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Quantitative and Qualitative Variations in *Eucalyptus* Essential Oils Depending on Species and the Cultivation Location

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Abstract

Objectives: This study examined the impact of various abiotic factors, specifically temperature and precipitation, on the yield and composition (volatile profile) of essential oils (EO) from four *Eucalyptus* species, including *Eucalyptus salmonophloia*, *Eucalyptus torquata, Eucalyptus lesouefii* and *Eucalyptus astringens*. Additionally, the antimicrobial properties of these EO were assessed. **Methods:** The species were collected from five arboreta in Tunisia belonging to two climatic conditions (arid and semi-arid). EOs were extracted from the leaves using the hydrodistillation technique and analyzed by gas chromatography coupled with mass spectrometry (GC-MS). The antimicrobial activity was evaluated by measuring the diameters of inhibition zones using the agar well diffusion method and by determining the minimum inhibitory concentrations (MICs). **Results:** The yield of *Eucalyptus* EOs varied from 0.12% to 4.63% (w/w, dry weight) depending on the species and the plant's growing location. 1,8-cineole (29.71% to 67.16%) was by far the major compounds in EOs of *E. salmonophloia, E. lesouefii* and *E. astringens*, however *E. torquata* was torquatone chemotype (33.41% to 44.78%). In general, the aridity increased the extraction yield of EO. Higher temperature and lower rainfall conditions enhanced the production of key compounds such as, 1,8-cineole and α-pinene, however, it decreases others compounds like spathulenol and viridiflorol. A notable antimicrobial activity was observed against all microbial strains tested, demonstrating both microbicidal and microbiostatic effects, particularly against *Escherichia coli*, *Serratia marcescens*, and *Candida tropicalis*. The EOs derived from the studied *Eucalyptus* species represent a valuable source of bioactive compounds, including 1,8-cineole, α-pinene, spathulenol, and β-eudesmol. These compounds contribute to the oils' significant antimicrobial efficacy, offering the additional advantage of being a natural product. **Conclusion:** Our findings reinforce the notion that environmental factors may serve as a limiting factor in the production and availability of *Eucalyptus* EO for medicinal and industrial applications.

Keywords

Eucalyptus spp., essential oils, environmental factors, yield variation, chemical composition, antimicrobial activities

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Introduction

The genus *Eucalyptus* is endemic to Australia, New Zealand, and Tasmania,[1] is one of the largest and the most important aromatic and medicinal genera of the *Myrtaceae* family, comprising more than 900 species which are widespread throughout the world.[2] They have rapidly spread worldwide to countries such as India, France, Chile, Brazil, South Africa, and Portugal. The ability of certain eucalypt species to adapt to a wide range of climates (semi-desert, cold, temperate, or alpine) is one of the key factors contributing to their remarkable success as exotic trees.[3] Approximately 1% of the approximately existing species are used for industrial purposes. Currently, the timber is mainly

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used for producing hardwood fiber, as well as for constructing windbreaks, shelterbelts, and providing fuel. *Eucalyptus* plants have attracted significant global attention across a wide range of industries, including perfumery, pharmaceuticals, nutraceuticals, and furniture production. Consequently, they have become a rapidly growing source of both wood and essential oils, which are utilized for numerous applications. In fact, *Eucalyptus* volatile oils are present in various parts of the plant, with over 300 species known to contain these oils, primarily in their leaves.[4]

Owing to their wide-ranging biological activities, *Eucalyptus* EOs are extensively used across numerous industrial sectors. These oils find applications in fields such as medicine, food flavoring, spices, insecticides, and herbicides. In terms of their phytopharmacological potential, there has been a growing body of research dedicated to exploring their biological properties. Studies have revealed a diverse array of therapeutic effects, including antihyperglycemic, antioxidant, antibacterial, ulcer-healing, cytotoxic, anti-inflammatory, and analgesic properties.[5,6] Additionally, the biodegradable nature of the oil makes it a safe and effective option for the bioremediation of environmental pollutants.[7]

Previous phytochemical studies have identified the presence of various compounds, including oxygenated monoterpenes (such as 1,8-cineole, citronellol, piperitone, isopulegol, citronellal, α-terpineol, linalool, terpinyl acetate, citronellyl acetate, etc), monoterpene hydrocarbons (including α- and β-pinene, p-cymene, limonene, camphene, γ-terpinene, etc), oxygenated sesquiterpenes (such as spathulenol, caryophyllene oxide, etc), and sesquiterpene hydrocarbons (including β-caryophyllene, aromadendrene, α-copaene, bicyclogermacrene, etc).[8] The chemical composition of *Eucalyptus* oil is shaped by various factors, such as species, variety, geographical origin, and environmental conditions,[9] which in turn affect its quality, effectiveness, and intended uses. For example, the levels of important components like 1,8-cineole, α-pinene, and limonene can fluctuate considerably, influencing both the oil's therapeutic properties and its market value.[10]

Understanding the geographical and varietal origin of *Eucalyptus* EO is important for several reasons. Firstly, it helps ensure quality control and standardization, which are critical for maintaining the therapeutic effectiveness of pharmaceutical products.[11] Secondly, it supports the authentication of the EO, safeguarding consumers from adulteration and guaranteeing that they receive authentic products with consistent quality.[12] Lastly, knowledge of the origin is essential for optimizing cultivation practices, enabling the production of high-quality oil with the desired characteristics, benefiting both producers and consumers.[12]

The growing prevalence of bacterial resistance to traditional antibiotics is a global concern. The antimicrobial resistance crisis has been linked to the improper use of these medications, and resistant strains have become widespread. It is estimated that the medical cost for each patient with an antibiotic-resistant infection can reach up to $29,069, and these infections are often life-threatening.[13] Similarly, fungi have developed resistance to polyenes, azoles, and echinocandins, with drug-resistant strains being reported in all fungal species.[14] *Eucalyptus*, which includes numerous species that produce essential oils, is known for its significant antimicrobial potential, as demonstrated in various species such as, *E. salmonophloia*, *E. torquata*, *E. lesouefii*, *E. astringens, E. sideroxylon*, *E. leucoxylon* and others.[15] In fact, the medicinal value of *Eucalyptus* EO is largely attributed to its primary component, 1,8-cineole (also known as eucalyptol).[10,16]

Since 1957, 117 species of this genus have been introduced to Tunisia and acclimated in 30 arboreta distributed from the north to the south of the country.[17] The area of eucalypt plantations in Tunisia is estimated at 55,000 ha, representing 5% of total forest cover.[18] They have primarily been used as fire wood, for the production of mine wood, and in the fight against erosion.[19] *Eucalyptus* trees are also melliferous and of great economic interest.[20] In addition, they are extensively employed in traditional medicine; certain species are utilized in Tunisian folk medicine as antiseptics for respiratory tracts throughout history. The medicinal properties of *Eucalyptus* have been utilized to address various health conditions, including arthritis, asthma, burns, fever, influenza, sore throat, malaria, wounds, and pharyngitis.[21]

Taking these considerations into account, in this present study, the yield and chemical composition of the EO of various *Eucalyptus* species and influential environmental factors were analyzed to explore the diversity of the EOs and determine the law of geographic variations. Additionally, the antimicrobial activity of these oils was examined.

Materials and Methods

Plant Material

Clean and mature leaves of four *Eucalyptus* L'Hér. species, namely *E. torquata* Luehm, *E. salmonophloia* F. Muell, *E. astringens* Maiden, and *E. lesouefii* Maiden, were collected in November 2021 from five arboreta in Tunisia. The selections were based on differences in altitude and climate between the five areas. The sites can be grouped into two bioclimatic zones: semi-arid (Elhanya (SA1) and Henchir Naam (SA2)) and arid (Metouia (A1), Hamma (A2), and Zrig (A3)). Each studied species was obtained from the five arboreta, except for *E. lesouefii*, which was not collected from the Zrig arboretum, and *E. astringens*, which was not collected from the Metouia and Hanya arboreta. Geographical coordinates and climatic conditions of the stations are provided in Table 1. At each site, approximately 2 kg of leaves were collected from five trees, separated from the lignified parts, and air-dried in the shade for 15 days. The trees studied were all planted during the same period (1959-1960). Specimens were identified at the Regional Station of the National Research Institute of Rural Engineering, Water, and Forests (Gabes, Tunisia). Samples of these four species have been preserved in the station herbarium (Codes 41/2021, 42/2021, 43/2021, 44/2021, 45/2021, 46/2021, 47/2021, 48/2021, 49/2021, 50/2021, 51/2021, 52/2021, 53/2021, 54/2021, 55/2021, 56/2021 and 57/2021)

Table 1. The Geographic Characteristics and Climatic Conditions of the Studied Sites.

Site	Longitude	Latitude	Altitude (m)	Mean annual rainfall (mm)	Mean annual temperature (°C)	Bioclimatic condition
Metouia (A1)	N 33°57'52.29"	E 9°59'11.38"	30	152	20.35	Arid
Hamma (A2)	N 33°54'30.24"	E 9°39'50.46"	37	156	20.68	Arid
Zrig (A3)	N 33°43'54.23"	E 10°09'38.59"	37	150	19.48	Arid
Elhanya (SA1)	N 35°52'02.95"	E 10°21'38.13"	50	315	19.32	Semi-arid
Henchir Naam (SA2)	N 36°13'14.64"	E 9°10'35.79"	450	441	19.91	Semi-arid

Bacterial and Fungal Strains

The *in vitro* antibacterial activity of the EOs was tested against Gram-negative bacteria species *Escherichia coli* ATCC 25756 (Ec), *Pseudomonas aerogenosa* ATCC 27853 (Pa) and *Serratia marcescens* (Sm) as well as Gram-positive bacteria *Staphylococcus aureus* (Sa) and *Micrococcus luteus* (Ml). Antifungal activity was determined using two fungal species: *Candida albicans* (Ca) and *Candida tropicalis* (Ct). Mueller–Hinton media (BioRad, Mitry-Mory, France) and potato dextrose agar were used for the antibacterial and antifungal assays, respectively.

