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Chemical Composition and Antimicrobial Efficacy of Three Tunisian Oils Against Multidrug-Resistant Bacteria

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Abstract

The marine ecosystem was considered a significant receptor site for environmental contamination, particularly with bacteria. Microbiome from Posidonia oceanica leaves collected from the central-eastern of Tunisia (Coastal of Mahdia) was identified using 16S rRNA gene sequencing. Susceptibility to several antimicrobial agents was studied using the microdilution method. Essential oils extracted from three Tunisian plants, *Syzygium aromaticum, Juniperus phoenicea*, and *Cupressus sempervirens*, were tested against the identified strains. A microdilution checkboard assay displayed the combination of essential oils and amoxicillin. *Staphylococcus arlettae* (MN889255.1) and *Bacillus sp.* (MG591719.1) were isolated from the microbiome of seagrass meadows (Posidonia oceanica) leaves. These isolates were multidrug-resistant bacteria. Essential oils extracted from *J. phoenicea* exhibited the highest antibacterial activity. Combining this essential oil with amoxicillin showed an important action against *Bacillus sp* and *S. arlettae* isolates. These natural products showed promising activity in reating or preventing fish infections to reduce the use of conventional antibiotics in marine ecosystems. EOs could be used to avoid and/or treat fish infectious diseases and could promise a reduction in conventional antibiotics in aquaculture.

Keywords: Environmental pathogenic bacteria; multidrug-resistant bacteria; antibacterial effects; Essential oil

1. Introduction

The appearance of multi-resistant bacteria (ARB) has become a global health problem. The over use and misuse of antimicrobials in fish farming has resulted in an essential increase in ABR isolated in the aquatic environment. This environment is considered a reservoir of ARB and is implicated in spreading resistance genes and their transfer to humans [1].

In Tunisia, antibiotics used directly in seawater to treat bacterial infections in intensive aquaculture [2] increased antibiotic concentration in coastal zones. Drug residues and antibiotics are revealed in areas surrounding farms in sediment, water, fish, and aquatic plants [2-5]. Thus, there is growing interest in developing novel strategies based on effective natural substances against ARB. Some works reported the promoter antibacterial effect of EOs and their significant products on fish infection bacteria [6]. EOs could be used to prevent and/or treat fish infectious diseases and could promise the reduction of conventional antibiotics use in aquaculture.

Seagrass meadows can also be a reservoir of resistant bacteria. Posidonia oceanica (Neptune grass), a slow-growing endemic plant, is the Mediterranean Sea's most commonly present seagrass meadows. Seagrass meadows provide many ecosystem services as they are essential in controlling microbial pathogens in the water. They provide food, shelter, and nurseries for many species. Moreover, many microbial interactions may occur in their rhizosphere [7]. Data about the leaf microbiome is scant.

Due to the emergence of multidrug-resistance, alternatives to conventional antimicrobial therapy are needed. This study aims to evaluate the combined antibacterial activity of essential oils extracted from *Syzygium aromaticum*, *Juniperus phoenicea*, and *Cupressus sempervirens* against multidrug-resistant bacteria isolated from *Posidonia oceanica* leaves.

2. Materials and Methods

2.1. Plant material and isolation of essential oils

Essential oils were extracted from fresh aerial parts of three plants, *Syzygium aromaticum*, *Juniperus phoenicea*, and *Cupressus sempervirens*, collected in March (2022) using the hydro distillation method by Clevenger. Briefly, 800g of plants

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were added to 4 liters of distilled water in a 5 liter flask. The set was placed in a balloon heater attached to a refrigerator to ensure condensation of essential oils for 3 hours. Due to the effect of hot water, the essential oils are separated from the oil glands. The extraction temperature is consistently below 100°C to avoid the evaporation of both water and oil. Two phases were obtained: an aqueous phase (aromatic water) and an organic phase (essential oil). The organic phase was collected and dried under anhydrous sodium sulphate. Experiments were conducted thrice, and the mean values of the yield and standard deviation were determined.

