Biointerface Research in Applied Chemistry
Open-Access Journal (ISSN: 2069-5837)

Article
Volume 13, Issue 4, 2023, 301
https://doi.org/10.33263/BRIAC134.301

Investigation of the Antibiofilm Activity of Some Spices and Medicinal Plants Essential Oils

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Scopus Author ID 55036488700
Received: 5.07.2022; Accepted: 2.09.2022; Published: 8.09.2022

Abstract: Biofilms are the structure microbial that attach to different surfaces and enclave microorganisms in a matrix composed, protecting them from harsh conditions, including immune effectors and antibiotics. Therefore, this study aimed to assess the anti-biofilm activity of lavender, cloves, eucalyptus, rosemary, thyme, and oregano commercial essential oils, known for their biocidal properties against Gram-positive and Gram-negative ESKAPE isolates, exhibiting multiple drug resistance. The most efficient proved to be the oregano, thyme, and clove essential oils, which exhibited the lowest minimal biofilm eradication concentrations values against all bacterial species. Although lavender, eucalyptus, and rosemary had a weaker effect than the first three oils, they have also inhibited biofilm development at concentrations low enough to be considered effective. This experimental approach may open new perspectives for developing efficient strategies to combat the emergent threat of antibiotic resistance and to control the formation of microbial biofilms, being also a valuable source of bioactive compounds and new anti-biofilm drugs.

Keywords: biofilms; volatile oils; nosocomial infections; minimal biofilm eradication concentration; anti-biofilm.

1. Introduction

Biofilms are communities of microorganisms attached to a surface and play a significant role in the persistence of bacterial infections. Microorganisms become resistant to antimicrobial treatments, different harsh environmental conditions, and, most important, change immunity. Biofilm formation greatly enhances bacteria's survival and favors chronic infections that result in persistent inflammation and tissue damage. ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) show a significantly increasing percentage of resistant clinical isolates involved in nosocomial infections and an extraordinary ability to develop biofilms on the inert and cellular substrate [1]. These strains are frequently isolated from Romanian hospitals and were proven to harbor multiple (adherence, biofilm formation, virulence, antibiotic susceptibility) and genetic determinants of virulence and resistance. In a previous study, *S. aureus* strains isolated from a Romanian hospital exhibited the highest capacity to produce soluble virulence factors and to develop biofilms *in vitro*, with

significant differences between methicillin-resistant and methicillin-susceptible isolates. Among enterobacterial isolates, *K. pneumoniae* strains expressed the highest capacity to develop biofilms. Moreover, all ESKAPE strains adhered to the cellular substrate, showing various adherence patterns. *A. baumannii*, a worldwide emerging nosocomial pathogen, is often extended drug-resistant. A recent study performed on *A. baumannii* strains isolated from Romanian hospitals proved to remain susceptible only to colistin, demonstrating the high diversity of resistance and virulence genes, as well as of mobile genetic elements [2, 3].

Therefore, novel anti-biofilm strategies are urgently required to fight the associated infections and the negative impact of biofilm development in other fields of human activity [4]. Some medicinal plants and vegetal extracts, such as lavender, eucalyptus, and rosemary essential oils, show promising potential for treating biofilm-associated pathologies [5, 6]. Many medicines also contain compounds isolated directly from plants or modified versions of natural products. Volatile oils, or essential oils, are secreted by specific secretory cells and tissues. Volatile oils are characterized by volatility, fat solubility, and water vapor trainability having disinfectant, antispasmodic, antiemetic, stomachic, and carminative properties. From vegetable products, volatile oils can be obtained by entrainment (distillation) with water vapor, volatile apolar solvents, fats, or by pressing.

Therefore, this study aimed to assess the anti-biofilm activity of lavender, cloves, eucalyptus, rosemary, thyme, and oregano essential oils, previously reported antibacterial, bacteriostatic, and antiseptic properties [7].

2. Materials and Methods

The study included 38 bacterial strains, both Gram positive and Gram negative, isolated from patients admitted to the Cardiovascular Surgery section of a hospital in Bucharest.

The analyzed strains belonged to *Klebsiella* spp. (16 strains), *Acinetobacter* spp. (12 strains), *Staphylococcus* spp. 5 strains) and *Pseudomonas* spp. (5 strains). The sources of isolation were specified in Table 1.

Table 1. Clinical origin of the tested bacterial strains.

