Essential Oil Composition and Antimicrobial Activity of Eucalyptus robusta Families (Postprint)

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Abstract

To screen for Eucalyptus robusta essential oil families with excellent antibacterial activity, this study utilized four 5-year-old E. robusta families from Nanning and Wuxuan as experimental materials. Essential oils were extracted from fresh leaves via steam distillation, their chemical components were identified, and their antibacterial activity against Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Salmonella typhi was determined. The study explored family-specific differences in essential oil yield and composition, variations, and the influence of components on antibacterial activity. The results showed that: (1) The essential oil composition of the four E. robusta families was dominated by monoterpenoids (73.695%–84.535%), with the main components being pinene, phellandrene, and p-cymene. α-Pinene content in E. robusta Family No. 1 reached 41.629%. Common components across families included α-pinene, β-pinene, α-phellandrene, d-limonene, p-cymene, γ-terpinene, 4-terpineol, and α-terpineol. The oil yield of the 1,8-cineole chemotype was consistently low. (2) Different eucalyptus essential oils exhibited varying responses to environmental changes, with α-phellandrene, 1,8-cineole, d-limonene, α-terpineol, and p-cymene identified as correlated components in E. robusta family essential oils. (3) E. robusta essential oil demonstrated antibacterial activity against E. coli, S. aureus, and S. typhi, with Family No. 3 exhibiting anti-S. typhi activity surpassing that of streptomycin; however, no activity against P. aeruginosa was detected. Increased levels of alcohols, ketones, and aldehydes enhanced antibacterial activity. In conclusion, E. robusta essential oil is primarily composed of monoterpenoids, with terpene compounds being the most abundant. Some essential oil components were correlated among families, with shared common components. Alcohol, ketone, and aldehyde compounds in the composition contributed to enhanced antibacterial activity. However, the oil yield of the 1,8-cineole chemotype in E. robusta was consistently low, necessitating further selective breeding. These findings provide a theoretical basis for

the selective breeding of E. robusta essential oil varieties and their applications in fragrance and pharmaceutical industries.

Full Text

Essential Oil Components and Antibacterial Activity of *Eucalyptus robusta* Families

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Abstract: To screen for *Eucalyptus robusta* families with excellent antibacterial activity, this study examined four 5-year-old *E. robusta* families from Nanning and Wuxuan. Essential oils were extracted from fresh leaves via steam distillation, and their chemical components were identified. Antibacterial activity was measured against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhi*, while the relationships among family-specific oil yield, component variations, and antibacterial efficacy were analyzed. The results revealed: (1) The essential oils of the four *E. robusta* families were dominated by monoterpenoids (73.695%–84.535%), with pinene, phellandrene, and *p*-cymene as major constituents. α-Pinene reached 41.629% in Family 1, while common components across families included α-pinene, β-pinene, α-phellandrene, *d*-limonene, *p*-cymene, γ-terpinene, 4-terpineol, and α-terpineol. The 1,8-cineole chemotype exhibited consistently low oil yields. (2) Different leaf essential oils responded differently to environmental changes, with α-phellandrene, 1,8-cineole, *d*-limonene, α-terpineol, and *p*-cymene identified as correlated components. (3) *E. robusta* essential oils showed antibacterial activity against *E. coli*, *S. aureus*, and *S. typhi*, with Family 3 demonstrating even higher activity against *S. typhi* than streptomycin. However, no activity was observed against *P. aeruginosa*. Increased concentrations of alcohols, ketones, and aldehydes enhanced antibacterial efficacy. In conclusion, *E. robusta* essential oils are primarily composed of monoterpenoids, particularly terpene compounds. Correlated components and shared constituents exist among families, and increased alcohol, ketone, and aldehyde content improves antibacterial activity. However, the low oil yield of the 1,8-cineole chemotype necessitates further breeding efforts. These findings provide a theoretical basis for the selection

and breeding of *E. robusta* essential oil varieties for spice and pharmaceutical applications.