The yields of essential oil were given in g relative to 100 g of dry vegetable matter and calculated according to the following equation:

$$\textbf{Yield}\ (\%) = \frac{\textbf{amount of extracted oil (g)}}{\textbf{amount of dry vegetal matter mass (g)}} \times \mathbf{100}$$

2.2. Characterization of essential oils by Gas chromatography-mass spectrometry (GC-MS)

GC-MS (GC-MS 7890 system, Agilent, USA) was used to analyze and identify the chemical constituents of GEO. The mixture was separated into a DB-5MS column (30 m × 0.25 mm × 0.25 µm). The analysis conditions were as follows: The oven temperature was programmed at 50 ºC (1 minute) to 260 ºC at a rate of 5 ºC/min and held isothermally for 10 minutes. The injector temperature was set to 250 ºC, and helium was used as carrier gas. The spectrometer was operated in electron-impact (EI) mode, ionization energy was 70 eV, ion source temperature was 280 ºC, and scan time was 1 s and mass range 50–550 amu.

The different volatile compounds were identified by matching their mass spectral fragmentation patterns with corresponding data (NIST 14 Mass Spectral and Wiley Registry™ of mass spectral data) and by comparison with authentic components. The peak areas were used to obtain the total percentage composition of the essential oils.

2.3. Samples collection of Posidonia oceanica leaves.

Posidonia oceanica leaves were collected on the northern coast of Mahdia, Tunisia, at Rejiche cost (35°47'20.46" N, 11°05'37.14" E) in the winter of 2020 (Figure 1).

Samples were transported to the laboratory in seawater from the exact location in glass sterile bottles at +4°C. Bacterial analyses were performed a maximum of 1h after the sampling. Sampling was done in a tetraplicate at the same site.

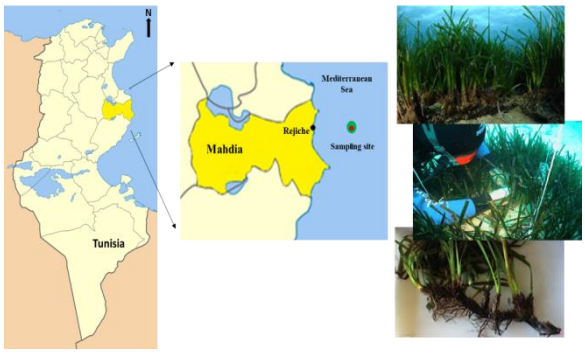

Fig. 1. Study area of the location along the Rejiche coast (Central eastern coast of Tunisia)

2.4. Bacterial isolation

The samples were subjected to a surface-sterilization protocol adapted from Garcias-Bonet [8]. Afterward, they were aseptically fragmented in small pieces, placed in 250 mL flasks containing the enrichment marine media such as H_3BO_3, 0.022 g/L; NH_4NO_3, 0.0016 g/L; $CaCl_2$, 1.8 g/L; $SrCl_2$, 0.034 g/L; Yeast Extract, 1 g/L; $C_6H_5FeO_7$, 0.1 g/L; $MgCl_2$, 8.8 g/L; Peptone, 5 g/L; KBr, 0.08 g/L; KCl, 0.55 g/L; NaCl, 19.4 g/L; NaF, 0.0024 g/L; $NaHCO_3$, 0.16 g/L; Na_2HPO_4, 0.008 g/L; Na_2SiO_3, 0.004 g/L; Na_2SO_4, 3.24 g/L; pH: 7.6±0.2, and incubated at 25°C on a rotary shaker at 120 rpm for 3 days. The pure bacterium was isolated from the enrichment culture using serial dilution onto marine agar plates.

2.5. Molecular identification of the isolates

Total genomic DNA from the pure strains was extracted, lysed in distilled water by phenol-chloroform method, and precipitated with ethanol, as previously described [9]. Molecular identification was based on 16S rRNA gene amplification by using the universal primers 5′-S-D-Bact-0008-a-S-20-3′ and 5′-S-D-Bact-1495-a-S-20-3′ [9]. The PCR reaction was performed in a 30 µL reaction mixture consisting of PCR buffer (1X), $MgCl_2$ (2 mM), 0.25 mM of each dNTP, 0.25 µM of each primer, 1 µg of chromosomal DNA, and 1 U of Taq DNA polymerase. The PCR program involved an initial step at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 45 s, annealing for 1 min at 55 °C and elongation for 2 min at 72 °C, followed by a final extension step at 72 °C for 8 min. PCR products were migrated on standard 1.5% agarose gel in 0.5× Tris–borate–EDTA buffer, stained for 30 min in 0.5 mg/l ethidium bromide solution, and visualized under UV light. The 16S