Bacterial strains	Source of isolation	Antibiotic resistance markers
S. aureus 1	Blood culture	P, CLD, T, E
S. aureus 2	Nasal swab	P, CLD, T, E
S. aureus 3	Wound secretion	P, CLD, T, E
S. aureus 4	Nasal swab	P, CLD, T, E
S. aureus 5	Nasal swab	P, CLD, T, E
P. aeruginosa 1	Tracheal secretions	CEFT, CEFE, CEFO, TZP, TIC, MEM, IMI, DORI, TOB, CIP, LEV, MINO, TIGE
P. aeruginosa 2	Central venous catheter	CEFT, CEFE, CEFO, TZP, TIC, MEM, IMI, DORI, TOB, CIP, LEV, MINO, TIGE
P. aeruginosa 3	Wound secretion	CEFT, CEFE, CEFO, TZP, TIC, MEM, IMI, DORI, TOB, CIP, LEV, MINO, TIGE,
P. aeruginosa 4	Urine culture	CEFT, CEFE, CEFO, TZP, TIC, MEM, IMI, DORI, TOB, CIP, LEV, MINO, TIGE
P. aeruginosa 5	Blood culture	CEFT, CEFE, CEFO, TZP, TIC, MEM, IMI, DORI, TOB, CIP, LEV, MINO, TIGE
A. baumannii 835	Tracheal secretions	CEFT, CEFE, CEFO, TZP, TIC, MEM, TOB, CIP, LEV, MINO, TIGE, TRIS
A. baumannii Mary-430	Wound secretions	CEFT, CEFE, CEFO, TZP, TIC, MEM, TOB, CIP, LEV, MINO, TIGE, TRIS
A. baumannii 510	Wound secretions	CEFT, CEFE, CEFO, TZP, TIC, MEM, TOB, CIP, LEV, MINO, TIGE, TRIS
A. baumannii 608	Wound secretions	CEFT, CEFE, CEFO, TZP, TIC, MEM, TOB, CIP, LEV, MINO, TIGE, TRIS

Bacterial strains	Source of isolation	Antibiotic resistance markers
A. baumannii 608	Wound secretions	CEFT, CEFE, CEFO, TZP, TIC, MEM, TOB, CIP, LEV, MINO, TIGE, TRIS
A. baumannii 1468	Tracheal secretions	CEFT, CEFE, CEFO, TZP, TIC, MEM, TOB, CIP, LEV, MINO, TIGE, TRIS
A. baumannii 588	Tracheal secretions	CEFT, CEFE, CEFO, TZP, TIC, MEM, TOB, CIP, LEV, MINO, TIGE, TRIS
A. baumannii 513	Tracheal secretions	CEFT, CEFE, CEFO, TZP, TIC, MEM, TOB, CIP, LEV, MINO, TIGE, TRIS
A. baumannii 629	Tracheal secretions	CEFT, CEFE, CEFO, TZP, TIC, MEM, TOB, CIP, LEV, MINO, TIGE, TRIS
A. baumannii 701	Tracheal secretions	CEFT, CEFE, CEFO, TZP, TIC, MEM, TOB, CIP, LEV, MINO, TIGE, TRIS
A. baumannii 822	Wound secretions	CEFT, CEFE, CEFO, TZP, TIC, MEM, TOB, CIP, LEV, MINO, TIGE, TRIS
A. baumannii 2015	Wound secretions	CEFT, CEFE, CEFO, TZP, TIC, MEM, TOB, CIP, LEV, MINO, TIGE, TRIS
K. pneumoniae 1536	Central venous catheter	TZP, AMOXI, CEFE, CEFU, CEFO, CEFT, IMI, DORI, AZTRE, GT, TOB, CIP, LEV, COL
K. pneumoniae 2437	Wound secretions	TZP, AMOXI, CEFE, CEFU, CEFO, CEFT, IMI, DORI, AZTRE, GT, TOB, CIP, LEV, COL
K. pneumoniae 2181	Urine culture	TZP, AMOXI, CEFE, CEFU, CEFO, CEFT, IMI, DORI, AZTRE, GT, TOB, CIP, LEV, COL
K. pneumoniae 2284	Urine culture	TZP, AMOXI, CEFE, CEFU, CEFO, CEFT, IMI, DORI, AZTRE, GT, TOB, CIP, LEV, COL
K. pneumoniae 5694/8824	Urine culture	TZP, AMOXI, CEFE, CEFU, CEFO, CEFT, IMI, DORI, AZTRE, GT, TOB, CIP, LEV, COL
K. pneumoniae 5853/2518	Urine culture	TZP, AMOXI, CEFE, CEFU, CEFO, CEFT, IMI, DORI, AZTRE, GT, TOB, CIP, LEV, COL
K. pneumoniae 9793	Pharyngeal exudate	TZP, AMOXI, CEFE, CEFU, CEFO, CEFT, IMI, DORI, AZTRE, GT, TOB, CIP, LEV, COL
K. pneumoniae 5778/4051	Central venous catheter	TZP, AMOXI, CEFE, CEFU, CEFO, CEFT, IMI, DORI, AZTRE, GT, TOB, CIP, LEV, COL
K. pneumoniae 2569	Anal portage	TZP, AMOXI, CEFE, CEFU, CEFO, CEFT, IMI, DORI, AZTRE, GT, TOB, CIP, LEV, COL
K. pneumoniae 5950/4053	Urine culture	TZP, AMOXI, CEFE, CEFU, CEFO, CEFT, IMI, DORI, AZTRE, GT, TOB, CIP, LEV, COL
K. pneumoniae 5993/252	Urine culture	TZP, AMOXI, CEFE, CEFU, CEFO, CEFT, IMI, DORI, AZTRE, GT, TOB, CIP, LEV, COL
K. pneumoniae 5993/252	Urine culture	TZP, AMOXI, CEFE, CEFU, CEFO, CEFT, IMI, DORI, AZTRE, GT, TOB, CIP, LEV, COL
K. pneumoniae 5671/4487	Pharyngeal swab	TZP, AMOXI, CEFE, CEFU, CEFO, CEFT, IMI, DORI, AZTRE, GT, TOB, CIP, LEV, COL
K. pneumoniae 5652/2514	Wound secretions	TZP, AMOXI, CEFE, CEFU, CEFO, CEFT, IMI, DORI, AZTRE, GT, TOB, CIP, LEV, COL
K. pneumoniae 8488/2387	Anal swab	TZP, AMOXI, CEFE, CEFU, CEFO, CEFT, IMI, DORI, AZTRE, GT, TOB, CIP, LEV, COL
K. pneumoniae 2372	Anal swab	TZP, AMOXI, CEFE, CEFU, CEFO, CEFT, IMI, DORI, AZTRE, GT, TOB, CIP, LEV, COL

