Effect of Silver Nanoparticles and Essential Oils against Multidrug Resistant Staphylococcus pseudintermedius: An In Vitro Study

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Article

Effect of Silver Nanoparticles and Essential Oils against Multidrug Resistant *Staphylococcus pseudintermedius*: An In Vitro Study

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Abstract: *Staphylococcus pseudintermedius* (SP) is an emergent zoonotic agent associated with multidrug resistance (MDR). This work aimed to describe the antibacterial activity of four essential oils (EOs) and silver nanoparticles (AgNPs) against SP. Fifteen SP strains were tested. The four EOs, namely *Rosmarinus officinalis* (RO), *Juniperus communis* (GI), *Citrus sinensis* (AR) and *Abies alba* (AB), and AgNPs were used alone and in combination to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) against SP. All strains presented MDR and methicillin resistance. Among the antibiotic cohort, only rifampicin, doxycycline and amikacin were effective. When EOs were tested alone, AB showed the lowest MIC followed by GI, RO and AR (0.23%±0.3; 0.5%±0.51; 0.79%±0.39; 0.93%±0.63). MBC was increased with the following order: AB, GI, AR and RO (3.56%±4.87; 4.93%±6.27; 8.53%±7.2; 10.34%±5.31). MIC and MBC values for AgNPs were 10.74 mg/L±4.23; 261.05 mg/L±172.74. In combination, the MIC and MBC values were lower for all compounds. In conclusion, EOs and AgNPs could be considered as promising antibacterial alternatives to antibiotics.

Keywords: MRSP; MDR; essential oil; silver nanoparticles

1. Introduction

The antimicrobial resistance (AMR) problem is seen through the lens of two complementary approaches, One Health and Global Health, which have been utilized to address issues related to infectious diseases in general, and AMR in particular. Both terms are transdisciplinary and based on the assumption that human and animal health are strongly related to the health of the ecosystems they are a part of [1,2]. One Health focuses on the role of linked ecosystems in the genesis and spread of AMR, and hence tackles AMR locally. Global Health, on the other hand, addresses the global conditions that facilitate the global spread of AMR and is based on the idea that controlling AMR necessitates integrated political and socioeconomic actions by countries, international organizations, and other global actors [3–5].

Over the last decade, *Staphylococcus pseudintermedius* (SP) has gained attention due to its zoonotic potential directly linked to the genetic acquisition of antibiotic-resistance genes (ARGs) and virulence factors. Up to 97.8% of Methicillin-resistant SP (MRSP) isolates showed multidrug resistance to three or more antibiotics commonly administered in veterinary medicine [6,7]. The colonization of SP is

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quite similar to that of *S. aureus* in humans, with the human nares being the most prevalent site of colonization in contrast to the pharynx and rectum in companion animals [8,9]. SP is noted for its opportunistic potential, particularly in immunocompromised hosts, and being a typical commensal of dogs. Furthermore, it has been linked to several cases of human colonization and infection, most of which were caused by intimate contact between companion dogs and people [10–13].

As a result, the need for alternatives to standard antibiotics is urgent. In this context, secondary metabolites of plants including essential oils (EOs), produced in response to environmental conditions such as herbivore assault, abiotic stress, or interspecific interactions, have emerged as significant potential choices [14]. EOs are the primary components of aromatherapy, and they are produced by up to 17,000 distinct plant species from 60 different families (e.g., Lamiaceae, Rutaceae, Myrtaceae, Zingiberaceae, and Asteraceae) [15].

A second rapidly-growing field is nanotechnology, aiming to synthesize and characterize nanoparticles (NPs) with several applications in different scientific disciplines [16]. As known, silver (Ag) is a noble metal used for centuries as an antibacterial agent and its chemical properties made it a valuable candidate as a metallic precursor for the synthesis of NPs [7].

This study aims to determine the antibacterial potential of chemically-synthesized silver nanoparticles (AgNPs) alone and in combination with EOs against MDR SP strains.

2. Materials and Methods

2.1. Bacterial strains, identification and culture conditions

Fifteen SP strains were chosen from the Department of Biomedical, Surgical and Dental Sciences (University of Milan) bacterial collection. Bacteria were isolated starting from 2018 from deep cutaneous pyoderma clinical samples and stored at -20°C in 25% (v/v) glycerol (Carlo Erba, Italy). Original isolation was performed using both phenotypic and molecular techniques. In brief, the phenotypic identification was made using Mannitol Salt Agar (Microbiol, Italy) as a selective and differential medium after first isolation on Columbia Base Agar (ThermoFisher, Italy) + 5% defibrinated sheep blood (Microbiol, Italy). Molecular final identification was done using a multiplex-PCR (mPCR) assay [17] coupled with Restriction Fragment Length Polymorphism (RFLP) assay [18]. After identification, each sample was stored in filtered (0.22 μm) glycerol at -20°C. The day before the experiment, each sample was thawed at room temperature and plated on Columbia Base Agar (ThermoFisher, Italy) + 5% defibrinated sheep blood (Microbiol, Italy) and incubated aerobically at 37°C for 18-24 h.

2.1. Determination of antimicrobial profiles

The Kirby-Bauer Disk diffusion assay was used to investigate the susceptibility of the strains against 22 antibiotic molecules following the Clinical and Laboratory Standard Institute guidelines [19]. Antimicrobials (in brackets, the abbreviation and concentration in μg) used were Oxacillin (OX; 5), Amoxicillin + clavulanic acid (AMC; 30), Amoxicillin (AMO; 30), Carbenicillin (CAR; 100), Cephalexin (CL; 30), Cefovecin (CVN; 30), Ceftiofur (EFT; 30), Ceftriaxone (CRO 30, Clindamycin (DA; 10), Lincomycin+Spectinomycin (LC-SP; 15), Doxycycline (DO; 30), Enrofloxacin (ENR; 5), Marbofloxacin (MAR; 5), Pradofloxacin (PRA; 5), Amikacin (AK; 30), Gentamicin (CN; 30), Neomycin (N; 30), Tobramycin (TOB; 10), Kanamycin (K; 30), Rifampicin (RD; 30), Azithromycin (AZM; 15), Erythromycin (E; 30).