rRNA fragments are sequenced and compared with those published in the NCBI database using the BLAST program, as by Hassen [9]. A phylogenetic dendrogram was developed utilizing the neighbor join method and tree topology for bootstrap analysis of 1000 datasets using MEGA 6 [10].

2.6. Antibiotic susceptibility testing

The MICs levels were obtained using the microdilution method according to the European Comity of Antimicrobial Susceptibility Testing (EUCAST, 2021). For *Staphylococcus sp*, the following antimicrobial agents were tested: Ciprofloxacin (CIP), Amikacin (AK), Kanamycin (K), Azithromycin (ATH), Erythromycin (ERY), Trimethoprim (TM), Cefotaxime (CTX), Rifampicin (RP) and Tetracycline (TET).

Bacillus sp. has shown suspect ability to Aztreonam (ATM), Imipenem (IMP), Ciprofloxacin (CIP), Chloramphenicol (C), Erythromycin (ERY), and Ceftazidime (CAZ).

2.7. Screening of EO activities

Antimicrobial activities of EOs were determined using the disc diffusion assay. Sterile blank discs of 6 mm diameter were impregnated with 10 µL of essential oil solution. They were placed on MH agar plates previously inoculated with fresh bacterial culture (106 CFU/mL). The plates were incubated at 30°C for 24 h. The diameter of the inhibition zone was measured.

2.8. Microdilution Assay

The MIC concentration, defined as the lowest concentration of EOs able to inhibit bacterial growth visibly was determined using the microdilution method described by Alibi [11].

2.9. Microdilution checkerboard assay

The microdilution checkerboard technique was used to assess the antimicrobial combinations as previously described [12, 13]. Serial dilution of EOs and amoxicillin were made, and then combined interactions of 2 EOs, 3 Eos, and EO with amoxicillin were examined. Plates were incubated at 37 °C for 24 hours, and then bacterial growth was evaluated. Fractional inhibitory concentrations (FIC) were determined using the following equation [14].

FIC= (MICA in combination with B)/(MICA alone)+ (MIC B in combination with A)/(MICB alone)

Results were interpreted as total synergism (FICI ≤0.5), partial synergism (0.5 < FICI ≤0.75), no effect (0.75 < FICI ≤2), or antagonism (FICI > 2). The FIC indices were calculated using EXCEL software. This assay was repeated three times (Where A and B are two different compounds).

3. Results and discussion

3.1. Chemical composition of Essential oils

The chemical composition of the three essential oils is shown in (Table 4). Thirteen constituents were identified in *Syzygium aromaticum* EO, accounting for 100% of the total essential oil composition, which was dominated by a high level of oxygenated monoterpenes (79.9%). Crysanthenone was the primary compound (33.9%), followed by eugenol (9.1%), Limonene (9.0%), and Piperitenone oxide (9.6%). Twelve constituents were identified in *Juniperus phoenicea* EO, making 100% of the total essential oil composition, dominated by oxygenated monoterpenes (30.0%) and monoterpene hydrocarbons (25.3%). The major components of this oil are menthone (17.3%), α-pinene (16.8%), germacrene D (16.5%), and pulegone (13.2%). *Cupressus sempervirens* EO was marked by a high amount of oxygenated monoterpenes (68.8%), succeeded by sesquiterpenes hydrocarbons (28.9%). Linalool (62.1%) and α-Farnesene (25.1%) were the major constituents among the seven identified volatile components, representing 100% of the total oil content.

It can be noticed that substantial amounts of monoterpenoids characterize the three studied EOs.

3.2. Identification of bacterial strains

Identification of the microbiome isolated from seagrass leaves showed a high microbial diversity. Sequencing results showed that leaves were mostly colonized by Staphylococcus arlettae MN889255.1 (99.28% identity level) (POL-1) and Bacillus sp. MG591719.1 (98.77 % identity level) (POL-2) (Figure 2).