Abbreviations: P=Penicillin; CLD=Clindamycin; T=Tetracycline; E=Erythromycin; CEFT=Ceftazidime; CEFE=Cefepime; CEFO=Cefotaxime; TZP=Piperacillin-Tazobactam; TIC=Ticarcillin; MEM=Meropenem; IMI=Imipenem; DORI=Doripenem; TOB= Tobramycin; CIP=Ciprofloxacin; LEV=Levofloxacin; MINO=Minocycline; TIGE=Tigecycline; TRIS=Trimethoprim-Sulfamethoxazole; AMOXI=Amoxicillin-Clavulanic acid; AZTRE=Aztreonam; GT= Gentamicin.

For the study, fresh bacterial cultures were obtained by seeding the studied strains on solid TSA media (*Trypticase Soy Agar*) and incubated for 18-20 hours at 37ºC.

The tested essential oils were achieved from commercial producers, according to Table 2.

Table 2. The tested essential oils.

Vegetal product	Commercial
Lavender EO	Fares
Cloves EO	Fares

Vegetal product	Commercial
Eucalyptus (EO containing *Eucalyptus globulus* and limonene leaf EO)	TrioVerde
Rosemary EO	Fares
Thyme EO	Fares
Oregano (contains *Origanum vulgare EO*+ carvacrol, linalol, thymol and limonene)	TrioVerde

2.1. Quantitative testing of the ability to inhibit adhesion to the inert substrate.

In order to determine the effect of the tested suspensions on the adhesion capacity to the inert substrate, we have used the crystal (purple) violet assay [8-10]. For this purpose, serial two-fold microdilutions were performed in 96 well plates in tryptic soy broth (TSB) liquid medium, starting from 1% oil in the first well, in a final volume of 150 µl liquid medium. The final tested concentrations are presented in Table 3.

Table 3. Schematic of the concentrations reached in each well after binary serial dilutions.

Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Well 7	Well 8	Well 9	Well 10
1%	0.5%	0.25%	0.125%	0.0625%	0.03125%	0.015625%	0.0078125%	0.00390625%	0.001953125%

The wells were then inoculated with 15 µl microbial suspension with standard density corresponding to Mac Farland 0.5 and incubated at 37^{o}C for 24 hours. For each test, we worked with microbial culture control (wells containing exclusively culture medium inoculated with microbial suspension) and with control of sterility of the environment (wells containing culture medium exclusively).

After incubating the plates at 37°C for 24 hours, the 96-well microplates were emptied, gently washed with phosphate-buffered saline (PBS) to remove the non-adherent bacterial cells, fixed with cold methanol for 5 min, stained with 1% violet crystal solution for 30 min, washed to remove the excess of staining solution and then resuspended with 33% acetic acid solution. The suspensions thus obtained were used for establishing the minimal biofilm eradication concentrations (MBEC) based on the spectrophotometric reading of the absorbance of the colored suspension at 490 nm.

