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Chemodiversity and antimicrobial activities of *Eucalyptus* spp. essential oils

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ABSTRACT

The essential oils extracted from the leaves of five *Eucalyptus* species: *E. astringens, E. camaldulensis, E. lehmannii, E. leucoxylon*, and *E. sideroxylon*, were investigated for their antimicrobial properties. These species were growing in the same plantation area, exposed to identical conditions, and subjected to uniform agronomic practices. Processed and analyzed under consistent parameters, the essential oil yields ranged from 0.14 to 0.96% (w/w). Chromatographic analysis were resolved into 48 compounds, with 11 common to all oils. Terpenoids (oxygenated mono- and sesquiterpenes) dominated the oil profiles, constituting 55.66–76.67% of the composition. Major components identified included 1,8-cineole (21.97–50.93%), α-pinene (2.18–15.95%), *p*-cymene (0.83–15.94%), spathulenol (0–20.49%), globulol (4.09–14.26%), and aromadendrene (2.37–15.03%). Genetically driven interspecific variation in composition was observed through Principal Component Analysis (PCA), Hierarchical Cluster Analysis (HCA), and heatmap clustering. Moreover, distinctive components were identified for each essential oil, offering a valuable tool for discriminating between *Eucalyptus* species and ensuring authentication and quality control in commercial samples. Results from antimicrobial disc-diffusion assays indicated robust antimicrobial activity in all essential oils, with those derived from *E. camaldulensis, E. lehmannii*, and *E. leucoxylon* exhibiting the highest effectiveness.

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1. Introduction

The genus *Eucalyptus* encompasses approximately 900 species and subspecies distributed worldwide (Barbosa et al., 2016). Members of this genus are multipurpose trees cultivated for their ornamental characteristics, timber production, and cut foliage (Caputo et al., 2020). *Eucalyptus* leaves, a by-product of tree cutting, are particularly rich in essential oil to which the antioxidant, antimicrobial, repellent, insecticidal, herbicidal, and nematicidal activities, among others are attributed (Barbosa et al., 2016; Batish et al., 2008; Mossi et al., 2011). Due to their numerous biological activities, *Eucalyptus* essential oils are widely used in various industrial sectors, including cosmeceuticals, fragrances, foods, pharmaceuticals, agrochemicals, and household products (Goldbeck et al., 2014). They are also employed in different traditional medicine systems to treat conditions such as colds, coughs, influenza, sore throat, and sinus congestion (Dogan et al., 2017). Recent applications of *Eucalyptus* essential oils include the treatment of gastrointestinal disorders (diarrhea, colic, and dysentery) and respiratory diseases (asthma, laryngitis, trachealgia, and pharyngitis), in addition to their anti-inflammatory, wound-healing, analgesic,

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anti-nociceptive, cytotoxic, and anti-diabetic properties (Aleksic Sabo & Knezevic, 2019).

Due to these intriguing activities and relevant applications, the main representative species of the genus *Eucalyptus* are extensively studied for the essential oil composition of their foliage. Previous phytochemical investigations have pointed to the presence of common compounds, including oxygenated monoterpenes (1,8-cineole, citronellol, piperitone, isopulegol, citronellal, α-terpineol, linalool, terpinyl acetate, citronellyl acetate, etc.), monoterpene hydrocarbons (α-, β-pinene, *p*-cymene, limonene, camphene, γ-terpinene, etc.), oxygenated sesquiterpenes (spathulenol, caryophyllene oxide, etc.), and sesquiterpene hydrocarbons (β-caryophyllene, aromadendrene, α-copaene, bicyclogermacrene, etc.) (Aleksic Sabo & Knezevic, 2019; Ameur et al., 2021; Barbosa et al., 2016; Limam et al., 2020).

However, the quality of *Eucalyptus* spp. essential oils and their subsequent biological activities can be somewhat variable, depending on factors such as plant species/subspecies, origin, season, organ, extraction, and analytical conditions. Consequently, different chemotypes within various populations of the same species have been described (Barbosa et al., 2016). Given their wide range of medicinal, agronomic, and industrial applications, analyzing *Eucalyptus* spp. essential oils and understanding their chemodiversity is crucial for defining potential applications and devising the best strategy for their conservation and naturalization.

In Tunisia, the genus *Eucalyptus* is represented by 117 species naturalized into 30 arboretums (Ben Jemâa et al., 2012). Most of them are cultivated for ornamental and honey trees, as well as for timber and firewood production. In folk medicine, *Eucalyptus* leaves are used to treat colds, coughs, and respiratory disorders, including pharyngitis, bronchitis, and sinusitis (Ameur et al., 2021). This fast-growing species has adapted well to the Tunisian climate and has been used to stabilize the coastal dunes of northwest Tunisia, reduce erosion, and protect roadsides (Elaieb et al., 2019).