Extraction of EOs

The dried leaf samples were hydro-distilled for three hours using a Clevenger-type apparatus. The EO samples were dried over anhydrous sodium sulfate Na_2SO_4 and stored in sealed amber vials at −4 °C until analyzed. The yield of EO was expressed as a percentage (w/w) of the dry material. The EO yield was quantified calculated using this following formula:

$$\text{EO yield (in \%)} = (m1 / m2) \times 100$$

With m_1 and m_2, the mass of extracted EO (g) and mass of dry leaves (g), respectively.

GC/MS Chemical Analysis of EOs

The EOs hydro-distilled from *Eucalyptus* species were analyzed by GC/MS. An Agilent GC system 7890B (Agilent, Santa Clara, CA, USA) equipped with a split/splitless injector and an Agilent MSD 5977B detector was used. One µL of EO dilution (0.01% in hexane; *w/v*) was injected in splitless mode at 300 °C on a 5%-phenyl-methylpolysiloxane based HP-5MS capillary column (30 m × 0.25 mm, d*f* = 0.25 µm). The temperature was maintained one min at 50 °C, and then increased at a rate of 5 °C/min until 300 °C. The final temperature was maintained for 5 min. The sources and quadrupole temperatures were set at 230 °C and 150 °C, respectively. The scan range was 40–400 m/z, and the carrier gas was helium at a flow rate of 1.2 mL/min. Compounds were identified by comparison of their Kovats index values relative to (C_{10}–C_{35}) n-alkanes obtained on HP-5MS column (Agilent, Santa Clara, CA, USA), with those provided in the literature[22] and by comparison of their mass spectra with those recorded in NIST (National Institute of Standards and Technology).

Agar Well Diffusion Method

To evaluate the antimicrobial potential of EOs, the agar diffusion technique was employed.[23] Before use, each bacterial suspension was adjusted to 1.2×10^8 CFU/mL, and the fungal suspension was adjusted to 10^8 spores/mL. Four dilutions of each EO (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) were prepared using dimethyl sulfoxide (DMSO). The surface of an agar plate was spread with 1 to 2 mL of the tested inoculum. Wells with a diameter of 4 mm were then punched into the inoculated agar medium, and 10 µL of each EO dilution was added to each well. The plates were incubated at 37 °C for 24 h. Antimicrobial activity was evaluated by measuring the diameters (mm) of the clear zones of growth inhibition. Piperacillin was used as the positive control and, DMSO was used as the negative control. Sensitivity to the different EOs was categorized as follows: not sensitive (diameter ≤8 mm); sensitive (diameter 9−14 mm); very sensitive (≥15 mm).[24]

Determination of the Minimum Inhibitory Concentration (MIC)

This method allows the determination of the Minimum Inhibitory Concentration (MIC) from a range of antimicrobial substance concentrations in a solid medium.[25] MIC testing is conducted using the standard dilution method on Mueller–Hinton agar for bacteria and PDA medium *Candida*. Serial dilutions of each EO (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) were prepared using dimethyl sulfoxide (DMSO). The lowest concentrations EO that inhibit microbial cultures in vitro are tested as follows: 10 µl of each dilution exhibiting activity is added to 100 µl of the appropriate liquid medium in a well plate. Optical density (OD) readings are taken at T0 (initial time = high OD). The plate is then incubated for 24 h at 37 °C, and OD readings are taken after 24 h of incubation (T24 = lower OD). The MIC of the extract is defined based on the Petri dish, either free from microbial growth (bactericidal effect) or exhibiting growth (bacteriostatic effect).[26]

Statistical Analysis

The yields data were analyzed using analysis of variance (ANOVA), and the significance of the differences between means was determined at $p < 0.05$ using Duncan's multiple range test.

Table 2. EO Content (%) in Dried Leaves of *Eucalyptus* Species.

Sites	Percentage Yield of EOs (% w/w)			
	E. salmonophloia	***E. torquata***	***E. lesouefii***	***E. astringens***
Metouia (A1)	$4.63^{h} \pm 0.27$	$0.12^{a} \pm 0.03$	$2.48^{de} \pm 0.00$	NT
Hamma (A2)	$3.74^{g} \pm 0.01$	$0.82^{b} \pm 0.11$	$2.30^{d} \pm 0.11$	$3.61^{fg} \pm 0.03$
Zrig (A3)	$3.36^{f} \pm 0.29$	$0.39^{a} \pm 0.12$	NT	$1.27^{c} \pm 0.12$
Elhanya (SA1)	$2.67^{e} \pm 0.12$	$0.27^{a} \pm 0.04$	$1.36^{c} \pm 0.13$	NT
HenchirNaam (SA2)	$2.21^{d} \pm 0.14$	$0.69^{b} \pm 0.01$	$1.17^{c} \pm 0.00$	$0.66^{b} \pm 0.06$

The different letters indicate a significant difference based on Duncan's multiple range tests at the 1% level
NT: not tested

To investigate the relationships between oil yield, chemical composition, and environmental factors (precipitation and temperature), we conducted a principal component analysis (PCA) on 12 compounds that accounted for an average concentration greater than 2% of the total oil content. The PCA was performed using functions from the "factoextra" and "FactoMineR" packages.[27] Additionally, Spearman correlation analysis was applied, and a correlogram was generated with the "corrplot" package (version 0.84).[28] in R software (version 4.3.1).

Results

Yields of EOs

The percentage yield of EOs extracted from the samples ranged from 0.12% to 4.63% (w/w), as detailed in Table 2. ANOVA revealed significant variation in oil yields based on species and cultivation site for the same species ($p < 0.05$). The highest and lowest EO yields were observed in the dried leaves of *E. salmonophloia* (4.63%) and *E. torquata* (0.12%), both cultivated in A1. For *E. salmonophloia*, EO yields ranged from $2.21 \pm 0.4\%$ in SA2 to $4.63 \pm 0.27\%$ in A1. In the case of *E. torquata*, EO yields varied from $0.12 \pm 0.03\%$ in A1 to $0.69 \pm 0.01\%$ in SA2. *E. lesouefii* produced EOs ranging from $1.17 \pm 0.00\%$ in SA2 to $2.48 \pm 0.00\%$ in A1, while *E. astringens* yielded EOs between $0.66 \pm 0.06\%$ in SA2 and $3.61 \pm 0.03\%$ in A2.

Chemical Composition

The analysis of the EOs by GC/MS identified 99 compounds, which accounted for 92.41% to 99.63% of the total oil (Suplementary materials (Figure S1)). The percentages of these compounds varied within species and across harvest zone. The EOs of *E. salmonophloia* and *E. astringens* were dominated by oxygenated monoterpenes (56.78% to 81.66%), with 1,8-cineole being the compound with the highest content in all locations. However, the EO profile of *E. torquata* showed a dominance of ketones, particularly torquatone (33.41% to 44.78%). The oxygenated monoterpene constituted about 43% and 50% of chemical composition of the EOs from *E. lesouefii* in the A2 and SA2 provenances, respectively. While those from A1 and SA1 were dominated by the oxygenated sesquiterpene (Table 3).

The analysis of *E. salmonophloia* oil led to the identification and quantification of 23, 29, 36, 53 and, 27 major compounds, corresponding to 97.44%, 98.81%, 99.63%, 96.03% and, 99.38% of the EO from leaves of locations A1, A2, A3, SA1 and SA2, respectively. According to the data presented in Table 3, the predominant compound in all five EOs analyzed was 1,8-cineole. Location A2 exhibited the highest percentage of 1,8-cineole (67.16%), while the lowest was found in SA1 (41.79%). Transpinocarveol (10.30%) and *p*-cymene (18.29%) were the second most abundant compounds in SA2 and SA1, respectively, while α-pinene held that position in the arid region sites (A1, A2, and A3). It is worth noting also that p-cymene was absent in A2 oil. Cryptone, from the ketone class, was comparatively abundant in SA1 (5.09%) and was a minor constituent in A3 (1.09%). Among different regions, SA2 recorded the best percentage of pinocarvone (2.47%) and spathulenol (3.32%). Myrtenal was only detected in A3 at 3.39%, and it was a minor constituent in SA1 (0.67%). other compounds, such as β-eudesmol, isopentyl isovalerate, terpinen-4-ol, torquatone, α-terpeneol, cuminaldheyde, 24-Noroleana-3,12-diene, globulol and p-Mentha-1(7),8-dien-2-ol were also detected in a few (1-3%).

In the case of *E. torquata*, the total numbers of compounds identified in the EOs from A1, A2, A3, SA1 and SA2 were 21, 24, 24, 27 and 24, respectively, representing 98.47%, 94.88%, 98.14%, 92.78% and 97.58% of the total oil. In all samples, torquatone (36.57% to 44.78%) was the major molecule. 1,8-cineole (11.05% to 18.56%), β-eudesmol (13.27% to 21.21%), α-pinene (2.69% to 11.62%) and transpinocarveol (2.64% to 14.90%) were also detected as main components in all analyzed oils. For instance, the oils from A1 and SA1 had the highest amounts of torquatone with similar percentages (44.48% and 44.78% respectively). The contents of 1,8-cineole and β-eudesmol were also similar (up to 12.00% and 13.00% respectively). *E. torquata* from A1 was characterized by the highest percentage of transpinocarveol (14.90%), while the highest synthesis of 1,8-cineole (18.56%) and α-pinene (11.62%) was found in A2. Oxygenated sesquiterpenes, such as globulol, were present in lower proportions, but still reached their maximum levels in SA1 and SA2 (3.47% and 2.82%, respectively). Furthermore, γ-eudesmol was found in appreciable proportions (6.29%) in A2, followed by SA2 (4.46%), but remained under

Table 3. Effect of Growth Locations on the EO Constituents of *E. salmonophloia*, *E. torquata*, *E. lesouefii* and *E. astringens*.