3. Results and Discussion

Microbial biofilm development on tissues or medical devices represents an advantage in the case of bacterial pathogens, as they protect them from immune effector and antimicrobial substances and, simultaneously, are permanent reservoirs of infections, especially in the case of a medical device [11]. Therefore, biofilm inhibition is a major drug target for treating various bacterial and fungal infections. The development of antibiofilm drugs now represents an extensively studied field in pharmaceutical chemistry.

Vegetal essential oils have been traditionally used to treat infectious diseases due to their large spectrum of antimicrobial and antiviral activity. They exhibit antimicrobial activity by acting simultaneously at the level of different microbial targets, including the microbial cell wall, plasma membrane, protein, and nucleic acids synthesis and functions. Their antibiofilm activity has also been revealed in the first decade [12].

Therefore, our purpose was to evaluate the anti-biofilm activity of seven commercial essential oils of vegetal origin against Gram-positive and Gram-negative resistant bacterial strains. In order to determine the adhesion capacity to the inert substrate, the purple (violet)

crystal staining method was used, which allowed the spectrophotometric determination of the MBEC values.

The determinations were performed for each tested essential oil and each clinical strain, and then the average MBEC value was established for each tested species. The results were centralized in tables 4-7.

The analysis of the MBEC values showed that the same three essential oils, oregano, thyme, and cloves, showed the lowest MBEC values against all tested bacterial species. Although the other oils, lavender, eucalyptus, and rosemary, had a weaker effect than the first three oils, they show anti-biofilm activity at concentrations low enough to be considered effective and used as an alternative in therapy.

Table 4. Average MBEC values determined for *S. aureus* clinical strains.

Tested essential oils	Average MBEC values expressed in percentage concentrations		
	24 hours	48 hours	72 hours
lavender essential oil	0.576273333	0.163563	0.732422
clove essential oil	0.012703542	0.026027	0.06021
eucalyptus essential oil	0.317381667	0.015253	0.024402
rosemary essential oil	0.165290467	0.040889	0.020342
thyme essential oil	0.01087345	0.018106	0.01077
oregano essential oil	0.005085	0.00263	0.004067
DMSO	7.816566667	4.73625	0.2156

Table 5. Average MBEC values determined for *P. aeruginosa* clinical strains.

Tested essential oils	Average MBEC values expressed in percentage concentrations		
	24 hours	48 hours	72 hours
lavender essential oil	0.687499	0.703125	0.375
clove essential oil	0.035154	0.056152	0.019041
eucalyptus essential oil	0.127301	0.328125	0.253416
rosemary essential oil	0.051267	0.234375	0.03662
thyme essential oil	0.013289	0.014159	0.024414
oregano essential oil	0.004392	0.024902	0.010252
DMSO	5.996092	10.78125	1.528068

Table 6. Average MBEC values determined for *A. baumannii* clinical strains.

Tested essential oils	Average MBEC values expressed in percentage concentrations		
	24 hours	48 hours	72 hours
lavender essential oil	0.570313	0.578125	1.625
clove essential oil	0.025878	0.02539	0.051244
eucalyptus essential oil	0.410156	0.363281	0.597656
rosemary essential oil	0.141601	0.113769	0.160156
thyme essential oil	0.023923	0.012693	0.028808
oregano essential oil	0.008786	0.00781	0.01074
DMSO	8.76464	1.684565	7.65625

Table 7. Average MBEC values determined for *K. pneumoniae* clinical strains

Tested essential oils	Average MBEC values expressed in percentage concentrations		
	24 hours	48 hours	72 hours
lavender essential oil	0.140625	0.205301	0.470702
clove essential oil	0.039304	0.078169	0.519529
eucalyptus essential oil	0.15039	0.103514	0.167969
rosemary essential oil	0.041014	0.036374	0.105467
thyme essential oil	0.003172	0.005002	0.014515
oregano essential oil	0.002196	0.002806	0.010008
DMSO	16.875	9.0625	3.75244

The clove oil has been very active against *S. aureus*, *P. aeruginosa*, and *A. baumannii* strains at all three tested time points, as well as against *K. pneumoniae*, at 24 and 48 hours (Figure 1).