Recent studies reported by Horst et al. (2022b) have raised questions about the use of *Eucalyptus foliage* as feed for ruminants due to its low crude protein and energy content. However, results reported by Horst et al. (2022a) suggest that some *Eucalyptus* species could be included in ruminant diets to modulate fermentation processes in the rumen. On the other hand, it is well assumed that natural extracts or essential oils rich in phytochemicals are perceived as safer and more environmentally friendly products (Beauchemin et al., 2022). Therefore, they have higher levels of acceptance, raising fewer animal and food safety concerns (Dey et al., 2021). Earlier compositional studies have reported interspecific and intraspecific variations in essential oil composition and its biological activities (Ameur et al., 2021; Ben Jemâa et al., 2012; Elaissi et al., 2012; Hamdi et al., 2015; Limam et al., 2020; Slimane et al., 2014; Yangui et al., 2017). However, most of these studies are focused on particular species, such as *E. camaldulensis* and *E. globulus* (Ben Jemâa et al., 2012; Hamdi et al., 2015; Slimane et al., 2014; Yangui et al., 2017), and little is known about the remaining species. The main objective of this present study was to determine the essential oil composition of five *Eucalyptus* species: *E. astringens*, *E. camaldulensis, E. lehmannii, E. leucoxylon*, and *E. sideroxylon,* and assess their chemodiversity. The antimicrobial activity against *Staphylococcus aureus, Enterococcus faecium, Escherichia coli, Salmonella typhimurium* and *Candida albicans* was also evaluated.

2. Materials and methods

2.1. Plant materials

Leaf samples were collected from five specimens of 52-year-old trees of *E. astringens, E. camaldulensis, E. lehmannii, E. leucoxylon,* and *E. sideroxylon* growing in the arboretum of Korbous (Northeastern Tunisia, latitude: 36°50′N, longitude: 10°23′E, Altitude: 180 m above sea level; climate: sub-humid). For each species, 10–15 trees were selected within each plot (based on health status and size), and a branch (approximately 3–4 m high and 1 m long) was cut from each tree, handpicking 100 g of fresh matter sample of mature foliage. Leaves were dried at room temperature (20 ± 2 °C), ground into fine powders, and examined for their essential oil composition.

2.2. Isolation of essential oils

The dried leaf samples underwent hydrodistillation (100 g/800 mL distilled water) for three hours using a Clevenger-type apparatus. The resulting essential oil samples were dehydrated using anhydrous sodium sulfate (Na_2SO_4) and stored in sealed amber vials at −20 °C until analysis.

2.3. Analysis of essential oils

Samples of essential oils were diluted 20-fold in hexane and analyzed using an HP 6890 (II) gas

chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an HP-5 (30 m × 0.32 mm ID, 0.25 µm film thickness; Supelco, Bellefonte, PA, USA) capillary column. Operating conditions were as follows: The oven temperature was programmed at 5 °C/min from an initial temperature of 40 °C (maintained isothermally for 10 minutes) to 280 °C, which was held for an additional 10 minutes. Injector and FID detector temperature were maintained at 230 °C; the injection volume was 0.5 µL; split ratio of 1:20, and the flow rate of the carrier nitrogen gas was 1.2 mL/min.

For gas chromatography-mass spectrometry (GC-MS) analysis, an HP 6890 gas chromatograph coupled to an HP 5973 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) was used. The analytical conditions and the column used for individual component separation were the same as those used for GC-FID analysis, except helium was used as the carrier gas. The mass spectrometer operated in electron-impact (EI) mode; ionization energy was set at 72 eV; ion source temperature was maintained at 270 °C; scan time was 1 second, and the mass range scanned was 50–550 amu.

The identification of constituents was based on the comparison of their retention indices (RI) relative to (C_7–C_{20}) n-alkanes (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) with those from the literature (Dey et al., 2021) and/or with those of authentic standards when available. Identification was also performed by matching against the NIST05a MS library. The relative content of the identified components was determined through electronic integration of the FID-peak areas without correcting for response factors.

2.4. Antimicrobial activity

The antimicrobial activity of *Eucalyptus* spp. essential oils was evaluated qualitatively using the disc-diffusion assay described by the National Committee for Clinical Laboratory Standards (NCCLS, 1997). The test microorganisms included the Gram-positive bacteria *S. aureus* (ATCC 6538) and *E. faecium* (ATCC 19434), the Gram-negative bacteria *E. coli* (ATCC 8739) and *S. Typhimurium* (ATCC 14028), and the yeast *C. albicans* (ATCC 10231). All microorganisms were obtained from the culture collection center of the Institut National de Recherche et d'Analyse Physico-Chimique (INRAP, Sidi Thabet, Tunisia). Bacterial strains were cultured in sterile Mueller Hinton agar (MHA) medium and incubated at 37 °C for 24 h, while fungal strains were cultured in Sabouraud dextrose agar (SDA) at 30 °C for 48 h.

