.5 indicates synergism (S). 0.5 < FICI < 1 means partial synergism (PS); FICI = 1 indicates additive effects (AD); 1 < FICI ≤ 4—indifference (I); FICI > 4 is antagonism (ANT) [27]; 4 < FICI ≤ 10 - low antagonism; 10 < FICI ≤ 15—moderate antagonism; 15 < FICI ≤ 20 - strong antagonism; FICI > 20 - very strong antagonism. FICI1 - determined by the disc technique (DDM); and FICI2 - determined by the cylinder technique (CT). *Sa* - *S. aureus*, *Ec* - *E. coli* and *Pa* - *P. aeruginosa*. FICI values ≤ 4 are marked in green and the color intensity decreases from dark green (synergism) to light green (indifference), FICI values > 4 are marked in brown and the color intensity increases from light brown (low antagonism) to dark brown (very strong antagonism).

Figure 3.2. Heatmap of FICI values for double and triple combinations.

4. Conclusions and personal contributions

General conclusions

In the performed studies, the antibacterial/antibiofilm efficacy was analyzed by the microplate method for the five types of essential oils available on the Romanian market. 2 to 4 variants of each essential oil were used to select the essential oil variants that showed the best antibacterial/antibiofilm efficacy for the next stages of the study. Also, the results obtained by the essential oils were compared with the results of some broad-spectrum antibiotics: gentamicin, streptomycin and amoxicillin & clavulanic acid.

Before performing the associations in binary combinations (essential oil 1 + essential oil 2) and triple combinations (essential oils + antibiotics), the components were chemically analyzed. Testing of antibacterial/antibiofilm efficacies for the binary and triple combinations was performed by both the microplate method and the adapted diffusimetric antibiogram method with two techniques: the disc technique and the cylinder technique.

After the studies were carried out, the following conclusions can be identified:

- All essential oils individually tested showed antibacterial and antibiofilm effect on Gram-positive and Gram-negative bacteria with a MIC value > 25 mg/mL;
- Among the bacteria tested, *E. coli* showed the lowest susceptibility;
- Essential oils with a moderate content of bioactive compounds showed a substantial decrease in antibacterial potential with increasing dilution. In contrast, essential oils with a high content of active metabolites showed insignificant differences at all decimal dilutions;
- Samples of the same essential oil produced by different manufacturers generally showed similar activities except for PEO. The 4 PEO samples showed large variations in antibacterial and antibiofilm effects, due to the significant differences in terms of content in bioactive constituents;
- In the next stage of testing, the essential oils (OEO, CEO, EEO, PEO and LEO) from "Laboratoarele Fares Biovital SRL" (Oraștie, Romania) were selected;
- Analysis of essential oils using gas chromatography coupled to a mass spectrometer (GC/MS) identified and quantified their constituents:

  CEO: Eugenol 86.23%, Caryophyllene 6.88%, Eugenol acetate 5.75%;

EEO: Eucalyptol 83.75%, Limonene 5.45%, o-Cymene 4.13%;

LEO: Linalol 52.93%, Linalyl Acetate 32.31%, α-Pinene 3.16%;

PEO: Isomenthone 26.53%, Eucalyptol 5.05%, Limonene 2.09%;

OEO: p-Thymol 72.10%, o-Cymene 16.27%, γ-Terpinene 2.98%;