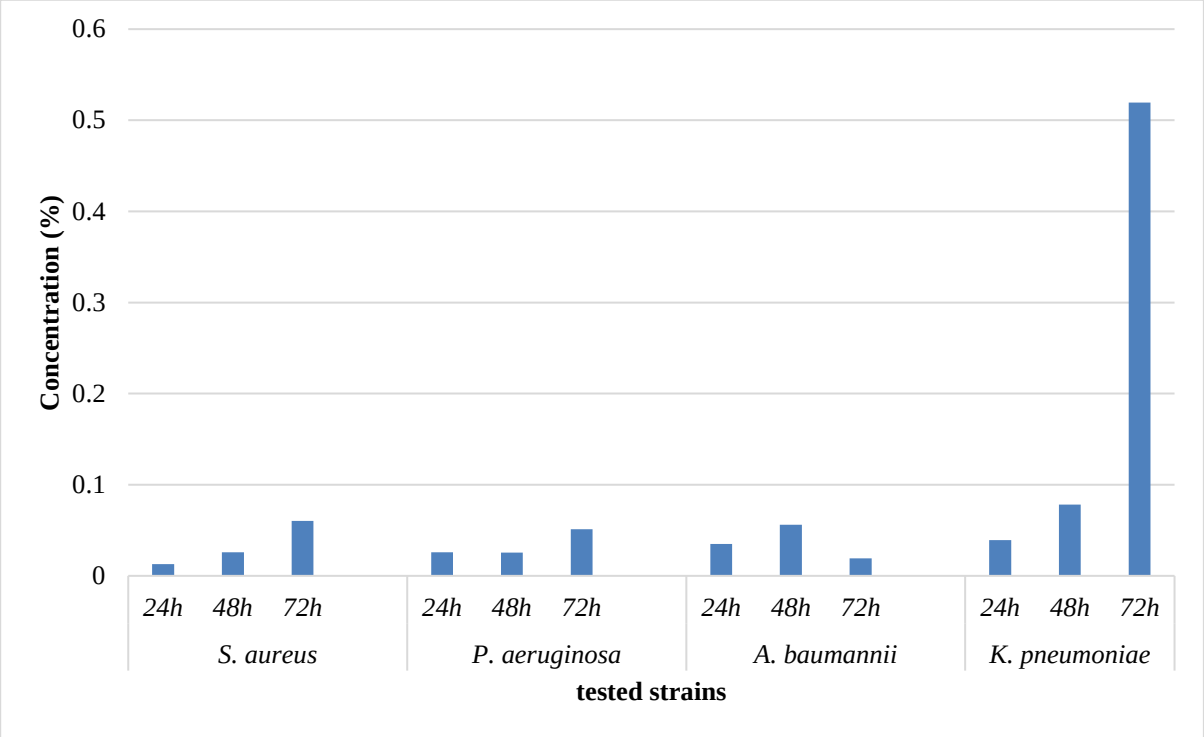

Figure 1. Graphic representation of the efficiency of clove essential oil against the tested strains at different time intervals.

The lavender and eucalyptus oils exhibited very similar anti-biofilm profiles, being more efficient against *K. pneumoniae* strains, while *P. aeruginosa* proved to be the most resistant (Figures 2, 3).