Keywords: *Eucalyptus robusta*, family, essential oil, ESKAPE pathogens, antibacterial activity

Introduction

Essential oils are volatile, oil-like liquids extracted from plant organs using modern techniques. They exhibit favorable properties including ease of extraction, biodegradability (Zygadlo & Grow, 1995), and low toxicity to vertebrates (Isman, 2000), making them widely applicable in antibacterial and medicinal contexts. The current overuse of single-mode antibiotics has increased bacterial resistance, particularly among drug-resistant ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) (Naveed et al., 2013; Chouhan et al., 2017), which seriously threaten the survival of higher organisms. The compounds abundant in essential oils hold significant potential for developing novel antibacterial drugs to alleviate pressure from resistant bacteria (Hyldgaard et al., 2012; Dhakad et al., 2018). Research has shown that eucalyptus essential oil ranks first in international essential oil trade, representing a colorless or pale yellow oil containing mixtures of monoterpenes, sesquiterpenes, alcohols, esters, aldehydes, and ketones (Khan et al. 2020) with remarkable biological activity (Dhakad et al., 2018). Although essential oil composition varies among different plant organs, eucalyptus possesses specialized secretory structures in leaves where essential oils are primarily stored. Consequently, eucalyptus oil in the market is mainly derived from leaf oil (Filomeno et al., 2016), underscoring the importance of eucalyptus leaf essential oil research. Current studies on eucalyptus leaf essential oil primarily focus on seasonal climate effects, oil yield under different extraction conditions, and differences in antibacterial and antioxidant capacity, with minimal investigation of variations among different families. However, superior family selection constitutes a crucial component of eucalyptus genetic improvement, necessitating further research on essential oils from eucalyptus families.

Eucalyptus robusta, a species in the Myrtaceae family native to southeastern Australia, was introduced to China around 1890. Its leaves and bark possess medicinal properties for sterilization and detoxification, with the primary active component—volatile oil or essential oil—exhibiting high antibacterial activity (Patrícia et al., 2007; Nagpal et al., 2010; Jian et al., 2012; Chen et al., 2018). While the eucalyptus oil market is dominated by *Eucalyptus citriodora* and *Eucalyptus globulus* essential oils, *E. robusta* is phylogenetically closest to *E. globulus* (Filomeno et al., 2016; Dhakad et al., 2018; Gao et al., 2021). Investigating *E. robusta* family essential oils could broaden and enhance the application value of eucalyptus leaf essential oils in spices and medicine. Previous studies on *E. robusta* essential oil extraction have identified water as the optimal solvent for extracting phenols, flavonoids, and proanthocyanidins, yielding higher quanti-

ties of phenolic and flavonoid compounds (Ozen et al., 2011). Eucalyptus leaf aqueous extracts demonstrate higher antioxidant values than other eucalyptus species (Bhuyan et al., 2016), and fresh leaf extraction yields phenolic compound concentrations dozens to hundreds of times higher than dry leaf extracts (Pinto et al., 2022). Therefore, *E. robusta* is more suitable for essential oil extraction from fresh leaves using the industrially common steam distillation method (Verdeguer et al., 2009; Puig et al., 2018). Based on these findings, this study employed steam distillation to extract essential oils from fresh *E. robusta* leaves. By analyzing the essential oil components of four different *E. robusta* families planted in Nanning and Wuxuan, and using *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhi*, and *Escherichia coli* (recognized as major ESKAPE pathogens) as test organisms for antibacterial assays, we examined family-specific oil composition, the relationship between composition and antibacterial capacity, and provide theoretical foundations for eucalyptus selection, breeding, and applications in spices or medicine.

1.1 Study Site Overview

The *E. robusta* family trial forest was established in June 2019 as a randomized block experiment with altitude as the influencing factor (elevation range <10 m). The experiment comprised three blocks, each containing 18 *E. robusta* families, with four replicates per family plot and a planting density of 2 m × 3 m. Sample plot information for Nanning Liangfengjiang and Wuxuan Liufengshan is presented in Table 1 .