Briefly, 100 µL of microbial suspension comprising 1–2 × 10^8 CFU/mL of bacterial cells or 1–5 × 10^6 CFU/mL for yeast were spread onto petri plates containing MHA or SDA culture mediums, respectively. Sterile filter paper discs (6 mm in diameter) were impregnated with 10 µL of essential oil (10 mg/mL in DMSO) and placed on the inoculated plates, left to stand for 2 h at 4 °C before being incubated at 37 °C for 24 h for bacteria and 30 °C for 48 h for yeast. The diameter of the inhibition zone was accurately measured. DMSO (10 µL per filter paper disc) was used as negative control while, Gentamycin and nystatin (10 µg/mL) were used as positive controls for bacteria and yeast, respectively.

2.5. Statistical analysis

The Principal Component Analysis (PCA), hierarchical cluster analysis using Euclidean distance and unweighted group method, and the heatmap clustering based on the entire composition of essential oils were conducted to elucidate the inter-relationships among all species. Antimicrobial activity data were presented as mean ± SD of triplicates. All analyses were performed using the statistical R 2.14.1 packages (Wirtschaftsuniversität Wien, Vienna, Austria).

3 Results and discussion

3.1. Yields and chemical composition of essential oils

From the leaves, pale yellowish essential oils with average yields of 0.96%, 0.35%, 0.55%, 0.32%, and 0.14% (w/w) were obtained for *E. astringens, E. camaldulensis, E. lehmannii, E. leucoxylon,* and *E. sideroxylon*, respectively (Table 1). These values align with those reported for *E. oleosa* (Marzoug et al., 2011), *E. camaldulensis, E. saligna* (Barbosa et al., 2016), *E. gomphocornuta, E. paniculata* (Limam et al., 2020), *E. bosistoana, E. mellidiora, E. odorata,* and *E. paniculata* (Kouki et al., 2022). However, they are considerably lower than those observed in *E. globulus, E. cinerea, E. citriodora* (Barbosa et al., 2016), *E. accedens, E. cladoalyx, E. lesouefi, E. melliodora, E. punctate, E. robusta, E. wandoo* (Ameur et al., 2021), *E. melliodora,* and *E. maidenii*, among others (Limam et al., 2020). These discrepancies could be attributed to genetic factors, pedoclimatic conditions, season, plant age, processing, and extraction methodology. In our case, differences in essential oil yields were unequivocally attributed to genetic differences (species) as they were cultivated and processed under the same conditions.

Table 1. Chemical composition (% total peak area) of the leaf essential oil of *Eucalyptus* spp.