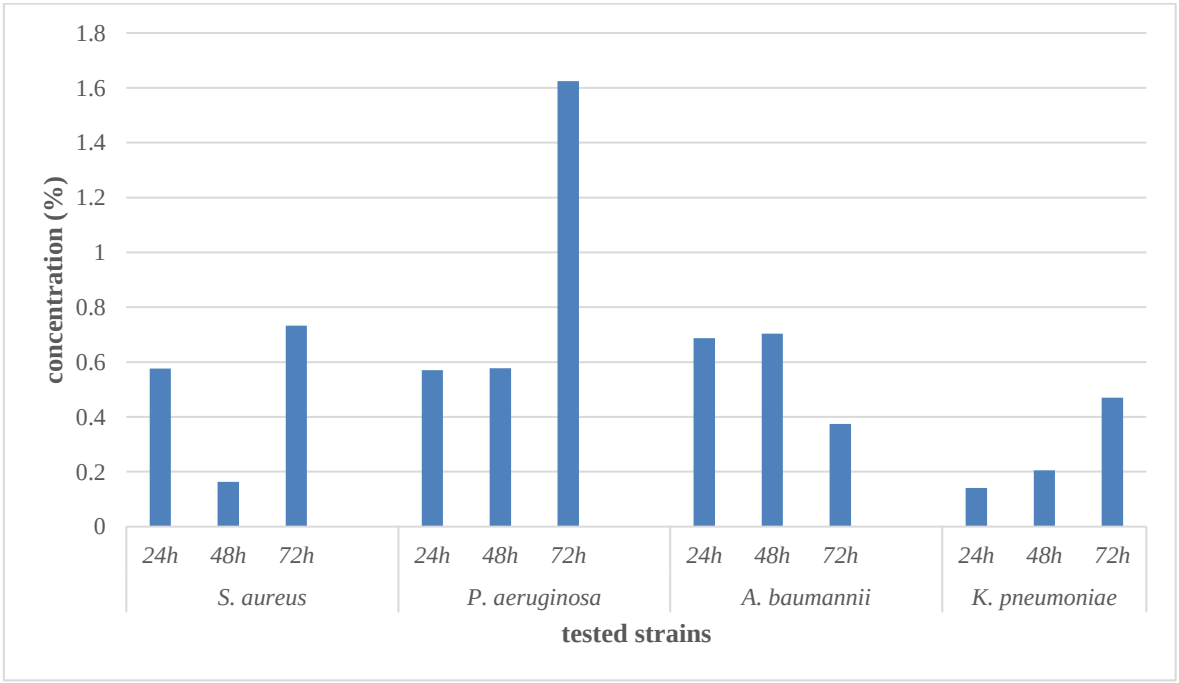

Figure 2. Graphic representation of the efficiency of lavender essential oil against the tested strains at different time intervals.

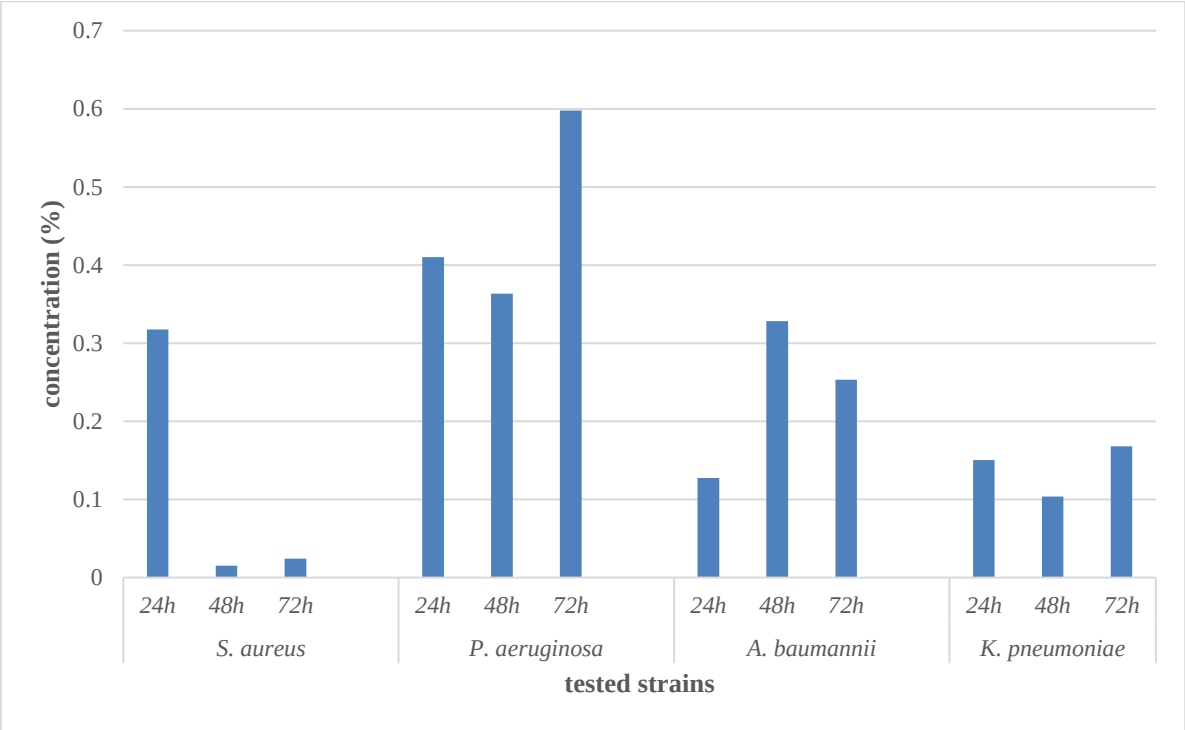

Figure 3. Graphic representation of the efficiency of eucalyptus essential oil against the tested strains at different time intervals.

The rosemary oil was active against *K. pneumoniae*, followed by *A. baumannii* and *S. aureus*, at specific time intervals (Figure 4).