ARGs were detected using two sets of mPCR as previously described [15].

2.2. Synthesis and characterization of silver nanoparticles

AgNPs were chemically synthesized starting from silver nitrate (AgNO3, Carlo Erba, Italy) as the metal ions donor, as described [20]. Briefly, a 100 mM solution of AgNO3 was prepared by dissolving 8.49 g of salt in 500 mL of distilled water and heated at 90°C for 5 minutes. By dripping, 12.5 mL of 1% Tri-Sodium Citrate (TSC) in water was added. The reduction of metal ions was ascertained by the colour change of the solution from transparent to brown. The volume was

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transferred into a separatory funnel and darkened for 24 hours. After eluting, the volume fraction containing the NPs (approximately 50 mL) was centrifuged (4,000 RCF, 15 minutes), washed twice with distilled water, and freeze-dried (CoolSafe Basic, Labogene, Scandinavia) for 24 hours at -54°C.

The reduction from Ag^{+} to Ag^{0} was monitored by measuring the ultraviolet-visible absorption spectrum UV-Vis (SpectraMax 340 PC, Molecular Devices, Germany) at wavelengths from 310 to 770 nm (with 50 nm path). Readings were taken twice within 15 minutes. Using a transmission electron microscope (EFTEM Leo 912ab (Zeiss, Milan)) at a voltage of 100 kV the nanoparticles' morphology was determined. Samples were briefly sonicated (30 kHz, 15 sec on and 45 sec off), and immediately a drop of the aqueous AgNPs suspension was mounted on a carbon grid and placed on a filter paper to absorb excess solvent. The morphological analysis (diameter and particle size distribution) was calculated with Image J2, v. 150 software.

2.3. Characterization of essential oils

The four EOs *Rosmarinus officinalis* (RO), *Juniperus communis* (GI), *Citrus sinensis* (AR) and *Abies alba* (AB) were purchased from an Italian company (Vitalis, Italy).

EOs were diluted in methanol, and the characterization was performed using an Agilent 5975C Series GC/MS and FID as detector. Volatiles were separated using an apolar capillary column Zebron-Semivolatiles (Zebron, Phenomenex,USA) of 30 m×0.25 mm (ID) and a film thickness of 0.25 µm. Carrier gas was helium at a flow rate of 1 mL/min. Two µL of each sample was injected in the GC MS using CTC PAL system in triplicates. The injector was at 230°C under splitless mode. The temperature program was isothermal for 3.5 min at 40°C, then two different temperature ramps were done to raise 140°C, 150°C (hold time of 7 min) and 220°C (hold time of 23 min) at a rate of 7°C/min and 20°C/min, respectively. The transfer line to the mass spectrometer was maintained at 150°C. The mass spectra were obtained by electronic impact at 70 eV, a multiplier voltage of 1294 V and collecting data at a m/z range of 35-500. The retention indices were determined in relation to a homologous series of n-alkanes (C7-C30, Sigma Aldrich, Milan, Italy) under the same operation conditions. The chromatograms were elaborated using the open-source MS-DIAL by using the NIST14 library as reference and by considering as cut-off a matching between the RI of the peak and of the library < 70% as suggested by the Metabolomics Standards Initiative of the International Metabolomics Society [21]. Indeed, the analytical data were expressed as a percentage of the sum of the retained peaks' total ion current (TIC). The results were expressed as average± standard deviation.

2.4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of AgNPs and EOs

The MIC of AgNPs and EOs was determined by following the microdilution method according to the Clinical and Laboratory Standard Institute guidelines [19]. As found in the literature, EOs were initially diluted (3:1) in dimethyl sulfoxide (DMSO, Merck, Italy) to allow two-fold dilution in Mueller-Hinton broth (Microbiol, Italy), reaching a linear oil gradient from 18.75% (v/v) to 0.04% (v/v). AgNPs were dissolved in distilled water to reach an initial concentration of 2.048 mg/mL and create a gradient from 512 µg/mL to 1 µg/mL. When combined with EOs, AgNPs were diluted in DMSO to maintain the same antimicrobial gradient. Before testing EOs and AgNPs against field strains, ATCC cultures were used (*S. aureus* ATCC 6358, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* subsp. *ozaenae* ATCC 11296, *Micrococcus yunnanensis* ATCC 7468, *Enterococcus casseliflavus* ATCC 12755, *Providencia rettgeri* ATCC 9250, *Proteus vulgaris* ATCC 7829, *Streptococcus agalactiae* ATCC 13813, *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 25928). DMSO alone was tested to rule out its potential antibacterial activity. All the strains were tested in triplicates; appropriate positive and negative controls were inserted into each 96-well plate. After 24 h of incubation at 37°C, MIC was visually determined as the lowest concentration that inhibits bacterial growth.

The MBC was determined by inoculating the entire volume of the wells (200 µL) in tubes with sterile Mueller-Hinton broth and incubating at 37°C for 24 h. The MBC was indicated as the lowest

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concentration capable to kill the bacteria in the broth. The lack of growth in the tubes was verified with a densitometer (BioSan Densitometer) and compared with the positive and negative controls.

2.5. Statistical Analysis

GraphPad Prism version 8.00 was used for the statistical analysis and graphical depiction (GraphPad Software®, San Diego, CA, USA, www.graphpad.com). The following tests were used to determine the distributions' normality (or non-normality): the D'Agostino and Pearson omnibus normality test, the Shapiro-Wilk normality test, and the Kolmogorov-Smirnov normality test. The Mann-Whitney test was employed to compare groups with non-parametric distributions; otherwise, the usual Student's t-test was used.