No.	Compounds	RI	*E. astringens*	*E. camaldulensis*	*E. lehmannii*	*E. leucoxylon*	*E. sideroxylon*
1	α-Pinene	939	10.26	2.41	15.95	12.47	2.18
2	Fenchene	951	–	0.16	–	–	–
3	Camphene	953	–	–	0.58	–	–
4	β-Pinene	980	–	–	–	0.38	–
5	β-Myrcene	988	–	–	–	0.19	–
6	α-Phellandrene	1005	–	0.51	–	0.1	–
7	4-Carene	1018	–	0.28	–	–	–
8	*p*-Cymene	1026	1.6	15.94	1.96	5.34	0.83
9	1,8-Cineole	1031	22.52	21.97	31.8	50.93	40.64
10	γ-Terpinene	1061	–	0.22	0.77	7.7	–
11	Terpinolene	1087	–	–	–	0.11	–
12	*p*-Cymenene	1089	–	0.39	–	–	–
13	Fenchol	1117	0.26	0.14	1.55	–	–
14	α-Campholenal	1125	–	–	0.42	–	–
15	*trans*-Pinocarveol	1139	7.19	2.99	4.73	1.07	2.62
16	Pinocarvone	1162	1.87	1.13	0.96	0.29	0.56
17	Borneol	1167	0.51	0.44	2.73	0.22	–
18	Terpinen-4-ol	1178	–	1.09	0.62	1.95	–
19	Isocarveol	1187	0.27	3.52	–	0.12	0.37
20	α-Terpineol	1189	0.53	–	6.72	1.79	–
21	2-Methyl-3-phenyl-propanal	1244	–	2.32	–	–	–
22	Piperitone	1251	–	–	–	0.18	–
23	Phellandral	1280	–	2.56	–	–	–
24	Thymol	1295	–	2.08	–	–	–
25	*p*-Cymene-7-ol	1291	–	0.35	–	–	–
26	Carvacrol	1299	–	–	0.44	–	–
27	α-Terpinyl acetate	1351	–	–	11.65	–	–
28	α-Gurjunene	1414	0.41	–	–	–	0.32
29	β-Caryophyllene	1418	0.83	–	0.3	–	0.53
30	γ-Maaliene	1435	0.3	–	–	–	–
31	Calarene	1440	0.71	0.32	–	–	0.56
32	Aromadendrene	1444	15.03	4.14	3.26	2.37	12.98
33	*allo*-Aromadendrene	1461	2.42	2.76	1.1	0.32	2.4
34	γ-Gurjunene	1469	0.49	–	–	–	–
35	γ-Selinene	1479	0.94	0.95	–	–	0.82
36	Viridiflorene	1483	1.36	0.49	0.4	–	1.24
37	*cis*-Calamenene	1511	0.35	0.8	–	–	–
38	Epiglobulol	1539	2.95	1.5	0.97	1.39	3.12
39	Selina-3,7(11)-diene	1543	0.47	–	0.43	–	1.39
40	Spathulenol	1577	–	20.49	1.82	–	4.55
41	Globulol	1583	11.37	4.09	4.12	8.59	14.26
42	Viridiflorol	1590	3.61	0.66	1.35	1.5	4.15
43	Rosifoliol	1603	1.49	0.2	–	0.63	1.73
44	Copaborneol	1606	–	1.37	–	–	–
45	Humulene epoxide II	1608	0.65	0.28	–	0.29	0.71
46	Selina-6-en-4-ol	1623	1.93	0.56	0.38	0.74	2.24
47	Isospathulenol	1641	0.51	0.9	–	–	0.64
48	Eudesmol	1654	–	–	1.41	0.88	1.15
	Group components						
	Monoterpene hydrocarbons		11.86	19.91	19.26	26.29	3.01
	Oxygenated monoterpenes		33.15	36.27	61.62	56.55	44.19
	Sesquiterpenes hydrocarbons		23.31	9.46	5.49	2.69	20.24
	Oxygenated sequiterpenes		22.51	30.05	10.05	14.02	32.55
	Miscellaneous		–	2.32	–	–	–
	Total identified		90.83	98.01	96.42	99.55	99.99

The chromatographic analysis identified 48 components covering more than 90% of the total peak area. Typical chromatographic profiles are shown in Figure 1. Irrespective of the *Eucalyptus* species, all oil samples are terpenoid-rich essential oils (Table 1). Terpenoids (oxygenated mono- and sesquiterpenes) are particularly abundant in *E. lehmannii* (71.67%), *E. leucoxylon* (70.57%), and *E. sideroxylon* (76.74%).

The oxygenated monoterpene 1,8-cineole (eucalyptol) was by far the major component (22–51%) in all investigated essential oils. Therefore, the studied *Eucalyptus* species could be categorized as the 1,8-cineole chemotype. Other significant compounds, including aromadendrene, globulol, pinocarvone, and α-pinene, were identified in *E. astringens*. Aromadendrene and globulol were also detected in