			E. salmonophloia					*E. torquata*					*E. lesouefii*				*E. astringens*		
			Peak area (%)																
Component	RI^{cal}	RI^{lit}	A1	A2	A3	SA1	SA2	A1	A2	A3	SA1	SA2	A1	A2	SA1	SA2	A2	A3	SA2
α-thujene	924	927		0.35	0.06	0.17	0.10						TR	0.29	0.27	0.19			
α-pinene	931	936	13.97	12.00	11.70	7.43	7.13	2.69	11.62	6.69	4.91	6.19	4.56	14.83	9.97	6.24	24.1	17.58	6.93
camphene	945	943	0.10	0.19	0.11	0.06	0.11	TR	0.06	0.08	TR	TR		TR	TR	TR	0.26	0.18	0.12
thuja-2,4(10)-diene	951	950	0.08	0.05	0.20	0.07	0.06										0.08	0.07	0.06
sabinene	971	968				0.06													
β-pinene	973	972	0.36	0.42	6.05	0.75	0.64	0.06	0.40	0.11	0.13	0.23	0.44	1.33	0.86	0.49	0.43	0.24	0.12
β-myrcene	989	991				0.19													
α-phellandrene	1003	1005			0.29	0.27							0.80	0.55	1.34	1.54			
isobutylisovalerate	1005	1004				0,17													
p-cymene	1026	1025	6.90		9.07	18.29	2.29		1.16					7.57	9.41	4.51			
1,8-cineole	1032	1030	63,78	67,16	48,97	41,79	63,17	12,51	18,56	14,49	12,93	11,05	21,72	39,68	29,71	43,19	45,30	54,35	46,32
limonene	1090	1032			0.08														
isopentylisovalerate	1104	1106	0.51	0.62		1.32													
fenchol	1113	1114	0.85	0.30	0.22	0.10	0.66	0.18	0.05	0.52	0.15	TR		TR	TR	0.09	0.18	0.28	0.39
(+)-3-thujone	1116	1106				0.08													
trans-p-mentha-1(7),8-dien-2-	1121	1120		0.15		0.24													
α-campholenal	1125	1126	0.08	0.05	0.17	0.15		0.10		TR		TR							TR
transpinocarveol	1139	1141	6.33	6.81	5.73	4.86	10.3	14.9	5.25	8.16	5.56	2.64	0.18	0.51	1.48	3.21	8.78	10.4	19.3
trans-verbenol-	1145	1143			0.23	0.07													
camphor	1145	1149	0.31	0.15			0.24												
camphene hydrate	1148	1150		0.06	0.09				0.07	0.12	0.05							0.11	0.32
pinocarvone	1163	1164	1.32	1.37	1.66	1.12	2.47	5.10	1.26	1.99	1.34	0.71		TR	0.21	0.48	1.77	2.21	4.70
(-) borneol	1166	1166	0.29	0.72	0.31	0.18	0.64		0.35	0.45	0.50	0.19	TR	0.10	0.08	0.24	0.56	0.77	1.65
terpinen-4-ol	1178	1179	0.12	0.41	0.59	1.02							2.16	0.48	0.77	1.67			
cryptone	1188	1185			1.09	5.09													
α-terpeneol	1191	1191		1.57	1.61	1.11										0.61	0.05	1.09	
myrtenal	1197	1193	0.83		3.93	0.67	1.18	0.62	0.34	0.64	0.49	0.23		0.14	0.09				0.95
cis sabinol	1199	1203				0.15													
verbenone	1211	1212			0.24	0.07													
cis carveol	1220	1220	0.32		TR	0.14	0.35												
trans-pinocarvyllacetate	1242	1297													0.06				
p-mentha-1(7),8-dien-2-ol	1229	1230	0.37			0.42	1.02												
myrtenylacetate	1241	1332		0.05			0.28										0.14	0.18	0.50
cuminaldheyde	1242	1242			1.10	1.58													
piperitone	1256	1255				0.29													
phellandral	1276	1278			0.17	0.37													
nonanoicacid	1284	1269				0.09													
p-cymene- 7-ol	1294	1288			0.78	0.36													
carvacrol	1304	1298	0.59	0.09		0.82								0.11	0.41				
methylgeranate	1320	1317			0.15	0.08							TR						
thymol	1324	1293														0.23			
2-hydroxycineole acetate	1343	1339		0.07	0.07	0.09	0.18	0.06	0.10	0.08	0.06	0.10	2.71	2.68	0.67	0.69		TR	
β-elemene	1394	1390											0.09	TR					
isoamyl benzoate	1439	1439				0.18													
α-gurjunene	1412	1412											0.08		TR				
caryophellene	1422	1420							TR			TR	0.11	0.11	0.24	0.15	0.62	TR	TR
aromadendrene	1442	1442	0.59	0.05	TR		0.08	0.16	0.24	0.14	0.34	0.34	0.45	0.12	0.12	0.30	2.03	1.01	0.55
dehydroaromadendrene	1456	1454											0.20	0.17	0.15	0.19			
humulene	1457	1450															0.06		
eudesma-1,4(15),11-triene	1463	1471						TR						0.21					
β-selinene	1490	1485	TR					0.14	0.25	0.19	0.21	0.23	0.06						
γ-himachalene	1492	1476													0.12				
γ-maaliene	1499	1431										0.13	TR						

(continued)

Table 3. Continued.

			E. salmonophloia					*E. torquata*					*E. lesouefii*				*E. astringens*		
			Peak area (%)																
Component	RI^{cal}	RI^{lit}	A1	A2	A3	SA1	SA2	A1	A2	A3	SA1	SA2	A1	A2	SA1	SA2	A2	A3	SA2
bicyclogermacrene	1500	1495					0.13						10.61	0.28	0.67	0.90	0.81	0.19	0.64
calamenene	1526	1527			0.07	0.10									0.07				
δ-cadinene	1527	1523											0.06						
α-elemol	1553	1549						TR	0.08	0.06	0.06	0.17							
epiglobulol	1565	1573	TR		TR		0.06	0.18	0.15	0.15	0.37	0.37		0.13	0.14	0.15	0.30	0.64	0.53
maaliol	1573	1569	TR		0.05		0.16	0.06	0.12	0.11	0.17	0.22	2.17	0.29	0.69	0.51	0.28	0.21	0.36
spathulenol	1583	1578	TR	0.21	1.24	0.64	3.23		0.07	0.25	0.12	0.20	15.83	10.94	17.41	12.75	1.52	0.36	2.38
globulol	1590	1587	0.21	0.32	0.58	0.85	1.43	2.22	1.22	1.59	3.47	2.82	12.48	TR		4.52	6.48	7.97	9.28
viridiflorol	1598	1595		0.01	TR	0.23	0.53	0.29		0.33	0.42	0.30	10.00	1.11	2.69	2.22	1.28	0.56	1.81
germacra-4(15), 5,10(14)-trien-1 –ol	1604	1604											0.07	0.57	0.73				
ledol	1609	1607	TR		0.05	0.08					0.12								
ledeneoxide (iii)	1616	1604						0.07			0.10		0.09	6.77	0.18				
eremophilene	1621	1488									0.07	TR	0.16					0.12	0.13
rosifoliol	1629	1622							TR			0.11				0.77			
1,10-di-epi-cubenol	1634	1624			0.07	0.13													
γ-eudesmol	1637	1630	0.16	0.17	0.11	0.16		0.75	6.29	2.66	1.42	4.46		1.20		0.10			
isospathulenol	1644	1640			TR		0.29						2.77	0.91	1.76	1.17	0.19		0.25
isoaromadendreneepoxide	1677	1688						TR					0.20	0.80	9.39	0.11			
guaiol	1646	1614							0.05	0.37	0.85	0.09	0.13				0.13		
6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-naphthalen-2-ol	1652	1690											0,23	TR					
β- eudesmol	1657	1650	1.48	1.20	0.64	0.89	1.20	13.47	13.42	21.21	13.27	17.78	2.94	0.89	0.54	1.54	0.27	0.23	0.32
4-isopropyl-6-methyl-3,4,4a,7,8,8a-hexahydronaphthalene-1-carbaldehyde	1658	1502											0.28						
α-cadinol	1661	1655											1.31					0.05	
neointermedeol	1661	1656						0.06							0.63				0.21
benzene, 13,5-tris(1-methylethyl	1662										4.78								
10-epi-gamma eudesmol	1664	1620										7.39							
tetradecane	1698	1663				0.10													
ylangenal	1743	1370											0.12	2.54	1.70				
paeonol	1807	1477						0.31	0.36	1.18	0.89	0.83				0.32			
torquatone	1830	2370	0.08	0.06	0.76	1.70	1.45	44.48	33.41	36.57	44.78	40.43	0.66	2.44		8.50	0.32	0.11	0.25
methyl palmitate	1925	1919		0.07		0.25													
dibutylphthalate	1965	1960				0.05													
methyllinolenate	2101	2100				0.26													
methylstearate	2126	2123			TR	0.11													
24-noroleana-3,12-diene	2836	3057		2.79	0.66														
Total			**99.63**	**97.44**	**98.81**	**96.03**	**99.38**	**98.47**	**94.88**	**98.14**	**92.78**	**97.58**	**94.4**	**95.01**	**92.41**	**97.58**	**95.92**	**97.76**	**98.11**
Monoterpeneshydrocarbons			**21.41**	**13.07**	**27.56**	**27.22**	**10.33**	**2.75**	**13.31**	**7.00**	**5.09**	**6.42**	**5.80**	**24.57**	**21.85**	**12.97**	**24.87**	**18.18**	**7.55**
Oxygenatedmonoterpenes			**75.19**	**81.66**	**67.53**	**60.77**	**80.49**	**33.53**	**25.91**	**26.33**	**21.03**	**14.92**	**26.77**	**43.27**	**33.48**	**50.41**	**56.78**	**69.34**	**73.86**
Sesquiterpeneshydrocarbons			**0.59**	**0.05**	**0.07**	**0.10**	**0.21**	**0.30**	**0.57**	**0.39**	**0.61**	**1.24**	**12.90**	**0.91**	**1.22**	**1.54**	**3.52**	**1.19**	**1.19**
Oxygenatedsesquiterpenes			**1.85**	**1.91**	**2.74**	**2.98**	**6.90**	**17.1**	**21.32**	**26.67**	**20.38**	**33.74**	**48.27**	**23.82**	**35.86**	**23.84**	**10.45**	**10.14**	**15.26**
Others			**0.59**	**0.75**	**0.91**	**4.96**	**1.45**	**44.79**	**33.77**	**37.75**	**45.67**	**41.26**	**0.66**	**2.44**	**0**	**8.82**	**0.30**	**0.10**	**0.25**