3. Results

3.1. Phenotypic and molecular profiling of antibiotic resistance

All SP strains were found to be resistant to at least three pharmacological categories tested (*β*-lactams [B-LAC], Lincosamides [LIN], Tetracyclines [TET], Fluoroquinolones [FLQ], Aminoglycosides [AMN], Rifamycins [RIF], Macrolides [MAC]) and are therefore classified MDR, furthermore all were found to be resistant to methicillin which makes them MRSP (Figure 1).

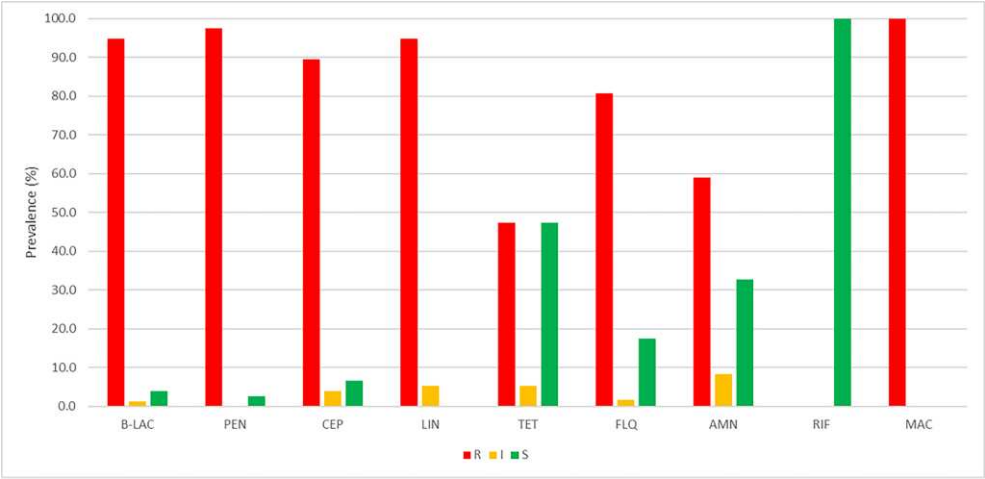

Figure 1. Prevalence of resistance by antibiotic class. Abbreviations: B-LAC=*β*-lactams; PEN=Penicillins; CEP=Cephalosporins; LIN=Lincosamides; TET=Tetracyclines; FLQ=Fluoroquinolones; AMN=Aminoglycosides; RIF=Rifamycins; MAC=Macrolides; R=Resistant; I=Intermediate; S=Susceptible.

Resistance to penicillins and fluoroquinolones was 97.4% and 80.7%, respectively. The cephalosporins (all 3rd generation except for 1st generation cephalexin) showed a slightly different trend, with resistance of 89.5%. Interestingly, among the 3rd generation molecules tested, ceftriaxone showed the same resistance as cephalexin (78.9%), a first-generation cephalosporin. Penicillins were effective in about 2.6% of cases (a worrying result aggravated by the total resistance to oxacillin), while cephalosporins in 6.6%. Lincosamides are the second antibiotic category with the greatest resistance detected (around 95%); clindamycin was completely resistant, while only two strains were susceptible to the combination lincomycin+spectinomycin. Doxycycline (belonging to the tetracycline family) is not an antibiotic traditionally used in treating skin infections (due to the molecule's photosensitivity and hepatotoxicity); nevertheless, approximately 47% of strains were susceptible to it. Fluoroquinolones (81% resistant) are the second antibacterial category used in the treatment of pyoderma, showing bactericidal activity since the DNA gyrase is the molecular target of this group of antibiotics. However, numerous point mutations (SNP) enabled SP to resist their biocide action in recent years. Among the three molecules tested, marbofloxacin (MAR) is the most active (32%), while the most prescribed enrofloxacin (ENR) and pradofloxacin (PRA) were resistant in 79% and 95% of

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cases, respectively. Among the tested aminoglycosides (which showed a sensitivity of 33%), amikacin was the molecule to which all strains were sensitive, followed by gentamicin and tobramycin with susceptibility of 37% and 26%, respectively. From a therapeutic point of view, amikacin should be considered the most reasonable pharmacological choice, its use must be limited to cases where no other molecule is usable, as it could cause renal damage. In this study, rifampicin is the molecule to which all strains are susceptible, opposite of the macrolides trend (both azithromycin and erythromycin), the class of antibiotic that showed resistance to all SP tested.

Genetically, all strains were positive for the *mecA* gene conferring resistance to methicillin (and all penicillins and cephalosporins except ceftaroline), with 84% positive for the *blaZ* gene conferring resistance to *β*-lactams (in particular ampicillin, amoxicillin, and amoxiclavulanate). The *aacA-aphD* gene (aminoglycoside resistance) was amplified in 78% of the bacteria, while the *tetM* and *tetK* genes were present in 5 and 7 strains, respectively.

All the strains were full-length sequenced using a third-generation sequencing machine [22]. The Multi-locus Sequence Typing (MLST) analysis was done using an online tool (https://pubmlst.org/organisms/staphylococcus-pseudintermedius/), and three different sequence types (STs) were found; ST71 was the most prevalent (11/15; 73%) followed by ST258 (3/15; 20%) and ST301 (1/15; 7%). Figure 2 represents the UPGMA cluster derived from the pangenome analysis with an online tool (http://pgaweb.vlcc.cn/). The black box includes the SP strains of ST71, which share the staphylococcal chromosome cassette (SCC)*mec* type II-III.