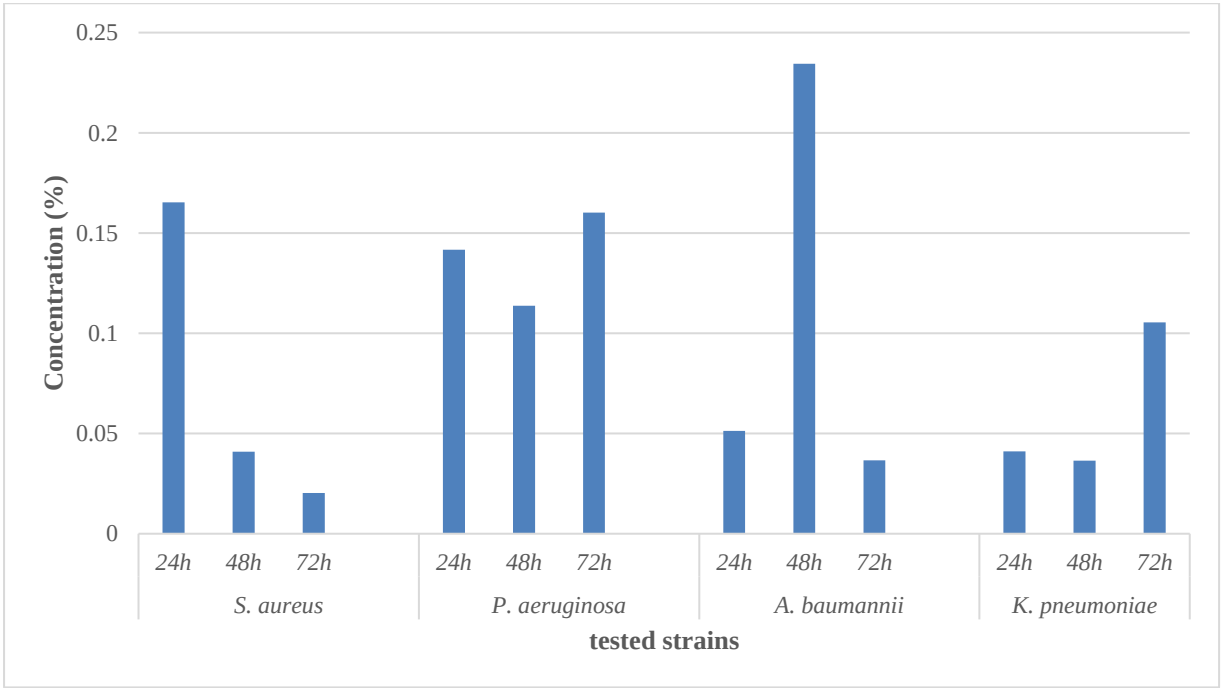

Figure 4. Graphic representation of the efficiency of rosemary essential oil against the tested strains at different time intervals.

The essential oregano oil has been active against *S. aureus* and *K. pneumoniae* biofilms (Figure 5).

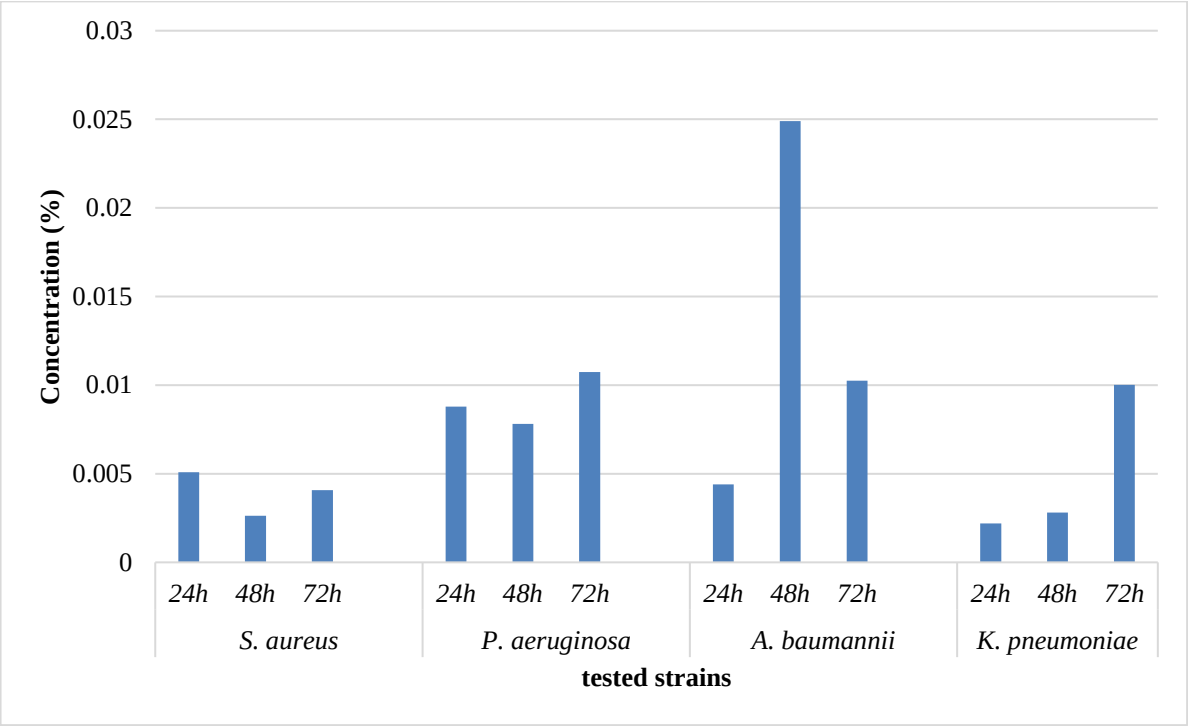

Figure 5. Graphic representation of the efficiency of oregano essential oil against the tested strains at different time intervals.