RI^{lit}, Retention index from literature[22,29]; RI^{cal}, Retention Index determined on HP-5 gas chromatography column using (C_{10}–C_{35}) n-alkanes series; TR, trace (<0.05)

3% in the other locations. 10-epi-gamma-eudesmol and benzene, 13,5-tris(1-methylethyl) were also exceptionally present in SA2 and SA1, respectively (7.39% and 4.78%). Other minor molecules, such as β-caryophyllene and α-humulene, were also found in analyzed oil.

The analysis of EOs hydrodistilled from the leaves of *E. lesouefii* allowed the identification and quantification of 31, 29, 31 and 30 major compounds, corresponding to 94.4%, 95.01%, 92.41% and 97.58% of the EOs from the leaves of A1, A2, SA1 and SA2, respectively. The oxygenated monoterpene 1,8-cineole (21.72% to 43.19%), spathulenol (12.75% to 17.41%), and α-pinene (4.56% to 14.83%) were by far the major components in all investigated oils. Other significant compounds, including globulol (12.48%), bicyclogermacrene (10.61%) and viridiflorol (10.00%), were identified exceptionally in *E. lesouefii* from A1. p-cymene was detected in A2 (7.5%), SA1 (9.4%), SA2 (4.51%) and absent in A1. Other compounds were detected as major components in some regions and as minor constituents in others, such as ledene oxide (6.77%) in A2, iso-aromadendrene (9.39%) and torquatone (8.50%) in SA2. Among the different regions, A1 recorded the highest percentages of β-eudesmol (2.94%), isospathuleol (2.77%), maaliol (2.17%), terpinen-4-ol (2.16%) and 2-Hydroxycineole acetate (2.71%).

In the case of *E. astringens*, 29 compounds were identified in the three oil samples, accounting for 95.92% to 98.11% of the total composition. The most abundant constituents were the 1,8-cineole, α-pinene, transpinocarveol and globulol. The first ranging from 45.30% in A2 to 54.53% in A3, α-pinene with the highest in A2 (24.10%) and the lowest in SA2 (6.93%), transpinocarveol was abundant which ranging from 8.78% in A2 to 19.3% in SA2 and finally globulol with the highest in SA2 (9.28%) while the lowest in A2 with 6.48%.

Principal Component Analysis (PCA)

To evaluate the impact of two distinct environmental factors (temperature and precipitation) on the volatile components and EO content of four *Eucalyptus* species, a biplot PCA was performed. The first two principal components (PC1 and PC2) explained 55.80% of the total variation in the dataset (Figure 1). The results suggest that temperature had a significant effect on the composition of the EOs, particularly influencing the content of 1,8-cineole, α-pinene, p-cymene, trans-pinocarveol, and pinocarvone. Additionally, EO yield was also affected by temperature. Precipitation, on the other hand, showed an impact on the EO profiles, with spathulenol, isospathulenol, viridiflorol, and globulol being the most prominent compounds, while γ-eudesmol, β-eudesmol, and torquatone were moderately influenced.

The PCA also revealed the presence of four distinct groups of *Eucalyptus* EO:

- **Group 1** in which, the EO from *E. torquata* (from locations A1, A2, A3, SA1, and SA2) are grouped together. These oils have similar chemical profiles, characterized by compounds like **torquatone, β-eudesmol, and γ-eudesmol**. These compounds are likely key markers of this species' oil.
- **Group 2**, this group includes EOs from *E. salmonophloia* (from locations A1, A2, A3, SA1, and SA2), *E. astringens* (from A2 and A3), and *E. lesouefii* (from A2). These oils are distinguished by higher levels of **1,8-cineole** and **α-pinene**. The higher **EO yield** seems to be another distinguishing factor for this group.
- EOs from *E. lesouefii* cultivated in A1, SA1, and SA2 form a separate **group 3** characterized by high levels of **spathulenol, isospathulenol**, and **viridiflorol**. These compounds are distinctive for this group, suggesting they are key chemical markers of *E. lesouefii* oils from these specific locations.
- **Group 4**, the EO from *E. astringens* from location SA2 is separated from the other samples. This oil is distinct due to its high content of **transpinocarveol** and **pinocarvone**, which are not as prominent in the other oils.

Correlation of EO Content and Components with Climatic Characteristics

The correlation between the EO yield of *Eucalyptus* species leaves, the relative contents of the twelve main compounds, and environmental factors was analyzed. The results illustrated in Figure 2 and Figure S2 (Supplementary materials) show that the oil yield was positively correlated with annual temperature ($r = 0.105$) and negatively correlated with annual rainfall ($r = -0.195$).

Among the EO components, α-pinene, transpinocarveol, pinocarvone, and 1,8-cineole showed a positive correlation with temperature ($r = 0.500$, $r = 0.301$, $r = 0.302$, and $r = 0.281$, respectively), whereas the same constituents contributed to a negative correlation with precipitation ($r = -0.280$, $r = -0.163$, $r = -0.183$, and $r = -0.073$). In contrast, spathulenol, viridiflorol, and isospathulenol exhibited a positive correlation with precipitation ($r = 0.286$, $r = 0.317$, and $r = 0.367$, respectively). Torquatone showed a moderate positive correlation with precipitation ($r = 0.121$). However, compounds such as γ-eudesmol, β-eudesmol, and globulol appeared to be more resilient to fluctuations in climatic conditions.

Antimicrobial Activity

According to our results presented in Table 4, the EO samples inhibited the growth of all tested microorganisms, with an inhibition zone diameter (IZD) increasing proportionally with the EO concentrations. With the exception of *E. salmonophloia* oils from A2 and SA1, all tested oils exhibited the strongest antibacterial activity against *M. luteus* at a dilution of 10^{-1}, with IZDs ranging from 15 mm to 30 mm. Indeed, the *E. lesouefii* oil from SA2 was the most active against this bacterium, with an IZD of 30 mm.