Table 1 Climate information of sampling sites in Nanning and Wuxuan

Location	Latitude (N)	Longitude (E)	Altitude (m)	Mean annual temperature (℃)	Annual rainfall (mm)
Nanning	22°36 49	108°17 46			
Wuxuan	23°45 23	109°42 17			

1.2.1 Sample Collection

Sampling was conducted on September 11, 2023, in Nanning and September 20, 2023, in Wuxuan. On the sampling day, Nanning experienced moderate rain with a temperature of 26°C, while Wuxuan was sunny with a temperature of 33°C. To meet component identification and antibacterial testing requirements, the study selected four *E. robusta* families (Families 1, 2, 3, and 4) based on superior growth performance and optimal sample preservation effects. Two well-growing trees were selected from each replicate block across different regions for each family. Leaves from entire lateral branches were collected within one hour and mixed into six-plant samples per family by region. After transport to the laboratory, samples were spread out to air-dry surface moisture, then chopped and bottled.

1.2.2 Essential Oil Extraction

Approximately 500 g of chopped fresh leaf samples were weighed and placed in a 2,000 mL round-bottom flask. Water was added to roughly level with the electric heating mantle to ensure adequate heating. Essential oils were extracted via reflux for 4 hours, with the oil-water mixture separated using an extractor. The separated essential oil was dehydrated with sufficient anhydrous sodium sulfate and preserved as essential oil samples.

1.2.3 Essential Oil Component Detection

Essential oil content was calculated using gas chromatography peak area normalization to determine the relative percentage content of each component. Component identification was performed through NIST standard spectral library searching and manual interpretation for qualitative analysis, with experimental methods and instrument conditions referenced from Liang et al. (2014).

(1) Gas chromatography-mass spectrometry (GC-MS) qualitative analysis conditions: Bruker TQ456 GC-MS instrument (USA) with an elastic quartz capillary column BR-5 (30 m × 0.25 mm × 0.25 m). Carrier gas was high-purity helium (99.99%), injection volume 1 L, split ratio 1:10. MS conditions: EI ion source, ionization voltage 70 eV, scan range 45–350 amu, full-scan mode. Solvent delay 2 min. Injection volume 1 L (5% ethanol solution). Temperature program: hold at 70°C for 5 min, ramp at $2°C \cdot min^{-1}$ to 150°C, hold 5 min, then ramp at $10°C \cdot min^{-1}$ to 230°C, hold 5 min.

(2) Gas chromatography (GC) quantitative analysis conditions: Agilent 7890A GC with an elastic quartz capillary column Rtx-5 (30 m × 0.25 mm × 0.25 m). Carrier gas was nitrogen. Temperature program: hold at 70°C for 5 min, ramp at $2°C \cdot min^{-1}$ to 150°C, hold 5 min, then ramp at $10°C \cdot min^{-1}$ to 230°C, hold 10 min. Injection port 250°C, vaporization chamber 250°C, split ratio 1:50, injection volume 0.3 L.

1.2.4 Antibacterial Activity Assay

The selection basis for antibacterial experimental subjects is shown in Table 2 . Bacterial suspension concentrations were adjusted to 0.5 McFarland standard. Sterile cotton swabs were dipped in the bacterial suspension, rotated and squeezed against the tube wall several times to remove excess fluid, and used to coat the entire agar plate surface. The coating was repeated several times, rotating the plate 60 degrees each time to ensure uniform distribution. After inoculation, drug sensitivity paper discs impregnated with 6 L of sample solution were applied. Tweezers were used to press the discs firmly onto the medium surface. Negative controls used paper discs soaked in normal saline, while positive controls used penicillin sensitivity discs for *S. aureus* and streptomycin sensitivity discs for *E. coli*, *P. aeruginosa*, and *S. typhi*. Within 15 minutes of disc application, plates were inverted and incubated at 37°C for 18–24 hours before

observation. Inhibition zone diameters (including disc diameter) were measured by visually inspecting each plate against reflected light with a ruler.