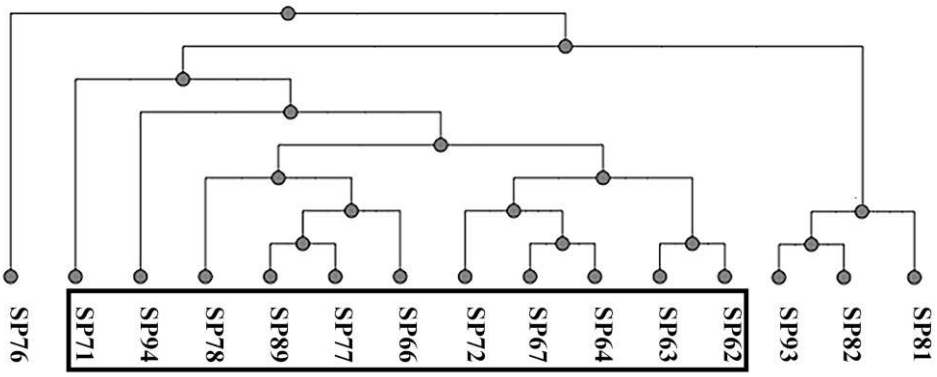

Figure 2. Pangenome-based UPGMA cluster analysis.

3.2. Characterization of antimicrobial molecules

3.2.1. AgNPs

AgNPs were obtained by chemical synthesis from silver nitrate (as Ag^+ donor) and TSC as reducing and stabilizing agent. The first macroscopic characterization indicative of the reduction of Ag^+ to Ag^0 was the observation (over time) of the solution colour change, which progressively turned from transparent to dark brown. Once the synthesis was completed, ultraviolet/visible spectroscopy (UV-Vis spectroscopy) confirmed the presence of metal nanoparticles. This technique makes it possible to derive qualitative-quantitative information by exploiting the ability of different substances to absorb a given wavelength, which in the case of AgNPs is 440 nm (Figure 3A).

During the synthesis, the pH of the reaction was not controlled, consequently, the morphology of the particles (Figure 3B) was not homogeneous but had different symmetries; triangular, pentagonal, hexagonal, filiform, spherical and cubic.

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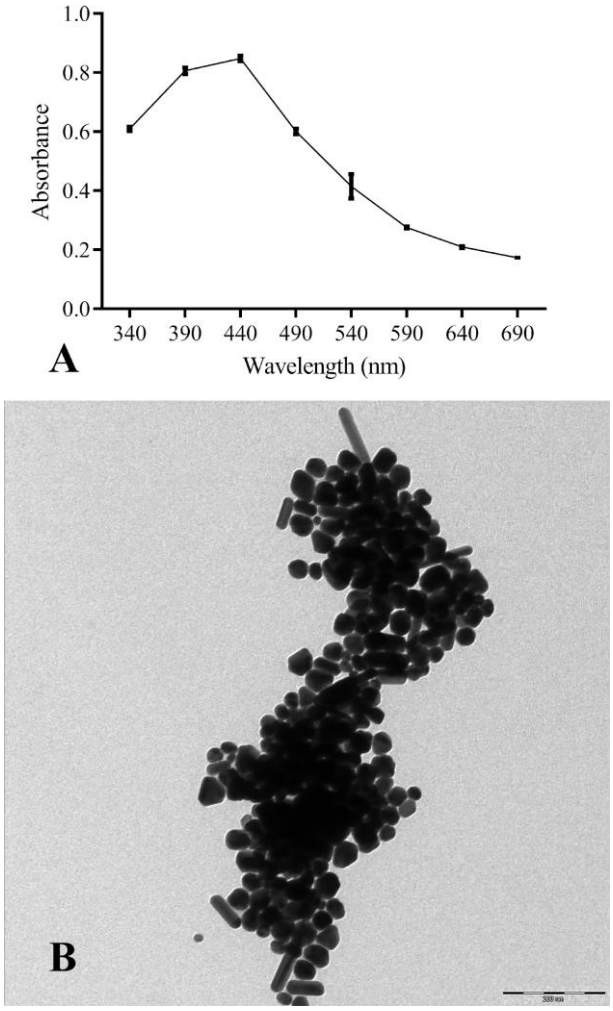

Figure 3. (A) UV-Vis absorption spectra of chemically-synthesized AgNPs; (B) TEM morphological analysis of NPs (scale bar corresponds to 200 nm).

The size analysis was performed with ImageJ software, which allowed the area of NPs with spherical symmetry to be derived (for the other symmetries, although it was possible to determine the area, the volume could not be calculated). The synthesized AgNPs had a size of 15±2.7 nm. A factor affecting their antibacterial capacity is the surface area/volume ratio (S/V), which links with the contact surface area between the particles and the pathogen. In this work, the S/V ratio was 0.56±0.09 nm^2/nm^3 (Figure S1).

3.2.2. EOs

The constituents of the four EOs are reported in Table 1, which are listed in ascending order of their linear retention indices (LRIs) on the apolar column. A total of 124 compounds belonging to different chemical classes have been identified. Of these, 35 were detected in AR, representing 96.68% of EO, 75 in AB (98.09%), 77 in GI (91.75%) and 57 in RO (98.65%). In all EOs, the monoterpenic fraction (40.55%, 39.68%, 39.56% and 60.17%, respectively) prevailed over the sesquiterpenic fraction (6.96%, 23.54%, 30.64% and 17.73%). Oxygenated monoterpenes were more abundant than monoterpene hydrocarbons in AR (22:1), lower in AB (1:2) and GI (1:1.2), in similar percentages in RO (1:1). In detail, cis- or trans-*p*-menth-2-en-1-ol characterized AR and AB (36.14% and 6.1%, respectively), GI (5.96%) together with 1,6-octadien-3-ol, 3,7-dimethyl- (6.12%), RO (7.22%) together with cis-α-terpineol (8.1%). Among the sesquiterpenoids, oxigenated sesquiterpenes were the main components in AR, AB and GI (6.62%, 12.57% and 21.98%) with α-sinensal (1.91%), α-santalene (3.71%) and guaiol (3.98%) as the major compounds, respectively. The sesquiterpene hydrocarbons

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predominated in RO (12.41%) and (+)-sativene was the most abundant (5.71%). Diterpenoids were present in very low percentages (0.55% to 3.58%) in three of the four samples, absent in RO.