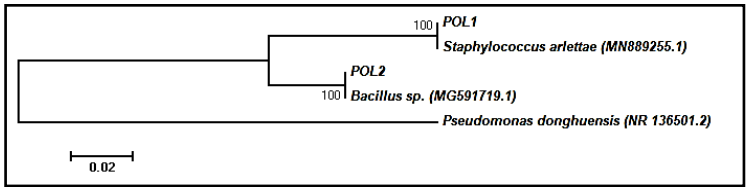

Fig. 2. Phylogenetic tree based on the 16S rRNA sequences of strains *Staphylococcus arlettae* and *Bacillus sp.* GenBank accession numbers are given in brackets. Bootstrap values obtained with 1000 repetitions were indicated as percentages at all branches.

3.3. Antimicrobial susceptibility

A total of twenty isolates of **Staphylococcus arlettae** (10) and *Bacillus sp*. (10) were randomly selected for antimicrobial assays (Table 1). All *S. arlettae* isolates were sensitive to vancomycin; however, they were highly resistant to Amikacin, Kanamycin, Rifampicin, and Tetracycline, while All *Bacillus sp.* strains were sensitive to vancomycin and daptomycin and resistant to Ciprofloxacin, Chloramphenicol, Erythromycin, and Ceftazidime.

Table 1. Antimicrobial susceptibility profile of the studied isolates

Isolates	Species	Antimicrobial resistance profile
SA-1	*S. arlettae*	CIP, AK, K, ATH, ERY, TM, CTX, RP, TET.
SA-2	*S. arlettae*	CIP, AK, K, ATH, ERY, RP, TET.
SA-3	*S. arlettae*	CIP, AK, K, ATH, ERY, RP, TET.
SA-4	*S. arlettae*	CIP, AK, K, ATH, ERY, TM, CTX, RP, TET.
SA-5	*S. arlettae*	CIP, AK, K, ATH, ERY, RP, TET.
SA-6	*S. arlettae*	CIP, AK, K, ATH, ERY, RP, TET.
SA-7	*S. arlettae*	CIP, AK, K, ATH, ERY, TM, CTX, RP, TET.
SA-8	*S. arlettae*	CIP, AK, K, ATH, ERY, TM, CTX, RP, TET.
SA-9	*S. arlettae*	CIP, AK, K, ATH, ERY, TM, CTX, RP, TET.
SA-10	*S. arlettae*	CIP, AK, K, ATH, ERY, TM, CTX, RP, TET.
B-1	*Bacillus sp.*	ATM, IMP, CIP, C, ERY, CAZ.
B-2	*Bacillus sp.*	ATM, IMP, CIP, C, ERY, CAZ.
B-3	*Bacillus sp.*	ATM, IMP, CIP, C, ERY, CAZ.
B-4	*Bacillus sp.*	ATM, IMP, CIP, C, ERY, CAZ.
B-5	*Bacillus sp.*	ATM, IMP, CIP, C, ERY, CAZ.
B-6	*Bacillus sp.*	ATM, IMP, CIP, C, ERY, CAZ.
B-7	*Bacillus sp.*	ATM, IMP, CIP, C, ERY, CAZ.
B-8	*Bacillus sp.*	ATM, IMP, CIP, C, ERY, CAZ.
B-9	*Bacillus sp.*	ATM, IMP, CIP, C, ERY, CAZ.
B-10	*Bacillus sp.*	ATM, IMP, CIP, C, ERY, CAZ.

CIP: Ciprofloxacin, AK: Amikacin, K: Kanamycin, ATH: Azithromycin, ERY: Erythromycin, TM: Trimethoprim, CTX: Cefotaxime, RP: Rifampicin, TET: Tetracycline, ATM: Aztreonam, IMP: Imipenem, C: Chloramphenicol, CAZ: Ceftazidime.

3.4. Determination of DIZs and MICs

The antimicrobial activity of the essential oils against studied microorganisms were assessed by determining the zones of inhibition and MIC values and the results observed are displayed in (Table 2).