- The NMR spectra as well as the IR spectrum confirm the chemical structure of Neomycin and Tetracycline Hydrochloride. As for Bacitracin, the NMR spectra are indecipherable due to the complexity of its molecule, but the IR spectrum confirms the chemical structure of Bacitracin;
- The samples were formulated as O/W emulsions having an essential oil concentration of 30% w/w; the emulsifier used was Poloxamer 407, 5% concentration in water. Each emulsion was diluted with double distilled water to obtain the final concentration for each essential oil stock solution of 25 mg/mL. Binary combinations of essential oils were prepared in a 1:1 ratio; Antibiotic medicines (neomycin, bacitracin and tetracycline hydrochloride) were dissolved in double-distilled water with a final stock solution concentration of 5120 μg/mL. The triple combinations contained equal parts of each constituent (1:1:1). Both binary and triple combinations showed adequate stability and homogeneity before entering testing;
- Following the tests carried out through the microplate method, the best antibacterial effects were achieved on *S. aureus* and *P. aeruginosa*, and the best antibiofilm effect was obtained on the *P. aeruginosa* biofilm;
- The microplate method revealed 6 triple combinations (EEO+PEO+TET, LEO+EEO+BAC, LEO+EEO+NEO, EEO+PEO+NEO, OEO+CEO+TET and CEO+PEO+TET) with antibacterial efficacy on *S aureus*, *E. coli* and *P. aeruginosa* higher than the binary combinations and antibiotics tested individually which indicates synergism of action;
- 12 triple combinations (EEO+PEO+BAC, CEO+EEO+NEO, LEO+EEO+BAC, EEO+PEO+BAC, LEO+EEO+NEO, EEO+PEO+NEO, OEO+CEO+TET, CEO+PEO+ TET, LEO+EEO+TET, EEO+PEO+TET, OEO+CEO+BAC and EEO+PEO+BAC) with antibiofilm activity on *S. aureus*, *E. coli* and *P. aeruginosa*, higher than binary combinations of oils essential and of the individually tested antibiotics were highlighted through the microplate method, which indicates synergisms of action;

- Testing through the adapted diffusimetric antibiogram method revealed significant antibacterial activity on *S. aureus* and *P aeruginosa* of the binary combinations of essential oils, as well as the triple combinations with conventional antibiotics;
- The diffusimetric antibiogram method revealed a synergistic binary combination (OEO+CEO) against *P. aeruginosa*, a partial synergism (EEO+PEO) against *S. aureus* and an additive antibacterial action (CEO+PEO) against *E. coli*;
- The triple combinations showed partially synergistic effects: EEO+PEO+NEO on *S. aureus* and EEO+PEO+TET on *E. coli*. The triple combination CEO+PEO+TET showed partial synergism on both *S. aureus* and *E. coli*.

Personal contributions

The degree of originality of the PhD thesis consisted in:

- The original design of the first study in which five essential oils from domestic producers were selected. In the pre-screening stage, for each essential oil, 2-4 products sold in our country, supplied by Romanian companies, were analyzed. Based on the GC-MS data provided by the manufacturers, their chemical composition was analyzed in accordance with the EMA regulations. Next, the antibacterial/antibiofilm efficacy was evaluated against Gram-positive and Gram-negative bacteria known for their multiple resistance to conventional antibiotics. This was correlated with the content of active constituents for each sample analyzed separately and thus the most active essential oils produced in Romania were selected
- The bioactive constituents in the selected essential oils were quantified by GC-MS analysis using internationally recognized standards. At the same time, the chemical structure of the tested antibiotics was confirmed with the help of NMR and IR spectra;
- The selected essential oils were associated in binary combinations as well as in triple combinations with conventional antibiotics that have been very little analyzed so far in the specialized literature accessed;
- Testing of antibacterial/antibiofilm efficacies for the binary and triple combinations was performed by both the microplate method and the adapted diffusimetric antibiogram method with two techniques: the disc technique and the cylinder technique;
- The multitude of methods used gives accuracy to the results obtained.

Research perspectives

The results obtained from the studies carried out during the doctoral thesis justify the continuation of the research in the following directions:

- The association in triple combinations of newer generation antibiotics used in the treatment of skin infections;
- Expanding research on other species of Gram-positive and Gram-negative bacteria;
- Evaluation of the antibacterial and antibiofilm effectiveness of the triple combinations through *in vivo* methods (experimental animal models);
- Evaluation of the toxicity of the triple combinations through *in vivo* methods (experimental animal models);
- Incorporation of the triple combinations in a pharmaceutical form with an increased bioavailability for cutaneous administration;
- Confirming the antibacterial/antibiofilm efficacy and establishing the safety profile of the triple combinations through clinical trials.

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