The thyme essential oil has been the most active against *K. pneumoniae* biofilm, followed by *S. aureus*, while for *P. aeruginosa* and *A. baumannii*, the MBEC was higher (Figure 6).

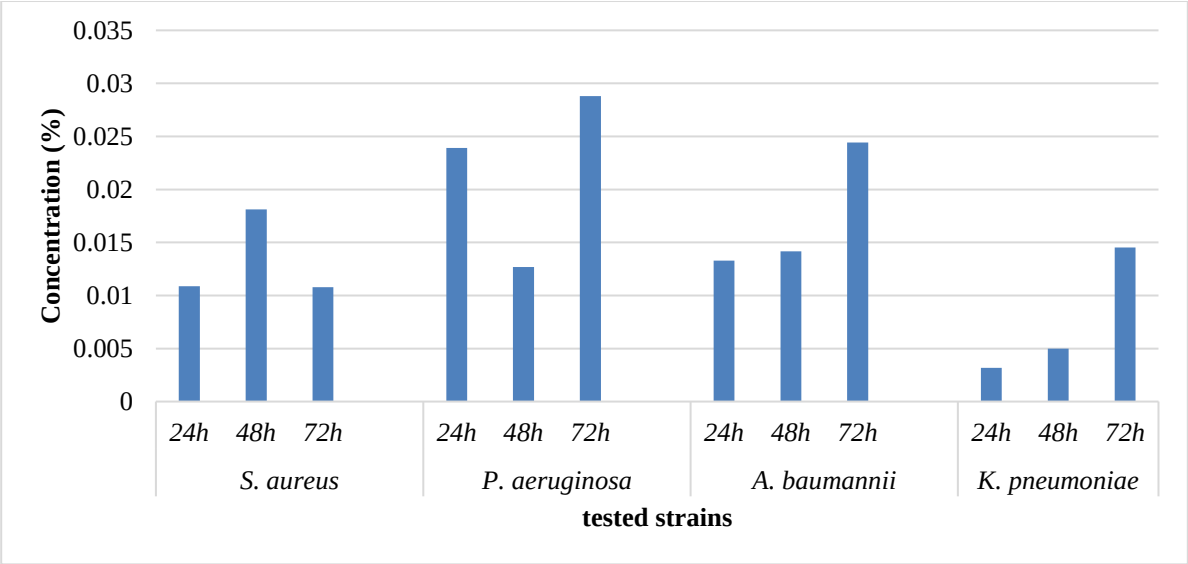

Figure 6. Graphic representation of the efficiency of clove essential oil against the tested strains at different time intervals.

The obtained results indicate that the tested oils were the most efficient against *K. pneumoniae* and *S. aureus* and less efficient against the Gram-negative, non-fermentative bacilli, *P. aeruginosa*, and *A. baumannii*, known for their complex natural and acquired resistance mechanisms.

4. Conclusions

Some of the essential oils evaluated in the present research exhibited very good antibiofilm activities against the tested nosocomial bacterial strains belonging to the ESKAPE group, being thus a viable alternative to control the formation of microbial biofilms and a valuable source of bioactive compounds and new drugs. The most efficient proved to be the oregano, thyme, and clove essential oils, which exhibited the lowest MBEC values against all bacterial species. Although lavender, eucalyptus, and rosemary had a weaker effect than the first three oils, they inhibited the biofilm development at concentrations low enough to be considered effective and used as an alternative in therapy. This experimental approach may open new perspectives for improving strategies to combat the emergent threat of antibiotic resistance phenomenon.

Funding

This research received no external funding.

Acknowledgments

I.G. acknowledges the support of CNFIS-FDI-2022-0675 and the Ministry of Research, Innovation, and Digitalization through Program 1—Development of the national R&D system, Subprogram 1.2—Institutional performance—Financing projects for excellence in RDI, Contract no. 41 PFE/30.12.2021. M.G. acknowledges the support of UEFISCDI, grant number PD 200/27/08/2020-PN-III-P1-1.1-PD-2019-1193.

Conflicts of Interest

The authors declare no conflict of interest.

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