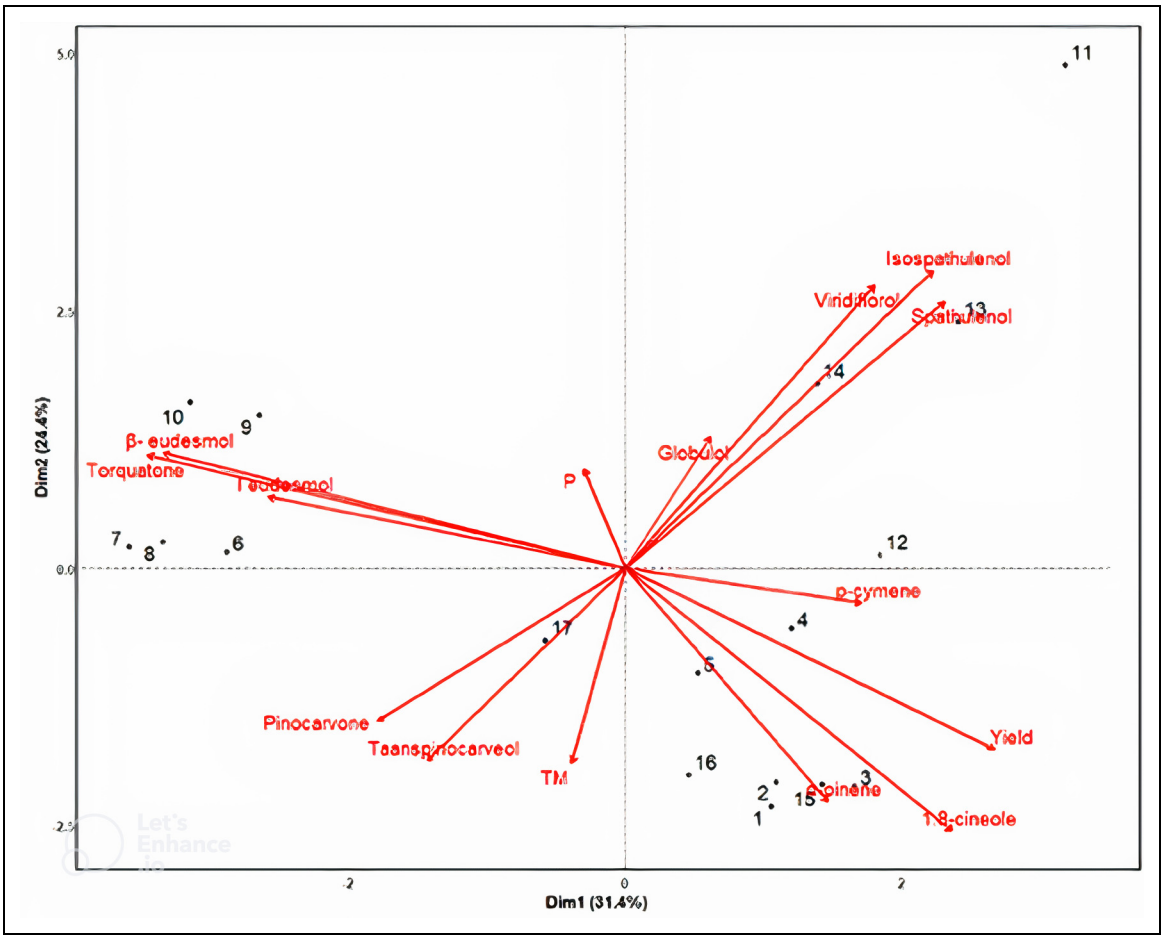

Figure 1. Principal Component Analysis (PCA) Results Carried Out with 12 Chemical Compounds Contents, EO Yield and Environmental Factors (Precipitation (**P**) and Temperature (**TM**)). Abbreviations of Species *E. salmonophloia* from A1, A2, A3, SA1 and SA2 (**1, 2, 3, 4** and **5**), *E. torquata* from A1, A2, A3, SA1 and SA2 (**6, 7, 8, 9** and **10**), *E. lesouefii* from A1, A2, SA1 and SA2 (**11, 12, 13** and **14**), *E. astringens* from A2, A3, SA2 (**15, 16** and **17**), refer Tables 1 and 3 for More Detail.

Also, *E. astringens* (SA2) exhibited the highest activity against *S. aureus* (23 mm, IZD), while *S. marcescens* showed greater sensitivity to *E. salmonophloia* (SA2) with IZD of 19 mm.

All tested EOs were able to inhibit the growth of *P. aeruginosa*, with IZDs ranging from 11 to 30 mm at a dilution 10^{-1}, which were greater than those observed with the antibiotic piperacillin (IZD of 10 mm). *E. salmonophloia* from A2 exhibited the best activity against *P. aeruginosa* (30 mm, IZD) and *E. coli* (29 mm, IZD) at a 10^{-1} dilution, remaining active even at a 10^{-4} dilution. The studied EOs also demonstrated antifungal activity against two yeast species, *C. albicans* and *C. tropicalis*, with IZDs ranging from 9 mm to 30 mm. *E. astringens* and *E. salmonophloia* EOs from SA2 showed the strongest effect against *C. albicans* and *C. tropicalis*, respectively, with IZDs of 30 mm and 20 mm.

The MIC values for the tested microbial strains ranged from dilutions of 10^{-1} to 10^{-4} (Table 5). To confirm the MIC, samples from the MIC tubes were plated onto agar media to check for the presence or absence of growth. The sensitivity of microorganisms may vary depending on the strain, as essential oil can be bactericidal (or fungicidal) against certain strains, bacteriostatic (or fungistatic) against others, or have no effect.[30] As shown in Table 5, no growth of *E. coli* was observed with *E. salmonophloia* (SA2), *E. lesouefii* (SA2), and *E. torquata* (SA2) at a CMI of 10^{-1} dilution, and with *E. salmonophloia* (SA1) and *E. lesouefii* (SA1) at a CMI of 10^{-2} dilution. Similarly, no growth of *S. marcescens* was observed with *E. salmonophloia* (SA2 and A2) and *E. astringens* (SA2 and A2) at an MIC of 10^{-1} dilution, and with *E. lesouefii* (SA1) at a 10^{-2} dilution. A fungicidal effect was only observed with *E. lesouefii* (SA1) against *C. tropicalis* at MIC of 10^{-2} dilution.

Discussion

The ANOVA indicated that the EO yields were significantly different between species and between arboreta ($p < 0.05$). For *E. salmonophloia*, the EO yields ranged from $2.21 \pm 0.40\%$ to $4.63 \pm 0.27\%$. This is in close agreement with previous studies, which reported leaf EO contents of 3.20% in central Tunisia[31] and 4.60% in southern Tunisia.[32] For *E. torquata*, EO yields varied between $0.12 \pm 0.03\%$ and $0.69 \pm 0.01\%$. Our results are

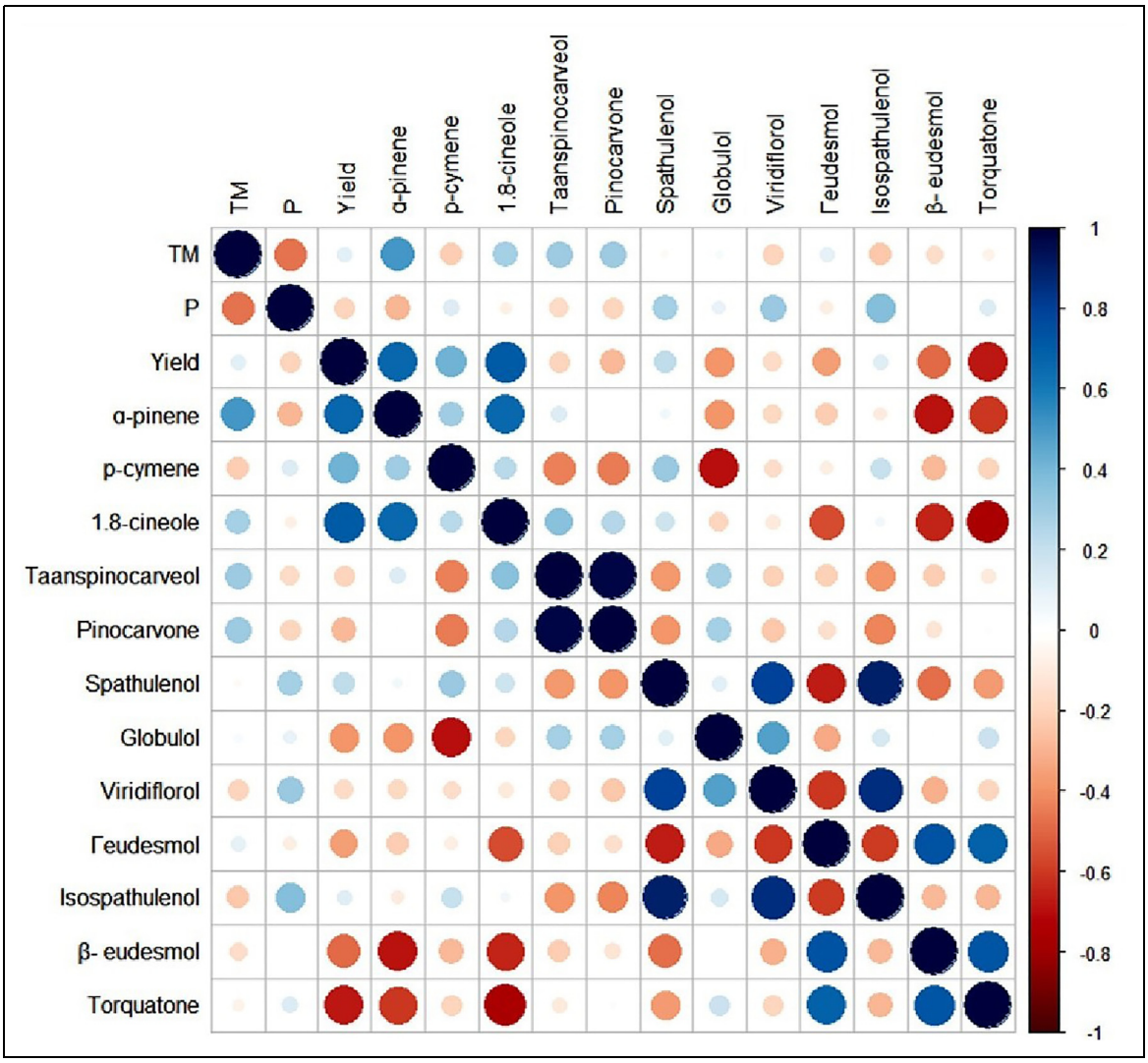

Figure 2. Correlation Between the Content of 12 Compounds in Four *Eucalyptus* Species, Essential Oil Yield, and Environmental Factors (Temperature (TM) and Precipitation (P)).

somewhat lower than those reported in the literature, where *E. torquata* from central Tunisia[31] yielded 3.2% EO and 2.6% in southern Tunisia.[33] However, in Iran[34] and Cyprus,[35] the EO yields were 1.60% and 1.70% respectively. In addition, *E. lesouefii* produced EOs ranging from 1.17% to 2.48%. However, In northern Tunisia (Henchir Naam),[36] EO production was reported to be 2.10% and 3.20%, but significantly lower than the 5.2% reported in Mjez Lbeb, Tunisia.[37]

E. astringens yielded EO between 0.66 ± 0.06% and 3.61 ± 0.03%. This result is nearly similar to the previous report that the leaf EO content of *E. astringens* species was 0.96% at Tunisia,[8] but also obtained with average yield of 3.2% in three different zone in Tunisia.[38]

When comparing the results from our research with those from other studies in the literature, although there are some minor differences in EO yields, the overall findings are generally consistent. The observed partial differences may be attributed to variables factors, including genotype, geographical location and environmental conditions. Other parameters affect performance, such as: phenological stage, the specific botanical organ, propagation methods, harvest timing, processing of the plant material, whether the material is fresh or dried, and the extraction techniques, all play a role in determining the quality and chemical composition of EOs.[39]

The leaf EO of *Eucalyptus* species was sub-jected to GC–MS analysis for the identification of the constituents. A wide variation was seen in the chemical composition among all agroclimatic zones. A total of 73 compounds were identified from all the zones for *E. salmonophloia*. 1,8-cineole was found to be major constituent ranging from 14.79% to 67.16%. The highest percentage was found in A2 (arid region), while the lowest was found in SA1 (semi-arid region). The second major constituent

Table 4. Inhibition Zone Diameters (mm) of Microbial Strains by the Eucalyptus EOs.