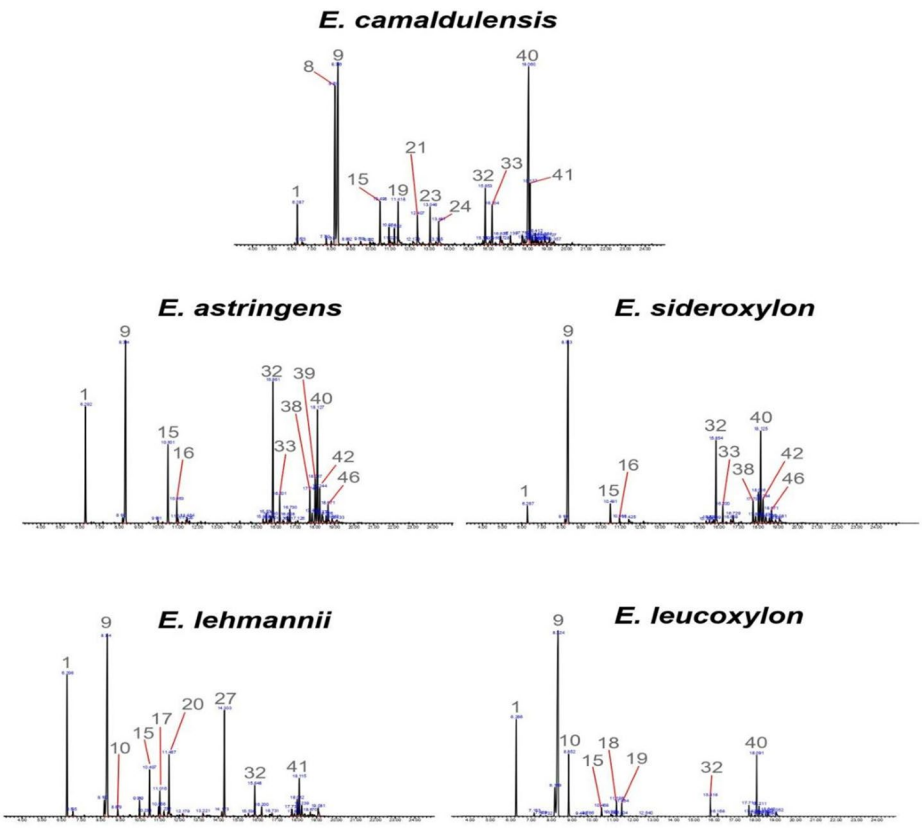

Figure 1. Typical GC-MS chromatograms of the leaf essential oil of *Eucalyptus* spp.

appreciable amounts in *E. sideroxylon*. In the essential oil of *E. camaldulensis*, spathulenol and *p*-cymene were abundant. *E. lehmannii* had the highest percentages of α-pinene, α-terpineol, and terpinyl acetate. The monoterpene hydrocarbons α-pinene, terpinolene, *p*-cymene, and the oxygenated sesquiterpene globulol were the most plentiful components in the essential oil of *E. leucoxylon*.

Compared with earlier compositional studies, the pattern of abundance of the main compounds has been reported in *Eucalyptus* species. For example, the profile 1,8-cineole > α-pinene has been previously described in Tunisian specimens of *E. leucoxylon* (Elaissi et al., 2012), *E. lehmannii*, and *E. astringens* (Limam et al., 2020). In contrast, the latter authors reported that spathulenol and *o*-cymene were dominants in the essential oil of *E. camaldulensis*. At this point, it can be inferred that this species represents different chemotypes. For instance, the *p*-cymene/1,8-cineole chemotype has been described in Turkish specimens of *E. camaldulensis* (Dogan et al., 2017).

Other chemotypes, including 1,8-cineole/*p*-cymene (Lucia et al., 2009); 1,8-cineole/limonene (Batista-Pereira et al., 2006); 1,8-cineole/α-pinene (Mediouni Ben Jemâa et al., 2013; Salem et al., 2016); α-phellandrene/β-pinene (Debbarma et al., 2013); linalool/1,8-cineole (Ghaffar et al., 2015); spathulenol/*p*-cymene (Verdeguer et al., 2009), and α-pinene/*p*-cymene (Chouhan et al., 2017), have been reported in *E. camaldulensis* specimens from Argentina, Brazil, Egypt, Tunisia, India, Pakistan, Spain, and Taiwan. The α-pinene/1,8-cineole chemotype has been recorded for Tunisian *E. astringens* (Hamdi et al., 2015) and *E. leucoxylon* (Ben Jemâa et al., 2012; Mediouni Ben Jemâa et al., 2013) specimens. Regarding *E. sideroxylon* from the same origin, the presence of at least two chemotypes, 1,8-cineole/globulol (in this study) and 1,8-cineole/α-pinene (Elaissi et al., 2012), may be confirmed. In contrast, it seems that the 1,8-cineole/α-pinene chemotype dominated the leaf essential oil of *E. lehmannii* species (Elaissi et al., 2012; Hamdi et al., 2015; Limam et al., 2020; Slimane et al., 2014). In general, it appears that the chemical composition of the essential oil of *Eucalyptus* spp. is particularly prone to qualitative and quantitative changes depending on genetic factors (species, subspecies, and cultivars), season, climate, soil type, and agronomic factors. Given their industrial importance as a source of essential oil, a better categorization of *Eucalyptus* species based on the definition of some distinctive specific markers will be of great importance for authentication purposes.

3.2. Specific markers and heatmap clustering

From the chemical composition of all essential oils, a list of chemically defined volatile markers has been established (Figure 2). As shown, the presence of γ-maaliene and γ-gurjunene is characteristic of the essential oil of *E. astringens*. Fenchene, 4-carene, *p*-cymenene, 2-methyl-3-phenyl-propanal, phellandral, thymol, *p*-cymen-7-ol, and copaborneol distinguish the essential oil of *E. camaldulensis*.

The presence of camphene, α-campholenal, carvacrol, α-terpinyl acetate, versus the absence of isocarveol, rosifoliol, and humulene epoxide II, distinguished the essential oil of *E. Lehmannii* from the remaining species. The essential oil of *E. leucoxylon* was characterized by the presence of β-pinene, β-myrcene, terpinolene, and piperitone, while being exempt from viridiflorene. The absence of borneol seems to be a characteristic of the essential oil of *E. sideroxylon*. From a practical standpoint, the mentioned chemical markers could provide baseline information for the quality assessment of the commercialized leaf essential oils of *Eucalyptus* species growing in the region of Korbous.