According to the classification reported by Rota and al. [15], it is considered strong activity when the inhibition zone ≥ 20 mm, moderate activity if the inhibition zone < 20-12 mm and weak or no inhibition when zone < 12 mm. Accordingly, the results of our experiments showed that both essential oils had weak to strong antibacterial potential against the tested microorganisms.

In fact, *Cupressus sempervirens* (Cypress) EO didn't show high antibacterial effect against both *S. arlettae* and *Bacillus sp* isolates (Figure 3) since the diameter inhibition zones (DIZs) were lower than 9 mm. the MICs ranged between 0.5µg/ml and 0.9µg/ml.

Syzygium aromaticum (Clove) exhibited weak to moderate antibacterial potency. As can be seen the DIZs ranged between 10 mm to 14 mm for all the isolates (Figure 3). The MICs ranged between 0.5 µg/ml and 0.06µg/ml. The activity of *Juniperus phoenicea* (Juniper) EO was significantly different from the above species. Indeed, This EO showed an important activity against *S. arlettae* isolates (Table 2). The DIZs were higher than 19mm and MICs ranged between 0.06 µg/ml and 0.001µg/ml (Figure 3). However, Juniper EO exhibited a low activity against *Bacillus sp* for which DIZs did not exceed 10mm and MICs were between 1 and 0.25µg/ml (Table 2).

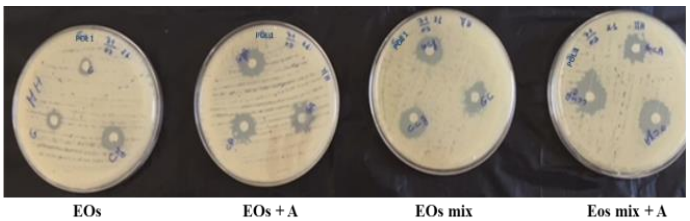

Fig. 3. Aromatograms of Essential Oils (Eos) combined with 100 mg/L Amoxicillin (A) obtained with *Staphylococcus arlettae* and *Bacillus sp*

Table 2. Diameter inhibition zones and minimal inhibitory concentrations obtained with *Cupressus sempervirens*, *Syzygium aromaticum* and *Juniperus phoenicea* Essential oils

	Essential oils					
	Cupressus sempervirens		*Syzygium aromaticum*		*Juniperus phoenicea*	
Isolates	**DIZ(mm)**	**MIC (µg/ml)**	**DIZ(mm)**	**MIC (µg/ml)**	**DIZ(mm)**	**MIC (µg/ml)**
SA-1	9	0.5	10	1	24	0.001
SA-2	8	1	13	0.06	20	0.001
SA-3	7	1	14	0.06	20	0.001
SA-4	9	0.5	12	0.25	19	0.001
SA-5	9	0.5	11	0.5	19	0.015
SA-6	8	1	10	1	23	0.001
SA-7	7	1	12	0.5	20	0.015
SA-8	7	1	13	0.25	21	0.015
SA-9	9	0.5	14	0.06	20	0.007
SA-10	8	1	12	0.25	19	0.007
B-1	8	1	14	0.06	9	0.5
B-2	7	1	11	0.5	11	0.1
B-3	8	1	13	0.125	10	0.5
B-4	8	1	10	1	8	1
B-5	8	1	12	0.25	9	1
B-6	9	0.5	12	0.25	10	0.5
B-7	8	1	13	0.125	12	0.25
B-8	9	0.5	11	0.5	10	1
B-9	7	1	13	0.125	10	1
B-10	8	1	14	0.06	9	1

DIZ: Diameter inhibition zones in (mm), **MIC:** minimal inhibitory concentrations in (µg/ml)

3.5. Microdilution checkboard assay

Studies with drugs in combination with natural products are now a common practice to try and revert bacterial resistance, which may permit an improvement in antibiotic activity or even revert the resistant condition [16].

FIC values observed a total synergetic effect for Cypress-Clove EOs and Juniper-Clove EOs combinations against all *Bacillus sp.* Strains, while no effect was observed for Cypress and Juniper EOs combinations tested for *Bacillus sp.* On the other hand, most of EOs combinations showed a partial synergy against *S. arlettae* strains. The following combinations: Juniper EO - amoxicillin, Clove EO- amoxicillin, and Cypress EO- amoxicillin exhibited a partial or total synergy against *Bacillus sp* and *S. arlettae* isolates (Table 3).