	Inhibition zone diameter, IZD (mm)																					
EOs	*E. salmonophloia* (SA2)							*E. astringens* (A2)							*E. lesouefii* (SA2)							
Microbial strains	Ec	Pa	Sm	Sa	Ml	Ca	Ct	Ec	Pa	Sm	Sa	Ml	Ca	Ct	Ec	Pa	Sm	Sa	Ml	Ca	Ct	
Dilution 10^{-1}	19	12	19	15	20	17	20	17	21	16	13	22	16	14	20	22	13	16	30	15	16	
Dilution 10^{-2}	0	9	0	0	11	0	0	0	12	0	11	19	0	0	0	15	12	13	13	0	0	
Dilution 10^{-3}	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0	11	0	0	0	0	0	
Dilution 10^{-4}	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	9	0	0	0	0	0	
Piperacillin	25	10	28	24	26	29	25	25	10	28	24	26	29	25	25	10	28	24	26	29	25	
DMSO	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

Piperacillin : Antibiotic standard.

	Inhibition zone diameter, IZD (mm)																					
EOs	*E. lesouefii* (A1)							*E. torquata* (A2)							*E. salmonophloia* (SA1)							
Microbial strains	Ec	Pa	Sm	Sa	Ml	Ca	Ct	Ec	Pa	Sm	Sa	Ml	Ca	Ct	Ec	Pa	Sm	Sa	Ml	Ca	Ct	
Dilution 10^{-1}	17	22	15	23	24	12	13	15	11	12	15	25	12	18	19	21	14	17	15	10	11	
Dilution 10^{-2}	12	12	0	15	19	0	0	12	0	0	10	14	0	11	11	0	9	10	10	0	0	
Dilution 10^{-3}	0	12	0	7	12	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	
Dilution 10^{-4}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Piperacillin	25	10	28	24	26	29	25	25	10	28	24	26	29	25	25	10	28	24	26	29	25	
DMSO	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

Piperacillin: Antibiotic standard.

	Inhibition zone diameter, IZD (mm)																					
EOs	*E. astringens* (SA2)							*E. torquata* (SA1)							*E. torquata* (SA2)							
Microbial strains	Ec	Pa	Sm	Sa	Ml	Ca	Ct	Ec	Pa	Sm	Sa	Ml	Ca	Ct	Ec	Pa	Sm	Sa	Ml	Ca	Ct	
Dilution 10^{-1}	16	17	17	22	27	30	19	12	16	12	19	22	13	17	13	14	10	14	20	17	14	
Dilution 10^{-2}	0	11	0	9	12	0	9	0	12	0	10	12	0	9	0	12	0	11	18	0	8	
Dilution 10^{-3}	0	11	0	0	0	0	0	0	12	0	0	0	0	0	0	12	0	0	0	0	0	
Dilution 10^{-4}	0	11	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	
Piperacillin	25	10	28	24	26	29	25	25	10	28	24	26	29	25	25	10	28	24	26	29	25	
DMSO	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

Piperacillin: Antibiotic standard.

	Inhibition zone diameter, IZD (mm)													
EOs	*E. lesouefii* (SA1)							*E. salmonophloia* (A2)						
Microbial strains	Ec	Pa	Sm	Sa	Ml	Ca	Ct	Ec	Pa	Sm	Sa	Ml	Ca	Ct
Dilution 10^{-1}	15	19	15	20	23	22	9	29	30	14	21	18	16	8
Dilution 10^{-2}	10	12	9	12	16	8	0	16	13	0	0	11	0	0
Dilution 10^{-3}	0	0	0	0	0	0	0	15	12	0	0	0	0	0
Dilution 10^{-4}	0	0	0	0	0	0	0	9	12	0	0	0	0	0
Piperacillin	25	10	28	24	26	29	25	25	10	28	24	26	29	25
DMSO	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Piperacillin: Antibiotic standard

Table 5. MICs of *Eucalyptus* EOs and their Actions (Bactericidal/Fungicidal or Bacteriostatic/Fungistatic).

EOs Microbial strains	EO1	EO2	EO3	EO4	EO5	EO6	EO7	EO8	EO9	EO10	EO11
Ec	10^{-1}/-	10^{-1} /+	10^{-1}/-	10^{-4}/+	10^{-2}/+	10^{-2}/+	10^{-2}/-	10^{-2}/-	10^{-1}/+	10^{-1}/+	10^{-1}/-
Pa	10^{-2}/+	10^{-4}/+	10^{-4}/+	10^{-4}/+	10^{-3}/+	10^{-1}/+	10^{-1}/+	10^{-2}/+	10^{-4}/+	10^{-3}/+	10^{-4}/+
Sm	10^{-1}/-	10^{-1}/-	10^{-2}/+	10^{-1}/-	10^{-1}/+	10^{-1}/+	10^{-2}/+	10^{-2}/-	10^{-1}/-	10^{-1}/+	10^{-1}/+
Sa	10^{-1}/+	10^{-2}/+	10^{-2}/+	10^{-1}/+	10^{-3}/+	10^{-2}/+	10^{-2}/+	10^{-2}/+	10^{-2}/+	10^{-2}/+	10^{-2}/+
Ml	10^{-2}/+	10^{-2}/+	10^{-2}/+	10^{-2}/+	10^{-3}/+	10^{-3}/+	10^{-2}/+	10^{-2}/+	10^{-2}/-	10^{-2}/+	10^{-2}/+
Ca	10^{-1}/+	10^{-1}/+	10^{-1}/+	10^{-1}/+	10^{-1}/+	10^{-1}/+	10^{-1}/+	10^{-2}/+	10^{-1}/+	10^{-1}/+	10^{-1}/+
Ct	10^{-1}/+	10^{-1}/+	10^{-1}/+	10^{-1}/+	10^{-1}/+	10^{-2}/+	10^{-1}/+	10^{-1}/-	10^{-2}/+	10^{-2}/+	10^{-2}/+

- = Absence of growth (bactericidal/fungicidal); + = Light growth (bacteriostatic/fungistatic)
EO1 :*E. salmonophloia*(SA2) ; EO2 : *E. astringens* (A2); EO3: *E. lesouefii* (SA2); EO4: *E. salmonophloia* (A2); EO5/ *E. lesouefii* (A1); EO6: *E. torquata* (A2); EO7: *E. salmonophloia* (SA1); EO8: *E. lesouefii* (SA1); EO9: *E. astringens* (SA2); EO10; *E. torquata* (SA1); EO11: *E. torquata* (SA2)

in arid zones was α-pinene, ranging from 11.70% to 13.97%, while in semi-arid areas, p-cymene (18.29%) and transpinocarveol (10.30%) were the second most abundant constituents.

Those results were supported by findings that EO of *E. salmonophloia,* from semi-arid of Tunisia, was reported to contain 1,8-cineole (37.80%) and p-cymene (29.40%) as major constituent.[31] Other from Gabes, in arid Tunisia, showed that major compounds were 1,8-cineole (59.30%) and α-pinene (10.7%).[32] Far from that, in Morocco, plants collected from two different localities revealed that the main components of the EOs were 1,8-cineole (63.50% to 69.90%) and bornéol (12.40% to 17.90%). Plants cultivated in Australia presented p-cymene (16.90%) as the major component, followed by cryptone (10.45%) and then 1,8-cineole (10.33%).[40]

Eucalyptus torquata was distinguished from the other species by the richness in torquatone as major component which ranged from 36.57% to 44.78%. For other compounds, β-eudesmol (13.27% to 21.21%), 1,8-cineole (11.05% to 18.56%), α-pinene (2.69% to 11.62%), transpinocarveol (2.64% to 14.90%) and γ-eudesmol (0.75% to 6.29%) were also the main compounds identified in this species, however their amounts vary from one region to another. As supporter to this findings, Elaissi and co-workers[31] reported the dominance of ketones in the leaf EOs of *E. torquata* growing in Tunisia with torquatone (42.00%) as the principal constituent, also contained 1,8-cineole (12.00%), α-pinene (10.50%), β-eudesmol (10.01%) and transpinocarveol (5.10%). Almost, similar results were reported for EOs from Australia with dominance of torquatone (42.00%), 1,8-cineole (11.21%), α-pinene (10.22%), γ-eudesmol (10.20%) and β-eudesmol (11.11%).[41] Also, in Cyprus,[35] the EOs presented torquatone (29.20%), 1,8-cineole (18.80%), α-pinene (18.60%), β-eudesmol (10.30%) and γ-eudesmol (6.80%) as major constituents. In contrast to the foregoing findings, research carried out in Iran and Morocco revealed that 1,8-cineole (24.20% to 69.60%) was the main compound in the leaf oil of *E. torquata,* and torquatone was completely absent.[42,43] The observed differences could be due to environmental factors, genetic variations, chemotypes, and other factors that influence the EO composition.