Additional analyses, including the heatmap clustering (with the highest percentage represented by intense blue color, while the lowest was indicated by light color) and hierarchical cluster analysis (HCA) based on Euclidean distance and the unweighted group method, allowed the separation of *Eucalyptus* spp. into three distinct groups: group 1—*E. lehmannii* and *E. leucoxylon*; group 2—*E. astringens* and *E. sideroxylon*; group 3—*E. camaldulensis* (Figure 2).

3.3. Principal component analysis (PCA)

To validate the aforementioned classification, a PCA analysis (Figure 3) based on the entire volatile profile was conducted. The PCA biplot, explaining 73.3% of the total variance (with 36.6% and 36.3% for PC1 and PC2, respectively), distinctly reveals three groups. The first group unites *E. lehmannii* and *E. leucoxylon*, characterized by high levels of α-pinene, γ-terpinene, and α-terpineol. These components, along with other monoterpenes, showed positive loading on PC1.

The second group, represented by the essential oils of *E. astringens* and *E. sideroxylon*, exhibits similar profiles primarily consisting of sesquiterpenes, both oxygenated and hydrocarbons. These include aromadendrene, globulol, viridiflorene, viridiflorol, rosifoliol, selina-6-en-4-ol, humulene epoxide II, α-gurjunene, and β-caryophyllene, all of which were negatively loaded on PC1. *E. camaldulensis* is distinctly separated from other species due to its elevated content of spathulenol, *cis*-calamenene, isocarveol, and *p*-cymene, in addition to the previously mentioned marker components.

Considering that all *Eucalyptus* species are of the same age and cultivated and processed under identical conditions (i.e. collection of leaves, drying, extraction of essential oils, and their analysis), the genetic dissimilarity between *Eucalyptus* spp. is reaffirmed based on their essential oil composition.

Given that the bioactivity of an essential oil is primarily determined by its chemical composition, it will be highly significant to evaluate the antimicrobial activity of *Eucalyptus* spp.

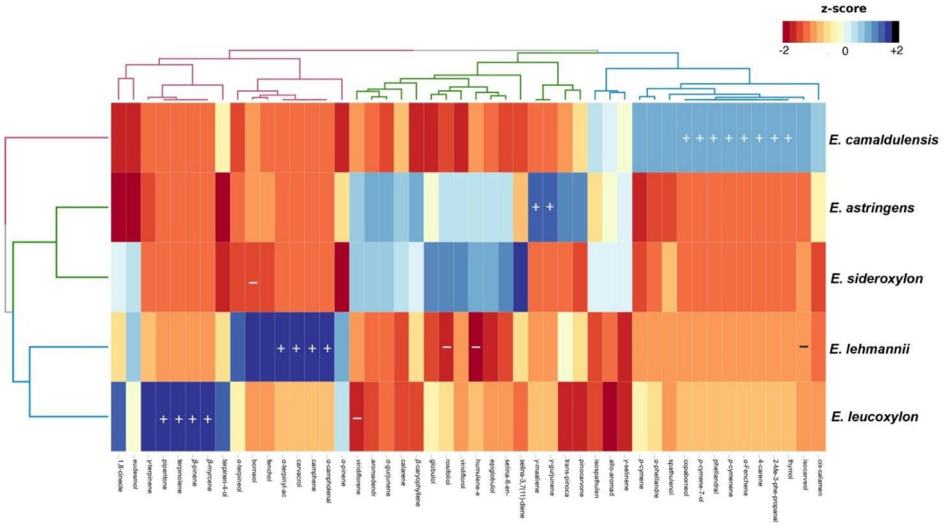

Figure 2. Heatmap clustering using Euclidean distance and unweighted group method, and distinctive chemical markers of leaf essential oils of *Eucalyptus* spp. (+): presence; (–) absence.