Table 3. Effects of combined essential oils together and amoxicillin on *Bacillus sp.* and *Staphylococcus arlettae* isolates

Combinations of Essential oils	***Bacillus sp.***	***Staphylococcus arlettae***
Cypress + Juniper	No effect	Partial synergy
Cypress + Clove	Total synergy	Partial synergy
Juniper + Clove	Partial synergy	Partial synergy
Cypress + Amoxicillin	Partial synergy	No effect
Juniper + Amoxicillin	Partial synergy	Partial synergy
Clove + Amoxicillin	Partial synergy	Partial synergy
Cypress + Juniper +Amoxicillin	Total synergy	Partial synergy
Cypress + Clove + Amoxicillin	Total synergy	Total Synergy
Juniper + Clove + Amoxicillin	Total synergy	Partial synergy

Seagrass meadows are ubiquitous marine habitats that have immense ecological and socioeconomic importance. Among their ecosystem services, they provide nutrients to fish, stabilize substrates of the sea floor, and serve as a niche for bacteria [17]. P. oceanica is the most commonly present seagrass meadows in the Mediterranean bowl. Unfortunately, the data about the leaf surface microbiome of *P. oceanica* are scant.

Previous works focused on bacterial endophytes [8] and the root system [18-20]. In 2017, two studies the northern hemisphere's coastal regions focused on the leaf microbiome of Zostera marina [21, 22].

Recently, Kohn et al. [23] studied the microbiome of *P. oceanica* leaves collected from Corsica Island in the western Mediterranean Sea. They found that young and aged *P. oceanica* leaf biofilms are mainly formed by species belonging to the *Planctomycetes* [23].

In our study, seagrass meadow biofilm indicated high variability in microbial species composition, which Firmicutes mostly formed. Bengtsson et al. (2017) and Ettinger et al. (2017) also found a low abundance of members of the phylum Planctomycetes in seagrass leaves. *S. arlettae* is an emerging opportunistic pathogen increasingly recognized as the etiological agent of human and animal infections [24]. *Bacillus sp*. are implicated in food poisoning, localized infections related to trauma, and significant soft tissue infections. Several strains are involved in serious diseases, notably *B. cereus*, associated with fulminant ocular infections [25, 26]. These bacteria have shown widespread resistance to several antibiotics, arising from substantial contamination of the Rejiche coastal with antibiotics. Antibiotics are attributed to different origins, such as hospital effluent and wastewater treatment plants that discharge their waste into the sea. The works of Afsa et al. [27] revealed that 90% of the contamination levels by antibiotic compounds confirmed this hypothesis, and their fate starting from a hospital in Rejiche City (Tunisia) towards the receiving coastal area is proven. Alibi et al. [28] detected multidrug-resistant *Streptococcus faecalis* in the same region. Resistance to available antibiotics in pathogenic bacteria is currently a global challenge since (i) the appearance of pathogenic and multi-resistant bacteria increased dramatically each year, (ii) the coast of Rejiche is very frequented by swimmers and can be infected by these pathogenic bacteria and (iii) fish can also be contaminated by these bacteria especially that this area is known by its fishing activities. Once infected, it is difficult for the patient to find the antibiotic to destroy these bacteria.

To unlock this problem, using natural compounds alone or combined with conventional antibiotics may be a promising alternative.

The application of EOs showed excellent efficacy as antibacterial agents against major aquaculture pathogens, which can be linked to their chemical composition [6]. The chemical constituents of EOs can be classified structurally into four groups: terpenes, terpenoids, phenylpropanoids, and other constituents [29]. In the current research, monoterpenoids were the prominent constituents of the three EOs (79.9%. in the profile of *Syzygium aromaticum*, 50.8%. in the profile of *Juniperus phoenicea* and 68.8%. in the profile of *Cupressus sempervirens*)-followed by terpenes (17.6%. in the profile of *Syzygium aromaticum*, 32.5%. in the profile of *Juniperus phoenicea* and 31.2%. in the profile of *Cupressus sempervirens*).