Table 1. Chemical composition (%) of *Citrus sinensis* (AR), *Abies alba* (AB), *Juniperus communis* (GI) and *Rosmarinus officinalis* (RO) essential oils determined by GC/MS.

#	Component[1]	LRI[2]	AR[3]	AB[4]	GI[5]	RO[6]
			% TIC			
1	α-Sinensal	855	1.91±0.06	tr	-	-
2	Ethanol, 2,2'-oxybis-	967	11.0±5.01	5.08±4.67	5.19±0.35	8.25±0.17
3	Methyl DL Leucate	968	3.01±1.14	2.63±1.38	2.14±1.09	2.73±0.84
4	Acetic acid, 2-ethylbutyl ester	976	0.28±0.05	tr	tr	tr
5	Pseudolimonene	994	0.17±0.04	2.92±5.43	4.10±5.59	6.54±4.69
6	Nonane, 3-methyl-	995	0.11±0.02	-	tr	tr
7	α-Phellandrene	996	0.22±0.04	tr	4.24±5.53	0.37±0.29
8	3-Butenoic acid, 3-methyl-, trimethylsilyl ester	1004	2.35±0.55	0.18±0.33	0.34±0.17	0.26±0.27
9	α-Terpinene	1008	tr	8.16±7.78	0.82±2.09	5.21±4.19
10	4-Carene	1009	tr	5.87±5.13	1.50±1.99	2.76±3.69
11	2-Carene	1009	tr	3.57±4.19	1.03±1.43	2.67±0.20
12	α-Tesabinenene	1014	tr	8.16±7.78	0.82±2.09	5.21±4.19
13	β-cis-Ocimene	1022	0.63±0.24	2.94±5.14	0.30±0.80	5.54±7.41
14	3-Carene	1029	tr	tr	tr	0.58±0.67
15	β-Terpinene	1036	tr	3.19±4.80	0.27±0.72	5.81±7.58
16	Crithmene	1054	0.74±0.30	tr	2.55±3.69	0.30±0.44
17	Benzene, butyl-	1056	tr	0.69±0.60	0.95±1.32	0.19±0.26
18	4-Thujanol	1065	0.47±0.02	-	0.15±0.22	-
19	Terpinolene	1080	tr	0.10±0.09	5.90±8.21	0.13±0.18
20	1,6-Octadien-3-ol, 3,7-dimethyl-	1094	-	0.32±0.30	6.12±8.51	2.00±2.61
21	Benzene, 1-ethyl-2,3-dimethyl-	1096	tr	0.14±0.14	0.35±0.87	tr
22	Benzene, 1,2,4,5-tetramethyl-	1101	tr	0.15±0.14	0.40±1.01	tr
23	5,6-dehydrocamphor	1104	0.22±0.09	0.15±0.27	3.19±8.42	0.10±0.14
24	p-Mentha-1,3,8-triene	1106	tr	tr	0.37±1.03	tr
25	Allo-Ocimene	1113	tr	0.24±0.44	0.15±0.21	0.37±0.57
26	Disulfide, methyl (methylthio)methyl	1119	4.12±0.49	tr	tr	-
27	(Z)-p-Menthen-2-en-1-ol	1125	36.14±0.43	0.15±0.25	0.41±1.06	7.22±5.89
28	Terpineol, cis-β-	1133	0.98±0.01	0.25±0.23	0.11±0.14	8.10±6.36
29	(E)-p-Menthen-2-en-1-ol	1137	tr	6.10±7.95	5.96±8.29	3.67±0.62
30	Benzene, 1-ethenyl-4-methoxy-	1158	tr	tr	0.20±0.44	0.36±0.44
31	Ethanone, 1-(2-methylphenyl)-	1176	tr	0.33±0.64	0.59±1.59	5.47±8.30
32	γ-Terpineol	1182	tr	tr	tr	0.18±0.23
33	Isopentyloxyethyl acetate	1184	tr	0.26±0.49	tr	tr
34	α-Terpineol	1191	tr	-	0.19±0.46	0.49±0.43
35	(L)-α-Terpineol	1191	tr	-	1.66±3.94	0.40±0.26
36	Ethanone, 1-(3-methylphenyl)-	1192	tr	0.31±0.60	0.73±1.58	tr
37	Phenol, 2,4,6-trimethyl-	1202	tr	-	1.61±4.47	0.20±0.31
38	(1S,4S)-Dihydrocarvone	1202	tr	0.24±0.46	tr	-
39	Undecanal	1290	tr	-	0.11±0.29	-
40	Citronellyl Acetate	1336	tr	0.10±0.09	tr	tr
41	2,3-Dimethyldodecane	1346	tr	tr	0.10±0.19	0.20±0.32
42	(E,Z)-jasmone	1349	tr	tr	0.29±0.62	tr
43	Tridecane, 7-methyl-	1353	tr	tr	0.19±0.19	0.23±0.34
44	α-Longipinene	1360	0.21±0.05	tr	0.44±1.20	tr