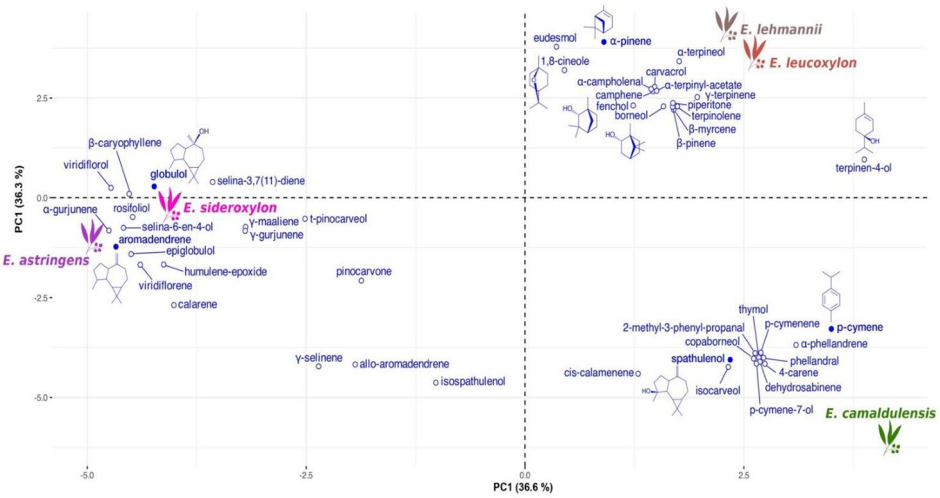

Figure 3. PCA biplot of the *Eucalyptus* spp. leaf essential oils.

Table 2. Antimicrobial activity (expressed as zone of inhibition in mm) of the leaf essential oils of *Eucalyptus* spp.

Microorganism	*E. coli* (ATCC 8739) G(–)	*S. typhimurium* (ATCC 14028) G(–)	*S. aureus* (ATCC 6538) G(+)	*E. feacium* (ATCC 19434) G(+)	C. albicans ATCC 10231
E. astringens	13 ± 0.7	11.75 ± 0.3	23.75 ± 0.5	21 ± 0.5	14.75 ± 0.3
E. camaldulensis	15.5 ± 0.3	15.75 ± 0.5	35 ± 0.0	29.5 ± 0.7	21 ± 0.0
E. lehmannii	14.75 ± 1	8.25 ± 0.7	31.5 ± 0.7	34.75 ± 0.3	15.5 ± 0.7
E. leucoxylon	15.75 ± 0.3	15 ± 0.0	27.75 ± 0.3	20.5 ± 0.7	13.75 ± 0.3
E. sideroxylon	13.75 ± 0.3	11.25 ± 0.3	25 ± 0.0	30.5 ± 0.7	8.75 ± 0.3
Gentamycin	21.75 ± 0.3	23 ± 0.0	25.75 ± 0.3	31.5 ± 0.7	–
Nystatin	–	–	–	–	21.5 ± 0.5

3.4. Antimicrobial activity of Eucalyptus spp. Essential oils

The results of the antimicrobial activity of the five *Eucalyptus spp.* essential oils are summarized in Table 2. All essential oils strongly inhibited the growth of the tested strains, with the Gram-positive strains *S. aureus* (inhibition zone diameter: 25–35 mm) and *E. faecium* (inhibition zone diameter: 29.5–34.5 mm) being the most sensitive. They were notably inhibited by the essential oils of *E. camaldulensis, E. lehmannii,* and *E. sideroxylon*. Additionally, the essential oils from *E. camaldulensis* and *E. leucoxylon* were particularly effective against the Gram-negative bacteria *E. coli* and *S. typhimurium*. The former essential oil (*E. camaldulensis*) also demonstrated high efficiency against the yeast *C. albicans,* with an inhibition halo similar to that of the standard antibiotic nystatin.

These results align with previous reports showcasing the potent antimicrobial activity of *Eucalyptus* essential oils against gram-positive bacterial strains, especially *S. aureus,* and the yeast *C. albicans* (Barbosa et al., 2016). The sensitivity of Gram-positive bacteria is attributed to the presence of a thick peptidoglycan wall associated with the lipophilic ends of lipoteichoic acid, facilitating the entry of hydrophobic components into the cell membrane (Chouhan et al., 2017). Numerous studies have linked the antimicrobial activity of *Eucalyptus* essential oils to their main components. For instance, it has been reported that the essential oil of *E. camaldulensis* strongly inhibits the growth of the Gram-positive *S. aureus* and *Bacillus cereus* (Dogan et al., 2017). *E. camaldulensis* essential oil, described as the most active among *Eucalyptus* species, has also been found effective against the yeast *C. albicans* (Aleksic Sabo & Knezevic, 2019), supporting our findings. Similar results have been reported for essential oils derived from *E. sideroxylon* (Ashour, 2008), *E. astringens, E. lehmannii* (Limam et al., 2020), and *E. leucoxylon* (Elaissi et al., 2012), among others.