Eucalyptus lesouefii leaves have shown quantitative and qualitative differences among regions. 1,8-cineole (21.72% to 43.19%), spathulenol (10.94% to 1.41%), p-cymene (4.51% to 9.41%), bicyclogermacrene (0.28% to 10.61%), α-pinene (4.56% to 14.83%), globulol (0% to 12.48%) and viridiflorol (2.22% to 10.00%) were mainly the identified compounds. A clear intraspecific variation was observed when the oils from A1 and SA1 were distinguished by the absence of p-cymene and globulol, respectively. Additionally, higher percentages of bicyclogermacrene (10.61%), globulol (12.48%), and viridiflorol (10.00%) were exceptionally recorded in A1.

Comparing to the literature, EOs of *E. lesouefii* from Australia was reported to contain bicyclogermacrene (18.49%), 1,8-cineole (17.23%), α-pinene (15.47%), aromadendrene (10.83%), phellandrene (4.40%) and globulol (3.00%).[44] To compare, spathulenol was detected at 1% in the previous study, which is lower than our findings (12.75% to 17.41%). However, aromadendrene and phellandrene, which were present in the previous study, were not detected in our research. The EO of *E. lesouefii* from the Henchir Naam arboretum, the same location as our study (SA2), has also been reported.[45] In contrast to our findings, the previous study identified β-eudesmol (44.90%) and α-eudesmol (20.20%) as the main compounds, however, the contents of 1,8-cineole (5.50%), α-pinene (2.70%), spathulenol (2.00%), and p-cymene (0.60%) were lower than those observed in our study (43.19%, 6.24%, 12.75%, and 4.51%, respectively). This variation could be explained not only by different climatic and edaphic conditions across regions but also by factors such as the collection date, the age of the tree, the extraction method used, the state of plant material (dried or fresh).[44–46]

In the case of *E. astringens*, the EOs extracted from plants cultivated in arid locations (A2 and A3) showed 1,8-cineole as the major constituent (45.30% and 54.35%, respectively) followed by α-pinene (24.10% and 17.58%, respectively), transpinocarveol (8.87% and 10.46%, respectively) and then globulol (6.48% and 7.79%, respectively). However, those obtained from semi-arid region (SA2) were characterized by 1,8-cineole (46.32%) as the major compound, followed by transpinocarveol (19.35%), globulol (9.28%) and then α-pinene (6.39%). It is

worth noting that EOs from the arid regions was characterized by the highest amount of α-pinene.

The richness of *E. astringens* EOs in 1,8-cineole, α-pinene, transpinocarveol and globulol has been described in the literature, with considerable differences in content depending on the origins.[8,46] Study carried out in Tunisia have also demonstrated this variability across three regions with different climatic conditions, belonging to lower humid and sub humid bioclimatic stages.[38] They found that 1,8-cineole, α-pinene, transpinocarveol, aromadendrene, and globulol varied as follow: 40.10%, 21.80%, 10.00%, 9.90% and 5.70%, respectively, for leaves collected from Mrifek (humid inferior); 39.10%, 30.00%, 3.70%, 3.80% and 4.50% for those from Korbous arboretum (sub-humid); and 47.60%, 14.00%, 9.30%, 3.80%, and 5.30% for those from Choucha arboreta (humid inferior). A study conducted in Morocco also confirmed this variation in the EO of *E. astringens*, particularly in the mean percentages of 1,8-cineole (59.30% to 61.40%), α-pinene (4.90% to 14.30%), and transpinocarveol (3.10% to 13.20%) for two provenances characterized by an arid climate.[42] On the other hand, the literature reports interesting contents of other compounds, such as aromadendrene (10.80% to 15.03%) in Tunisia (sub-humid climate),[38] also, p-cymene (17.72%) and spathulenol (12.61%) in Australia.[44] In comparison to our findings, spathulenol (0.36% to 2.38%) and aromadendrene (0.55% to 2.03%) were detected in smaller amounts, while p-cymene was not detected in our study.

In conclusion, the EO chemical composition of all the investigated oils shows variations according to the species and the different bioclimatic areas. These variations are related both to the relative proportion of the constituents and also to the presence or absence of specific components. This aligns with earlier studies in the literature, which have shown that cultivating *Eucalyptus* species in different locations, characterized by varying geographical and climatic conditions, significantly affects the chemical composition of the EOs. Generally, the observed differences can be attributed to multiple factors. This variability is mainly influenced environmental settings, which vary across geographical areas. Genetic factors also play a role in determining the chemical composition of EOs. The various extrinsic factors may include differences in harvest season, climate, soil type, age of the plants and extraction method.[47,48] Genetic and chemotypic variations also affect the main constituents of the EOs, as each species has a slightly different biosynthesis pathway, governed by gene expression.[49] In fact, combinations of external factors affect the internal mechanism of chemical biosynthesis through the interaction of gene expression, resulting in the synthesis of various compounds.

In this study, the production of EO was found to be affected by the environmental differences across various climatic regions. Notably, arid areas in Southern Tunisia (A1, A2 and A3), which are marked by high temperatures and low rainfall, demonstrated the highest EO yields. In contrast, the semi-arid regions (SA1 and SA2), with lower annual temperatures but higher levels of rainfall, yielded lower amounts of EOs. This pattern was noticeable for *E. salmonophloia, E. lesouefii* and *E. astringens* but not totally for *E. torquata*. However, the highest EO yield for *E. torquata* was still found in the arid region of Hamma (A2). After these results we can explain that climatic conditions of arid regions favored the maximum EO yield in leaves of studied *Eucalyptus* species.

Similar to the results of this study, the increase in the temperature level was also positively correlated with the essential oil of *E. cinerea* collected from the Giza, Egypt.[50] Additionnally, Manukyan and co-workers exposed *Thymus transcaucasicus* to different temperatures (15, 20, and 25 °C) and found that the highest EO yield was achieved at the highest temperature.[51] Indeed, the increase in EO production at relatively higher temperatures may be attributed to enhanced photosynthesis and the activation of enzymes involved in essential oil biosynthesis.[52] EOs possess a high heat capacity, which helps protect the plant from heat stress by storing excess heat.[53]

Water deficiency also creates an over-reduced state that triggers the production of secondary compounds, which in turn impacts the EO content. In our study, species showed an increase in EO content under aridity/drought conditions. In contrast, the period in which the lowest production by *E. citriodora, E. viminalis* and *E. globulus* was obtained is related especially to the water deficiency.[54] However, a review of existing literature revealed contradictory findings, which can be attributed to variations in drought conditions, duration, the physiological state of the plants, different species, and even cultivars within the same species.[55] Similarly, in *Sideritis perfoliata* and *Melissa officinalis*,[56] the EO yield also increased with decrease of water availability; however it was decrease in *Thymus vulgaris*[56] and *Juniperus Communis*.[57]

This analysis PCA also provides a clear distinction between the EOs, based on the main twelve identified compounds, which is useful for understanding the chemical diversity and potential applications of these oils. Therefore, four excellent types should be screened in *Eucalyptus* EO to determine its potential use. Those rich in torquatone, β-eudesmol and γ-eudesmol (Group1), other chemotype with higher 1,8-cineole and α-pinene content (Group 2), other identified type with elevated amount of 1,8-cineole, spathulenol, isospathulenol and viridiflorol (Group 3) and finally type with higher amount of 1,8-cineole and transpinoacrveol (Group 4). However, this chemotaxonomic variation shown in the results could be attributed to exogenous factors such as precipitation, temperature, light, soil type, altitude light etc, and to endogenous ones, related mainly to the anatomical, physiological and genetic characteristics of the plant, controlling the EO biosynthesis.

Clearly, our results underline the need to understand the potential impact of environmental factors on the compositional dynamics of *Eucalyptus* EOs, they highlight those environmental factors, in particular annual temperature and precipitation levels - key climatic parameters that vary according to the location of sampling areas - play an important role in shaping the chemical composition of EOs. Indeed, the results suggest that higher temperatures and decreased rainfall may promote the synthesis or concentration of certain compounds, such as α-pinene,

transpinocarveol, pinocarvone, and 1,8-cineole specially in *E. salmonophloia, E. lesouefii* and *E. astringens*. On the other hand, higher precipitation levels and lower temperature may be associated with an increase in the concentration of such compounds like spathulenol, viridiflorol, and isospatulenol particularly in *E. lesouefii* However, some compounds like γ-eudesmol, β-eudesmol, torquatone and globulol, which mainly synthesized by *E. torquata,* appear relatively resilient to climate variation represented by these two factors.

Similarly, Figueiredo and co-workers mentioned that the weather conditions can be crucial determinants for the EO properties of various medicinal plant species.[58] Another study suggests that the relative citral content in *Cinnamomum bodinieri* EOs was positively correlated with the annual average temperature. Also, the relative elemol, cymol, and camphor contents were positively correlated with the annual average sunshine length.[59] Another study demonstrated a positive correlation between high temperature and the accumulation of 1,8-cineole, the main component of *Lavandin intermedia* EO.[60] Also, α-pinene in *Mentha longifolia* EOs showed a notable positive correlation with the mean monthly temperature of the habitat.[61]

Overall, it is an important process to obtain high EO yields and desired component ratios from plants with medicinal and aromatic properties to meet the requirements of the sector. For this, it is necessary to determine the factors that can affect those properties. For instance, *Eucalyptus* EOs rich in 1,8-cineole and α-pinene are primarily appreciated for their therapeutic and industrial applications.[62] For this study, the correlation coefficients between 1,8-cineole and α-pinene content with EO yield ($r = 0.708$ and $r = 0.661$ respectively) indicated that there is a strong positive relationship between EO contents and its major constituents 1,8-cineole and α-pinene. The species studied, particularly those of **Group 2** (*E. salmonphloia* (from A1, A2, A3, SA1 and SA2), *E. lesouefii* (A2) and *E. astringens* (from A2 and A3)) were found to be the abundant sources of 1,8-cineole and α-pinene, with concentrations ranging from 39.68% to 67.16% for 1,8-cineole, and from 7.13% to 24.10% for α-pinene. The species also yielded the highest EOs which ranging from 1.27 ± 0.12 to 4.63 ± 0.27 (w/w). Importantly, the highest production and quality of those species were certainly optimized under arid conditions (high temperature with low precipitation). However, we cannot ignore the importance of other studied species and the other identified compounds. While 1,8-cineole and α-pinene are key contributors to the therapeutic and industrial value of EOs, other compounds present in these oils may also play significant roles in their overall efficacy and applications. The chemical composition of *Eucalyptus* EOs is complex, and the presence of additional bioactive constituents could enhance or complement the properties of 1,8-cineole and α-pinene, potentially expanding their uses in various sectors. Therefore, a broader understanding of how different species and their respective chemical profiles contribute to the overall quality and functionality of EOs is crucial for optimizing their production and ensuring they meet the specific demands of the industry.