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45	Pentanoic acid, heptyl ester	1364	tr	tr	0.14±0.40	tr
46	Cyclosativene	1367	0.13±0.00	0.83±1.60	0.49±1.36	tr
47	2-Octenal, 2-butyl	1367	1.60±0.05	0.20±0.35	0.15±0.23	0.11±0.15
48	Copaene	1367	tr	0.17±0.24	0.58±1.59	tr
49	α-Cubebene	1374	tr	0.90±1.71	0.48±1.29	tr
50	Di-epi-α-cedrene	1384	tr	0.88±1.56	0.18±0.49	tr
51	α-Cedrene	1384	tr	tr	0.19±0.53	0.47±0.76
52	β-Cubebene	1394	tr	tr	0.22±0.59	0.56±0.74
53	Acetic acid, decyl ester	1397	0.42±0.02	1.93±3.45	tr	0.40±0.61
54	2H-Pyran-2-one, 6-pentyl-	1408	tr	2.96±5.53	tr	2.74±4.38
55	β-Patchoulene	1408	tr	0.14±0.17	0.20±0.50	tr
56	Terpinyl propionate	1418	tr	2.67±5.10	tr	-
57	Lynalyl butyrate	1429	tr	tr	tr	0.84±1.28
58	β-Gurjunene	1429	tr	tr	0.44±1.20	tr
59	β-Santalene	1432	tr	0.32±0.39	tr	tr
60	β-Selinene	1432	tr	0.37±0.64	tr	-
61	α-Caryophyllene	1443	tr	0.24±0.22	tr	0.13±0.17
62	Spathulenol	1444	tr	tr	0.46±1.26	tr
63	α Himachalene	1450	tr	0.50±0.66	-	tr
64	Acetophenone, 4'-hydroxy-	1453	tr	0.41±0.72	-	tr
65	β-Humulene	1458	tr	0.52±0.71	tr	tr
66	α-Santalene	1459	0.42±0.1	3.71±5.86	0.16±0.16	0.97±1.47
67	11-Dodecenol	1461	tr	0.56±0.74	tr	0.13±0.21
68	γ-Gurjunene	1470	tr	0.39±0.73	tr	0.12±0.12
69	Butanoic acid, 3-methyl-, 1-ethenyl-1,5-dimethyl-4-hexenyl ester	1471	20.78±0.66	3.74±6.58	0.25±0.40	1.59±1.32
70	(E)-Isoeugenol	1474	tr	0.15±0.19	0.12±0.29	0.12±0.18
71	β-Chamigrene	1475	tr	0.20±0.29	0.10±0.27	1.42±2.23
72	10-Dodecenol	1479	tr	1.02±1.74	tr	0.24±0.34
73	β-Guaiene	1484	tr	0.25±0.43	0.14±0.25	1.52±1.96
74	γ-Cadinene	1488	tr	tr	0.14±0.24	0.66±0.99
75	α-Bisabolene	1494	tr	0.76±1.00	tr	0.64±1.01
76	Valencene (isomer R)	1495	tr	0.33±0.38	tr	0.56±0.84
77	Valencene (isomer S)	1497	tr	0.77±0.81	tr	tr
78	β-Bisabolene	1500	tr	0.64±1.18	tr	tr
79	2,4-Dodecadienal, (E,E)-	1502	0.34±0.00	0.26±0.37	tr	tr
80	(Z,E)-α-Farnesene	1505	tr	0.52±0.49	0.41±1.08	tr
81	α-Muurolene	1508	tr	tr	2.09±5.77	tr
82	δ-Guaiene	1508	tr	0.14±0.19	tr	tr
83	Epizonarene	1522	tr	tr	2.21±6.14	tr
84	Sesquiphellandrene	1522	0.21±0.04	0.80±1.34	0.12±0.32	tr
85	(+)-Sativene	1523	tr	1.63±2.51	-	5.71±9.07
86	Hedycaryol	1528	tr	tr	tr	0.56±0.88
87	Isocadiene	1534	tr	tr	2.72±7.53	tr
88	Butanoic acid, 3,7-dimethyl-6-octenyl ester	1536	tr	1.08±1.07	tr	0.14±0.08
89	Eudesma-3,7(11)-diene	1538	tr	tr	2.83±7.41	tr
90	β-Himachalene	1561	tr	0.12±0.19	0.58±0.79	0.25±0.38
91	Nerolidol	1567	tr	0.28±0.51	tr	2.00±3.16
92	Caryophyllene oxide	1576	tr	0.36±0.58	0.16±0.20	tr
93	β-Elemenone	1578	tr	0.51±0.91	0.22±0.48	tr
94	Carotol	1583	-	0.11±0.11	1.13±3.16	tr