Table 2 Characterization and application types of selected bacteria for antibacterial experiments

Bacterium	Classification	Representative direction	Application direction
Escherichia coli	Gram-negative bacteria	Biofilm bacteria	Food preservation and gastrointestinal infections
Salmonella typhi	Gram-negative bacteria	Epidemic pathogens	Epidemic prevention and control
Staphylococcus aureus	Gram-positive bacteria	Biofilm bacteria	Antiseptic agent
Pseudomonas aeruginosa	Gram-negative bacteria	Clinical most resistant bacteria	Human infection

2.1 Essential Oil Yield of *E. robusta* Families

The leaf dry-weight essential oil yields of four *E. robusta* families planted in Nanning and Wuxuan are shown in Figure 1

. Oil yields ranged from 0.313% to 0.793% across different families, with Family 1 demonstrating the highest yields in both Nanning (0.717%) and Wuxuan (0.793%), significantly higher than other families ($P<0.05$). Family 2 showed substantial variation in essential oil yield between the two regions, while Families 3 and 4 exhibited consistently low yields in both locations.

Note: Different lowercase letters indicate significant differences ($P<0.05$) in dry-weight oil yield among different families in the same region.

Figure 1 Yield of essential oils from different families

2.2.1 Leaf Essential Oil Component Differences

GC-MS and GC qualitative and quantitative analysis results for leaf essential oils from four *E. robusta* families (1, 2, 3, and 4) planted in Nanning and Wuxuan are presented in Table 3 . A total of 37 volatile components were identified, accounting for 92.00%–97.24% of total composition. Seventeen components exceeded 1% content: α-pinene, β-pinene, myrcene, α-phellandrene, α-terpinene, *d*-limonene, β-phellandrene, *p*-cymene, 1,8-cineole, γ-terpinene, terpinolene, 4-terpineol, isoborneol, α-terpineol, *cis*-sabinol, piperitone, and β-caryophyllene. Major constituents (pinene, phellandrene, *p*-cymene, 1,8-cineole, terpineol, and *d*-limonene) comprised 80.772%–86.774% of volatile compounds, with α-pinene reaching 41.629% in Family 1 from Wuxuan. Eight compounds exceeded 1%

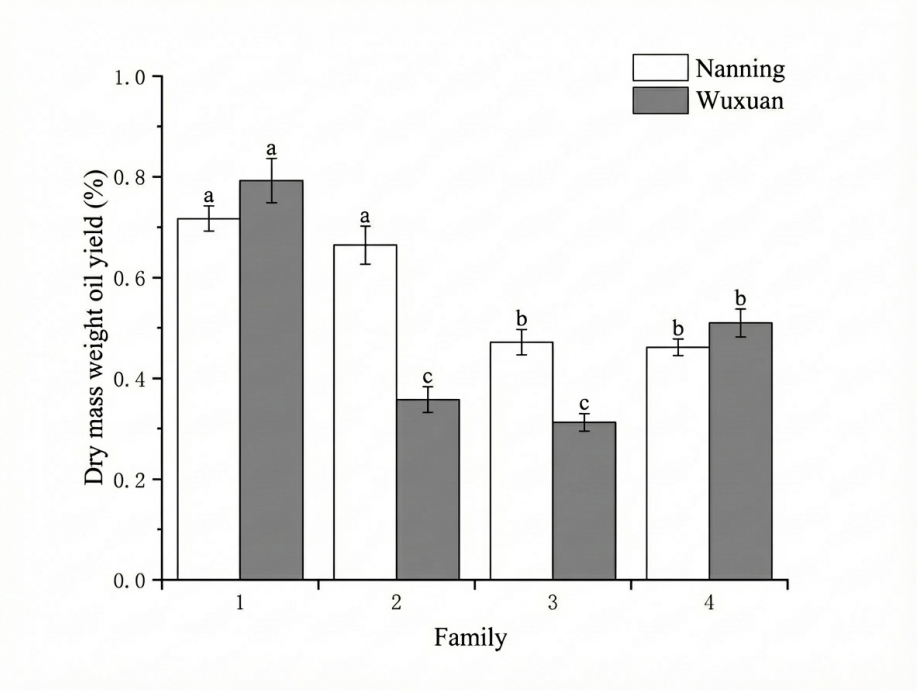

Figure 1: Figure 1

across all families: α-pinene, β-pinene, α-phellandrene, *d*-limonene, *p*-cymene, γ-terpinene, 4-terpineol, and α-terpineol. Families 1 and 4 showed lower α-pinene content in Nanning than Wuxuan, while Family 2 showed the opposite pattern. Family 2 exhibited higher β-pinene content in Wuxuan than Nanning. Except for Family 3, all families showed higher phellandrene content in Nanning than Wuxuan, while Family 3 had higher 1,8-cineole content in Nanning.