Direct evidence of the antimicrobial activity of the main components of *Eucalyptus* essential oils has also been provided. Particularly active compounds include 1,8-cineole (Wang et al., 2022), α-pinene (Dhar et al., 2014), terpinyl acetate (Badr et al., 2021; Fidan et al., 2019), α-terpineol (Li et al., 2014), globulol (Tan et al., 2008), aromadendrene (Mulyaningsih et al., 2010), *p*-cymene (Marchese et al., 2017), spathulenol (Dzul-Beh et al., 2019), *trans*-pinocarveol (Viljoen et al., 2002), and terpinen-4-ol (Cordeiro et al., 2020), among others. The synergistic and

additive effects of these compounds have been described for the essential oil of *E. globulus* (Mulyaningsih et al., 2010). In their checkerboard assay, the study authors successfully demonstrated that the combination of 1,8-cineole and aromadendrene greatly enhanced the antimicrobial effect against methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *E. faecalis* through additive or synergistic interactions. Four years later, Johansen et al. (2022) showed that combinations involving *p*-cymene, terpinen-4-ol, α-terpineol, and linalool exhibited an additive antibacterial effect against some food-borne pathogens, including *E. coli* O157:H7, *S. aureus, S. mutans, S. sanguinis, S. enterica, Listeria monocytogenes*, and *Vibrio parahaemolyticus*. More recently, it has been demonstrated that the combination of terpinen-4-ol and α-terpineol synergistically inhibits the growth of *S. aureus,* MRSA, *E. coli*, and *Pseudomonas aeruginosa* (Johansen et al., 2022).

From a mechanistic standpoint, the identified components (either individually or in combination) could exert their antimicrobial activity by interfering with the lipophilic core of the membrane, leading to increased fluidity, and ultimately causing the leakage of vital macromolecules (e.g. nucleic acids and proteins), potassium ions, and protons (Zomorodian et al., 2017). Other mechanisms of action include the alteration of fatty acid composition, impairment of metabolic pathways, inhibition of the cellular respiratory chain with a concomitant interruption of oxidative phosphorylation, a decrease in ATP pool, interference with glucose and oxygen uptake, denaturation of cell proteins, disruption of nucleic acid synthesis, installation of oxidative stress, and inhibition of enzyme activity (Angane et al., 2022; Dhar et al., 2014; Li et al., 2014; Melkina et al., 2021; Xiang et al., 2018). The disruption of biofilm formation and basic bacterial metabolism have been proposed as the main mechanisms underlying the antibacterial activities of 1,8-cineole-rich essential oils of *E. bicostata, E. gigantea, E. intertexta, E. obliqua, E. pauciflora*, and *E. tereticornis* against *S. aureus, L. monocytogens, acinetobacter baumannii, Pseudomonas aeruginosa* and *E. coli* (Polito et al., 2022).

Although, the exact mechanism of the antimicrobial effect of *Eucalyptus* essential oils is not fully understood, the implication of one or more of the mechanisms mentioned above could explain the strong antimicrobial activity of the studied *Eucalyptus* species. In any case, these data provide evidence for the current use of their essential oils as a natural antiseptic and food preservative.

4. Conclusions

The compositional analysis of the leaf essential oils of *Eucalyptus* spp. revealed significant chemical polymorphism, primarily determined by genetic factors (species effect). Essential oils rich in 1,8-cineole were categorized using distinctive chemical markers, enabling differentiation between various *Eucalyptus* spp. and their corresponding essential oils extracted under the same conditions. This chemical identification method can serve as a means to determine the specific *Eucalyptus* species of origin for the oils.

The studied oils exhibited robust antimicrobial activity, likely attributed to their high 1,8-cineole contents and/or other potential compounds acting synergistically or additively. Based on these findings, it is suggested that the essential oils from the studied *Eucalyptus* spp. could serve as candidates for natural flavors and preservatives in food/feed, cosmetic, pharmaceutical, agrochemical, and household applications, particularly for highly perishable items and products susceptible to microbial contamination. Further studies exploring additional activities of *Eucalyptus* essential oils and providing details on the mechanisms of their actions should be conducted and reported.

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Conceptualization, H.A. and K.H.; methodology, H.A., Y.M. and K.H.; software, K.H.; validation, H.A., S.L. and K.H.; formal analysis, H.A., Y.M. and K.H.; investigation, H.A., Y.M., S.H., W.S., S.A., and K.H.; resources, H.A., S.H. and K.H.; data curation, H.A. and K.H.; writing—original draft preparation, H.A. and K.H.; writing—review and editing, H.A., S.H., M.C., M.d.H.-M., W.S., S.A., S.L. and K.H.; visualization, H.A., S.H., M.C., M.d.H.-M., S.A., S.L. and K.H.; supervision, H.A., M.C., M.d.H.-M. and K.H., project administration, H.A., W.S. and K.H.; funding acquisition, W.S. All authors have read and agreed to the published version of the manuscript.

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Data availability statement

The data presented in this study are available on request from the corresponding author.

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