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95	Boronia butenal	1586	tr	2.63±4.99	tr	tr
96	Germacrene B	1589	tr	0.28±0.25	0.20±0.31	tr
97	Dodecan-1-yl acetate	1590	0.39±0.01	0.11±0.10	0.20±0.35	tr
98	Guaiol	1597	tr	0.11±0.10	3.98±9.16	0.10±0.07
99	Cedrenol	1607	1.17±0.55	1.19±1.76	0.27±0.72	tr
100	α-Eudesmol	1607	tr	0.14±0.14	0.28±0.76	tr
101	Cubenol	1611	0.20±0.01	0.48±0.29	0.20±0.54	0.24±0.34
102	Hinesol	1620	tr	0.37±0.58	3.55±9.82	0.53±0.83
103	Ledol	1620	0.30±0.01	0.45±0.42	0.52±1.44	tr
104	γ-Eudesmol	1625	0.10±0.00	tr	3.88±5.41	tr
105	β-Homocyclocitral	1627	-	0.31±0.47	-	-
106	τ-Cadinol	1642	tr	0.32±0.60	3.67±9.02	tr
107	Geranyl valerate	1648	2.49±0.09	tr	tr	tr
108	Blumenol C	1673	tr	1.60±3.05	tr	tr
109	2(1H)-Quinolinone, 1-methyl-	1673	tr	tr	3.53±9.77	0.19±0.30
110	δ-Cadinol	1678	tr	tr	0.54±0.87	-
111	Phenol, 3-methyl-5-(1-methylethyl)-, methylcarbamate	1693	tr	0.72±1.17	tr	0.17±0.27
112	Cedren-13-ol, 8-	1692	0.22±0.01	0.22±0.40	tr	-
113	(Z,E) Farnesyl acetate	1699	1.04±0.23	0.51±0.50	tr	tr
114	1-Heptadecene	1711	tr	0.18±0.22	tr	tr
115	(E,E) Farnesal	1718	tr	0.32±0.59	-	-
116	Solavetivone	1817	tr	0.18±0.34	-	tr
117	(E,E) Farnesyl acetate	1833	tr	1.11±2.09	-	tr
118	Rimuene	1905	tr	tr	0.35±0.93	-
119	Kaur-16-ene, (8-β,13-β)-	2015	tr	0.15±0.19	tr	tr
120	Epimanool	2019	2.25±0.07	tr	0.10±0.27	tr
121	(E,E) Farnesyl lactone	1920	0.21±0.08	-	tr	-
122	Farnesylacetone	1926	0.29±0.07	1.70±3.26	-	tr
123	Geranyllinalool	2025	tr	0.38±0.44	tr	tr
124	Kaur-16-ene	2046	1.56±0.34	1.03±1.79	0.10±0.27	tr
	SUM		96.68	98.09	91.75	98.65
	Monoterpenoids		40.55	39.68	39.56	60.17
	Monoterpene hydrocarbons		1.76	26.92	21.66	29.91
	Oxygenated monoterpenes		38.79	12.76	17.90	30.26
	Diterpenoids		3.78	1.56	0.55	-
	Diterpene hydrocarbons		1.53	1.18	0.45	-
	Oxygenated diterpenes		2.25	0.38	0.10	-
	Sesquiterpenoids		6.96	23.54	30.64	17.73
	Sesquiterpene hydrocarbons		0.34	10.97	8.66	12.41
	Oxygenated sesquiterpenes		6.62	12.57	21.98	5.32
	Others		45.39	33.31	21.00	20.75

[1]The components are reported according to their elution order on apolar column; [2]Linear Retention indices measured on apolar column; [3]Percentage mean values of AR components; [4]Percentage mean values of AB components; [5]Percentage mean values of GI components; [6]Percentage mean values of RO components; Abbreviations: -=Not detected; tr=traces (mean value <0.1% TIC).

3.3. Antibacterial activity of EOs and NPs

In this work, the antibacterial action of AgNPs and EOs of Spruce (AB), Orange (AR), Juniper (GI) and Rosemary (RO) was investigated by determining the MIC and MBC (Figure 4). In all the tests performed, the viability control with DMSO alone showed that this substance (used to make the

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oils soluble and to disperse the nanoparticles) did not interfere with the biological action observed by the test molecules.

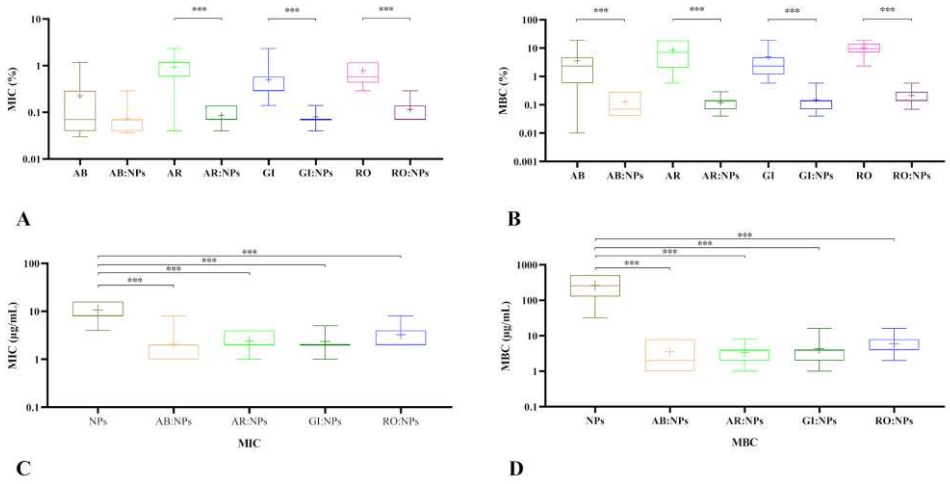

Figure 4. Determination of MIC and MBC. A) comparison of MIC values of EOs alone and associated with NPs; B) comparison of MBC values of EOs alone and associated with NPs; C and D) comparison of MIC and MBC values recorded with NPs alone and in combination with EOss. Results are expressed as % for EOs and µg/mL for AgNPs; each strain was analysed in triplicate. Results are statistically significant at $p<0.05$ (***: $p<0.0001$).

The results in Figure 4 show that both AgNPs and EOs succeed in inhibiting and killing SP strains. AB was statistically different from AR, GI, and RO, with *p*-values of 0.01, 0.0107, and 0.001, respectively. The mean values were found to be quite low, but the dispersion of the data must be considered due to the response of each strain studied (each triplicate MIC value was identical); in particular, AB, AR, and RO had an average MIC of 0.23±0.3%, 0.93±0.63%, and 0.79±0.39%, respectively.