Previous essential oils studies have demonstrated the antibacterial potentials of some of the individual monoterpenoids, including **eugenol, Limonene**, **Menthone, Linalool, Piperitenone Oxide, β-pinene**, **α-pinene, and pulegone**.

Marchese et al. [30] reviewed that many studies have highlighted the excellent antibacterial potential of eugenol (also called clove oil) on various microorganisms responsible for human infectious diseases. These inhibitory effects can be ascribed to the presence of free hydroxyl groups in the structure of eugenol, which makes this molecule a potential active ingredient in new and effective drugs curing of some bacterial infections. Eugenol has also significantly reduced bacterial biofilm growth in vitro and in vivo. Many multidrug-resistant bacteria isolates were found to be susceptible to eugenol.

Furthermore, numerous types of research find that *S. aromaticum* oil and its main active composition, eugenol, show safely beneficial advantages targeting common food source microorganisms, including gram-positive bacteria such as Staphylococcus, Streptococcus, and L. monocytogenes and gram-negative bacteria like E. coli, Salmonella, and P. aeruginosa and fungi as Aspergillus, Penicillium and Yeast via different mechanisms linked to the reduction of the migration and adhesion and the inhibition of virulence factors expression and biofilm formation [31].

D-limonene showed relevant clinical antibacterial activity for Gram-negative bacteria, and it had a synergistic effect when associated with gentamicin (standard antibiotic) [16, 32]. This may be due to terpenes' lipophilic character, which increases antibiotic influx into the cell by altering the cell membrane permeability. The lipophilic characteristic of terpenes causes the membrane to become more permeable to protons and ions due to structural disarrangement, including efflux proteins, which may lead to more significant interaction with the substances and cause bacterial death [16].

Božović et al. [33] have reviewed that The Mentha x villosa oil and its major component, **Piperitenone Oxide** as well as (+)-**pulegone**, showed antibacterial activity on a strain of methicillin-resistant Staphylococcus aureus.

Many studies have investigated the individual antibacterial activity of **1,8 cineole** against some gram-positive and multidrug-resistant bacteria or biofilms and proved that a combination between 1,8 cineole with other antibacterial agents demonstrated may enhance this activity in a synergistic or an additive way [34-36].

Wang et al. [37] have demonstrated that *R. officinalis L.* essential oil and its main component α-pinene possessed equal antibacterial activities on both gram-positive and gram-negative bacteria but a little bit greater than β-pinene and 1,8-cineole.

More recently, a positive isomer of α-pinene presented an inhibitory action on two antibiotic-resistant diseases, multi-resistant strains, *Staphylococcus aureus* and *Escherichia coli*. Authors recommended more detailed studies so that (+)-α-pinene can be used as a new therapeutic alternative to combat antimicrobial infections. Another research by Leite-Sampaio et al. [38], has demonstrated that (+)-α-pinene is a promising antibacterial compound and antibiotic resistance inhibitor since it significantly improved the activity of some conventional antibiotics against various multidrug-resistant bacterial strains.

Some studies have proved that *S. aureus* (gram-positive bacteria) strains were sensitive to basilic essential oil and lavender oil (linalool chemotypes) and **linalool** individually [39, 40].

A synergistic interaction of lavender essential oil and linalool combined with the antibacterial drug gentamicin was observed [40], while an additive interaction between S. aromaticum oil and linalool was observed against this bacterial strain [41].

In a recent study, Zhao et al. [42] reported menthone's antibacterial potency against methicillin-resistant *Staphylococcus aureus* (MRSA). The mechanism of action involved the alteration of membrane structural components and corresponding properties.

In the present work, *Juniperus phoenicea* and *Syzygium aromaticum* EOs showed strong and weak to moderate antibacterial potentials against *S. arlettae*. These oils exhibited a weak effect against *Bacillus sp*. On the other hand, *Cupressus sempervirens* EO revealed a weak antibacterial effect against both *S. arlettae* and *Bacillus sp* isolates.

Our findings are agree with the work of Ennajar et al. [43, 44], who found that Juniper EO exhibited a very effective bactericidal activity against many Gram-positive bacteria, including Staphylococcus aureus and Bacillus subtilis. This effect is due to their main composition, α-pinene.