Table 3 Chemical composition of essential oil from *E. robusta* families in Nanning and Wuxuan

Compound	Molecular formula	Retention time (min)	Nanning (%)	Wuxuan (%)
α-Thujene	$C_{10}H_{16}$			
α-Pinene	$C_{10}H_{16}$			
Camphene	$C_{10}H_{16}$			
β-Pinene	$C_{10}H_{16}$			
Myrcene	$C_{10}H_{16}$			
α-Phellandrene	$C_{10}H_{16}$			
α-Terpinene	$C_{10}H_{16}$			
d-Limonene	$C_{10}H_{16}$			
β-Phellandrene	$C_{10}H_{16}$			
p-Cymene	$C_{10}H_{14}$			
1,8-Cineole	$C_{10}H_{18}O$			
γ-Terpinene	$C_{10}H_{16}$			
cis-Linalool oxide	$C_{10}H_{18}O_2$			
Terpinolene	$C_{10}H_{16}$			
trans-Linalool oxide	$C_{10}H_{18}O_2$			
p-Cymenene	$C_{10}H_{12}$			
Linalool	$C_{10}H_{18}O$			
Iso-amyl valerate	$C_{10}H_{20}O_2$			
β-Fenchol	$C_{10}H_{18}O$			
cis-β-Terpineol	$C_{10}H_{18}O$			
cis-2-*p*-Menthen-1-ol	$C_{10}H_{18}O$			
Terpinen-4-ol	$C_{10}H_{18}O$			
Isoborneol	$C_{10}H_{18}O$			
α-Terpineol	$C_{10}H_{18}O$			
cis-Sabinol	$C_{10}H_{16}O$			
Sabinyl acetate	$C_{12}H_{18}O_2$			
Carvotanacetone	$C_{10}H_{16}O$			
Piperitone	$C_{10}H_{16}O$			
Benzyl isobutyrate	$C_{11}H_{14}O_2$			
Germacrene D	$C_{15}H_{24}$			
β-Caryophyllene	$C_{15}H_{24}$			
Aromadendrene	$C_{15}H_{24}$			
δ-Cadinene	$C_{15}H_{24}$			
Globulol	$C_{15}H_{26}O$			

Compound	Molecular formula	Retention time (min)	Nanning (%)	Wuxuan (%)
(+)-Viridiflorol	$C_{15}H_{26}O$			
Cubenol	$C_{15}H_{26}O$			
β-Senlineol	$C_{15}H_{26}O$			
Total				

Note: "—" indicates undetected or low content.

The classification of volatile components in leaf essential oils from four *E. robusta* families in Nanning and Wuxuan is shown in Table 4 . Monoterpenoids were the primary constituents (73.608%–84.535%), with monoterpene hydrocarbons comprising 46.283%–77.874% and monoterpenols 5.712%–27.799%. Aromatic compounds were the second major group (4.570%–18.370%). Except for Family 3, all families showed lower monoterpenol content in Nanning than Wuxuan. All families exhibited lower aromatic compound content in Nanning than Wuxuan, with this difference being less pronounced in Families 1 and 3 compared to Families 2 and 4. Families 2 and 4 showed higher monoterpene hydrocarbon content in Nanning than Wuxuan, while Family 3 showed the opposite pattern, and Family 1 maintained relatively stable monoterpene hydrocarbon content.