By combining the EOs with AgNPs, the MIC values decrease statistically significantly for all oils except AB. Comparing the MICs of the enhanced oils with each other, only the AB-RO pair results in a statistically significant difference with $p=0.0301$. Similarly, the MBC values for the oils analysed alone were significantly different when comparing AB-AR ($p=0.0488$), AB-RO ($p=0.0004$) and GI-RO ($p=0.0184$). Again, the oil with the highest bactericidal ability was AB, with mean MBC values of 3.56±4.87%, followed by GI, AR, and RO for which the respectively MBCs were 4.93±6.27%, 8.53±7.2%, and 10.34±5.31%. The association with AgNPs exerted a synergistic action improving the bactericidal abilities of all EOs, which were significantly different from those recorded before the enhancement. Comparing the MBCs of the blend EOs, only the AB-RO pair resulted in a statistically significant difference ($p=0.0343$). Finally, the MIC and MBC values for AgNPs examined alone were 10.74±4.23 g/mL and 261.05±172.74 g/mL, respectively.

4. Discussion

From a One Health and Global Health perspective, the global increase in prevalence of MDR and, more broadly, MRSP strains isolated from both cases of canine pyoderma and human infections are of growing interest in the public health landscape. If this fact is also associated with the limited pharmacological choice resulting from the lack of new antibiotic molecules, developing alternatives to classic antimicrobial therapy must be considered. Within the realm of novel therapeutic possibilities, the rediscovery of alternative treatments based on the use of natural ingredients and the creation and characterization of nanotechnologies are piquing the scientific community's attention.

SP is currently considered a zoonotic agent, despite its isolation in human medicine being greatly overestimated. Close contact between pets (particularly dogs and cats), owners, and other people (such as veterinarians who work with small animals) may enhance the pathogen's probability of

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adapting to humans. Most SP infections in humans are related to skin and soft tissue infections (SSTI), infections associated with medical equipment, and invasive infections, particularly in hospitalized patients who have had past contact with pets[8,23–26]. MRSP is recognized to have dogs as its major reservoir, as opposed to MRSA strains, which have humans as their reservoir. Compared to MRSA, the rise of MRSP in dogs and its zoonotic transmission to humans is a serious public health concern. This work revealed some fairly concerning epidemiological statistics on the spread of MDR strains. The antibiogram findings suggest a scenario of widespread resistance, making it difficult for the veterinarian to select the optimal antibiotic to administer. Because all strains were *mecA*-positive, no *β*-lactam antibiotics may be used to treat infection with this disease. The only two compounds all strains were susceptible to were amikacin (aminoglycoside) and rifampicin (rifamycins), both nephrotoxic and hepatotoxic. The resistance profile found in this work is consistent with that obtained in another Italian investigation that examined MRSP and MSSP strains isolated from dogs in Milan and Naples [27].

Plants have always played a major role in pharmacological panacea, with the earliest written evidence dating back to ancient civilisations (e.g., Greeks, Egyptians, and Romans). Their use as "complementary therapies" has never waned, and their use as a supportive therapy remains evident in human medicine today [28]. Due to their various biological features, they have recently been rediscovered as possible solutions to widespread antibiotic resistance.

The role of EOs against the most common pathogens has been investigated extensively[29–35]. On the other hand, data on their efficacy against SP are scarce. The results of this work are quite comforting promising and confirmed those of the little limited available literature. Indeed, in different studies, the MIC value of RO towards *S. pseudintermedius* was 0.5% [36]. For *S. aureus*, multiple results are present: 1.5% [37]; 1.5-3.6% [38]; 2% [39]. Fu et al., found a MIC value for *S. aureus* and *S. epidermidis* of 0.125% and 0.250%, respectively [40]. No data from similar studies using the same pathogen are available for other EOs used in this work. Only one Italian study (Nocera et al., 2020) demonstrated that MRSP strains isolated from dogs are susceptible to the action of some EOs, albeit different ones [41]. As regards the main constituents of the four EOs tested, although their specific activity against SP has not yet been documented, some of them, such as *p*-menthenols, have already shown a significant antimicrobial potential against some pathogenic strains [42] or have been recognized (e.g., 1,6-octadien-3-ol, 3,7-dimethyl-, cis-*β*-terpineol, *a*-santalene or guaiol) as responsible for the antibacterial activity of the EOs in which they were present in greater quantities [43–46]. However, it is now well established that minor active compounds coexisting in essential oils can contribute to giving them the registered antimicrobial activity [47]

Metallic silver, as a drug, has accompanied human life for centuries. Its use as a medicine was already known to the Persians, who covered water cisterns with thin sheets of this metal to limit the growth of pathogenic bacteria and ward off epidemics. The Romans first included this metal in their pharmacopoeia, describing its antibacterial and healing qualities. Today, this metal is used to synthesize NPs which might be applied as alternatives to antibiotics due to their microscopic nano-scale size (1-100 nm) and large surface area [7]. In the present study, NPs were synthesized using a chemical method which allowed to obtain particles of different symmetries. This leads to the second limitation found in this work that concerns the lack of homogeneity in the morphology of the particles obtained, as described in the results (and confirmed by other authors); the failure to control the pH of the reaction favored the synthesis of multiple morphologies which, nevertheless, exerted a bactericidal action certainly superior to one-dimensional colloidal solutions. This approach is not certainly free of potential errors, but offers better results when compared to plant- or bacteria-mediated biological synthesis [7].

5. Conclusions

In this *in vitro* study, we investigated the antimicrobial activity of four EOs and AgNPs, both individually and in combination, against multidrug (MDR) and methicillin-resistant (MRS) Staphylococcus *pseudintermedius* (SP). The promising results showed that either EOs, AgNPs, and EOs+AgNPs have antibacterial activity.

The widespread prevalence of MDR and MRSP strains isolated from humans and companion animals are growing public health concerns. The antimicrobial potential of EOs and plant-derived compounds with nanoparticles (NPs) to treat bacterial infections may be investigated from a One Health and Global Health perspective in order to broaden their application in all infectious diseases where antibiotics, even the most recent ones, are ineffective.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: S/V ratio.

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