Clove EO can be considered a promising inhibitor against fish bacterial pathogens [6]. The potency of clove EO is mainly due to eugenol, which showed a crucial antibacterial potency against a wide range of Gram-negative and Gram-positive bacteria, including multidrug-resistant clinical isolates [45].

Contrary to our findings, Ben Nouri et al. [46], reported that *Cupressus sempervirens* EO shows high antibacterial effect against *Bacillus subtilis*, *Escherichia coli*, *Klebsiella oxytoca*, *Morganella morganii*, *Shigella*, and *Vibrio cholerae*. Similarly, Selim et al. [47] showed that *Cupressus sempervirens* possess an antibacterial and antibiofilm activity. This potency is due to monoterpene hydrocarbons, in particular α-pinene [47].

The combination between the three EOs and amoxicillin (a penicillin antibiotic) showed a partial or total synergistic impact on the resistant bacterial isolates. As can be observed, these combinations are more antibacterial effective than their corresponding individual EOs. The synergistic effect was previously reported [48, 49] EOs can act by increasing the bacterial membrane permeability and causing damage in the cell wall [45] or by modifying its selective permeability and therefore enhances the effect of amoxicillin. These results are in agreement with the observations of Freitas et al. (2020) and Araújo and al. (2020) [50, 51].

Our results are promising in the field of formulation of new effective therapeutic agents against Gram-positive multidrug resistance bacteria for all studied EOs.

Table 4. Chemical and percentage compositions of the studied essential oils

No.	Compound[a]	Composition (%)		
		Syzygium aromaticum	*Juniperus phoenicea*	*Cupressus sempervirens*
1	2-Hexenal	-[b]	11.2	-
2	**α-pinene**	-	**16.8**	2.3
3	β-Pinene	-	4.4	-
4	1-Octen-3-ol	-	5.5	-
5	3-Octanol	-	-	3.0
6	**Limonene**	**9.6**	4.1	-
7	eugenol	**9.1**	-	-
8	**Linalool**	-	3.5	**62.1**
9	**Chrysanthenone**	**33.9**	-	-
10	Isopulegol	4.1	-	-
11	**menthone**	-	**17.3**	-
12	Isomenthol	3.9	-	-
13	**pulegone**	**7.4**	**13.2**	-
14	Piperitenone	6.7	-	-
15	**Piperitenone oxide**	**9.0**	-	2.2
16	α-Copaene	-	2.2	-
17	β-Bourbonene	-	2.7	-
18	β-Caryophyllene	3.6	-	0.8
19	α-Humulene	-	2.6	-
20	Massoia lactone	-	-	4.5
21	**Germacrene D**	2.0	**16.5**	-
22	α-Caryophyllene	2.4	-	-
23	**α-Farnesene**	-	-	**25.1**
24	Caryophyllene oxide	2.5	-	-
25	Cinerolon	5.8	-	-
26	Monoterpene hydrocarbons	9.6	25.3	2.3
27	Oxygenated monoterpeness	79.9	30.0	68.8
28	Sesquiterpenes hydrocarbones	8.0	24.0	25.9
29	Oxygentaed sesquiterpenes	2.5	0.0	0.0
30	Others	0.0	16.7	3.0
	Total oil composition	100	100	100

[a] Compounds listed in order of elution
[b] not detected

4. Conclusion

In summary, our data revealed the presence of *S. arlettae* and *Bacillus sp* in the microbiome of *Posidonia oceanica* leaves. These pathogens were highly resistant to different antimicrobial agents. Among all studied EOs, The *Juniperus phoenicea* EO exhibited the highest antibacterial activity. Combining of this EO with amoxicillin showed an essential action against *Bacillus sp* and *S. arlettae* isolates. Thus, further studies should be continued to explore the antibacterial effect of Juniper oil and its main constituents individually or and in combination with common drugs. Furthermore, other experimental procedures should be performed to find out new antibacterial agents regarding the effectiveness of combinations between essential oils and their ingredients with a broad list of common drugs.

5. Conflicts of interest

There are no conflicts to declare.

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