For this reason, EOs, selected for their distinct geographical origins and corresponding chemical compositions, were chosen to evaluate their antimicrobial activities. These oils include *E. salmonophloia* (collected from SA1, SA2, and A2), *E. torquata* (collected from SA1, SA2, and A2), *E. lesouefii* (collected from SA1, SA2, and A1), and *E. astringens* (collected from SA2 and A2). The *in vitro* antimicrobial activity of the tested EOs, as estimated by the diameter of inhibition zones, varied depending on species, the location of collection and microbial strains tested. This variability could be attributed to the chemical composition of the leaf oils.

At a dilution of 10^{-1}, a substantial antimicrobial activity was exhibited by all *Eucalyptus* EOs against the seven tested microbial strains, since the lowest inhibition zone registered was 8 mm against *C. tropicalis* from *E. salmonophloia* (A2) EO. These microorganisms were then classified as sensitive to very sensitive to the EOs.

The EOs demonstrated significantly stronger antimicrobial activity against *P. aeruginosa* compared to the standard antibiotic Piperacillin. The greatest activity was observed with *E. salmonophloia* (A2) oil (IZD = 30 mm) which also showed superior efficacy against *E. coli* (29 mm), outperforming Piperacillin (IZD = 10 mm) in this regard as well. This oil was characterized by the highest mean percentage of 1,8-cineole (67.16%). In contrast, Ben Marzoug and co-workers did not observe any activity (0 mm, IZD) from the same *Eucalyptus* species (*E. salmonophloia*) against the same bacterial strain (*P. aeruginosa*).[32] It has been reported also that the EOs from *E. camaldulensis* and *E. tereticornis* exhibited inhibition against this bacterium, but with an IZD of only 16 mm.[53] Similarly, EOs from *E. cinerea* showed weaker activity (IZD = 7 mm) against *P. Aeruginosa*.[54] On the other hand, *P. aeruginosa* was the most resistant bacteria species to *Eucalyptus* EOs[52] due to a very restrictive outer membrane barrier, being highly resistant even to synthetic drugs.[63]

E. astringens (SA2) oil exhibited strong antifungal activity against *C. albicans* (IZD = 30 mm), surpassing the effectiveness of the antibiotic Piperacillin (IZD = 29 mm). This oil was primarily characterized by 1,8-cineole (46.32%) and transpinocarveol (19.30%) as major components. Moreover, the MIC values recorded for the *Eucalyptus* EOs ranged from 10^{-1} to 10^{-4}, depending on the microbial strains tested. The determination of MIC as either bacteriostatic (fungistatic) or bactericidal (fungicidal) precisely reflects the potential of each EO as an antimicrobial agent.

As mentioned in the results above, The *E. lesouefii* (SA1) EO demonstrated the best antimicrobial activity, with both bactericidal and fungicidal effects against *E. coli*, *S. marcescens*, and *C. tropicalis*, as its MIC values were lower than those of the other species. Most of the antimicrobial activity of the *Eucalyptus* EOs has been attributed to the oxygenated monoterpenes, 1,8-cineole.[64,65] The results of the present study suggest that the observed antimicrobial activity cannot be attributed solely to the abundance of the major compound, 1,8-cineole. This is evident from the fact that *E. lesouefii* (SA1), which exhibited both bactericidal and fungicidal activity at lower MICs compared

to other tested EOs, did not contain the highest concentration of 1,8-cineole. Instead, the major compounds in *E. lesouefii* (SA1) were found to be 1,8-cineole (29.71%), followed by spathulenol (17.41%). Besides, numerous prior studies have consistently the antimicrobial activity of sesquiterpene spathulenol as show that it was bactericidal on drug-resistant and susceptible strains of *Mycobacterium tuberculosis*.[66] Also, spathulenol (21,36%), the major compound of *Eugenia calycina* leaf EO showed antimicrobial activity against anaerobic bacteria *Prevotella nigrescens* and *Porphyromonas gingivalis*.[67] In that case 1,8-cineole and its synergistic effect with the other compounds as the spathulenol could justify this antimicrobial property.

Additionally, the EO of *E. torquata* (SA2), rich in torquatone (40.43%) and β-eudesmol (17.78%), and contained the low percentage of 1,8-cineole (11.05%), but it exhibits bactericidal effects against *E. coli* as the same effect of *E. salmonophloia* which mainly rich in 1,8-cineole (63.78%). In that case, Marzouki and co-workers revealed that torquatone, a major component in the EO of *E. torquata*, *E. torwood*, and *E. woodwardi*, does not appear to have a direct effect on antibacterial activity. In fact, *E. torwood* EO, which contains the highest level of torquatone, demonstrated the lowest antibacterial activity.[33] Therefore, there are no studies in the literature regarding the effects of this compound on antibacterial properties. However, β-eudesmol and its isomers (γ- and α-eudesmol) extracted from the leaves of *Guatteria* species exhibited strong antimicrobial properties against gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus epidermidis*, *S. aureus*, *M. luteus*, and *Enterococcus hirae*), gram-negative bacteria (*E. coli* and *P. aerugionsa*), and fungi (*C. albicans*).[68]

Terpenes are the primary class of compounds found in the essential oils of *Myrcia* (Myrtaceae) and are well-documented in the literature for their inherent antimicrobial properties, as well as their synergistic effects against human pathogens.[69]

As result, the anti-microbial effects of EOs cannot be attributed solely to a single major compound, as minor constituents may also have a substantial impact through additive, synergistic or antagonistic interactions. Indeed, Studies linking the antimicrobial activity of *Eucalyptus* EOs to their main components are abundant. In this context, 1,8-cineole,[58] α-pinene,[70] aromdendrene,[71] p-cymene and spathulenol,[72] among others, have been studied for their antimicrobial effects. The synergistic and additive effects of these compounds were previously described on the EO of *E. globulus*, where combinations of 1,8-cineole and aromadendrene reduced the MIC in an additive manner against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecalis*.[73]

From a mechanistic standpoint, the identified components (either alone or in combination) may exert their antimicrobial activity by disrupting the lipophilic core of the membrane, leading to increased fluidity and, ultimately, the leakage of vital macromolecules such as nucleic acids and proteins, as well as potassium ions and protons.[74] Other proposed mechanisms of action include alterations in fatty acid composition, impairment of metabolic pathways, inhibition of the cellular respiratory chain with concurrent disruption of oxidative phosphorylation, depletion of the ATP pool, interference with glucose and oxygen uptake, denaturation of cellular proteins, disruption of nucleic acid synthesis, induction of oxidative stress, and inhibition of enzyme activity.[75] Although the exact mechanism of the antimicrobial effect of EOs is not fully understood, the involvement of one or more of the mechanisms mentioned above could explain the strong antimicrobial activity observed in the studied *Eucalyptus* species. Whatever the case, our findings confirm that the EOs of *Eucalyptus* species is a valuable source of bioactive compounds, including 1,8-cineole, α-pinene, p-cymene, spathulenol, and β-eudesmol. These results further reinforce the understanding that *Eucalyptus* EO possesses significant antimicrobial activity, with the added benefit of being a natural product. The antimicrobial potential of these oils suggests they may have practical applications as microbiostatic, antiseptic, or disinfectant agents. This confirms their potential use in the food and pharmaceutical industries and highlights their value as an alternative antimicrobial agent in natural medicine for treating various infectious diseases.

Conclusion

This study has proved that arid regions (with higher temperatures and lower rainfall) provide favorable environmental conditions for cultivating *Eucalyptus* species, such as *E. salmonophloia*, *E. lesouefii*, and *E. astringens*, and producing an acceptable essential oil content, primarily rich in 1,8-cineole, α-pinene, transpinocarveol, and pinocarvone. However, semi-arid regions (with lower temperatures and higher rainfall) promote the early synthesis of spathulenol, isospathulenol, and viridiflorol, primarily in *E. lesouefii* species. In contrast, the synthesis of torquatone, β-eudesmol, and γ-eudesmol by *E. torquata* species was more slowly influenced by fluctuations in climatic conditions.

The oils from the various *Eucalyptus* species also demonstrated significant antimicrobioal effect, importantly; these properties could not be attributed to a single major compound alone, indicating the complexity of interactions between different constituents in *Eucalyptus* EOs. These findings underscore the potential application of these oils in the food, pharmaceutical and natural medicine industries as effective, natural antimicrobial agents. Further studies are required to better understand the mechanisms of action and optimize their use in practical applications.

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Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Supplemental Material

Supplemental material for this article is available online.

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