Table 4 Classification of essential oil components in *E. robusta* families in Nanning and Wuxuan

Classification of compound	Nanning (%)	Wuxuan (%)
Monoterpene		
Monoterpenol		
Monoterpenketone		
Monoterpene Ester		
Total		
Sesquiterpene		
Sesquiterpene Alcohols		
Aromatic		
Ester		

2.3 Correlation Analysis of Essential Oil Components

Correlation analysis of major volatile components in *E. robusta* leaf essential oils is presented in Figure 2

. In the volatile components of family leaf oils, 1,8-cineole showed extremely significant ($P<0.01$) and significant ($P<0.05$) positive correlations with *d*-limonene and α-terpineol, respectively. *d*-Limonene exhibited extremely significant ($P<0.01$) and significant ($P<0.05$) positive correlations with α-terpineol and *p*-cymene, respectively. α-Terpineol showed significant ($P<0.05$)

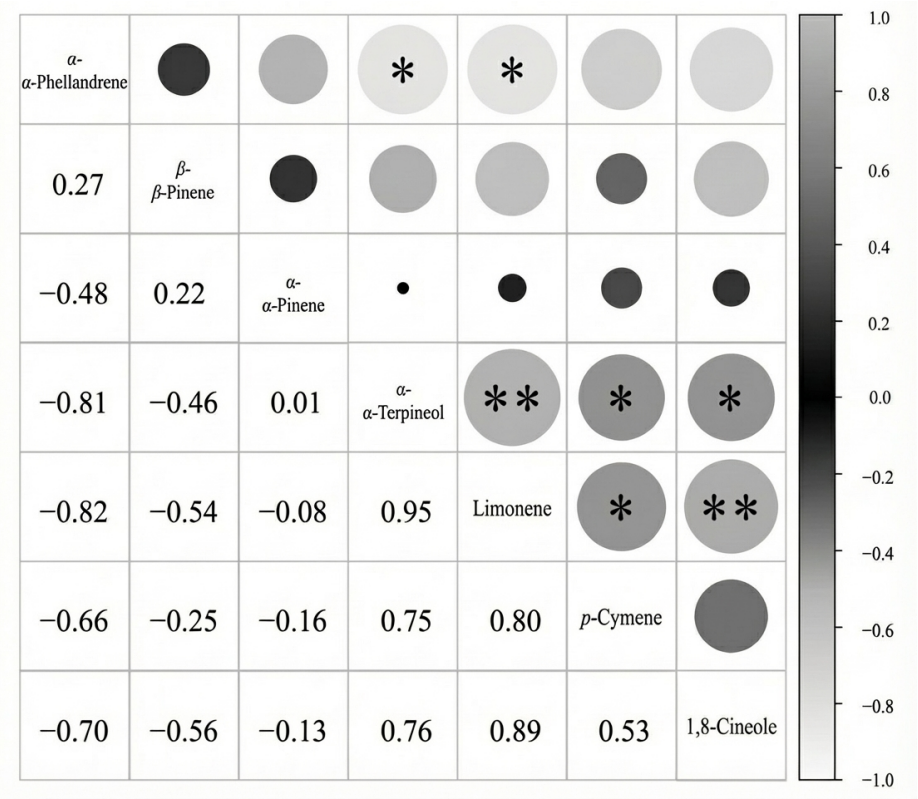

Figure 2: Figure 2

positive correlation with *p*-cymene, while α-phellandrene showed significant (P<0.05) negative correlation with α-terpineol and *d*-limonene. Overall, 1,8-cineole, *d*-limonene, α-terpineol, and *p*-cymene in *E. robusta* family essential oils demonstrated certain positive correlations.

Note: and ** indicate significant (P<0.05) and extremely significant (P<0.01) correlation levels, respectively.*

Figure 2 Heat map of correlation analysis of main components in essential oils

2.4.1 Antibacterial Activity of Essential Oils

Antibacterial activity test results for volatile components of *E. robusta* leaf essential oils are shown in Table 5 . *E. robusta* essential oil volatile components exhibited antibacterial activity against *E. coli*, *S. typhi*, and *S. aureus*, but no activity was detected against *P. aeruginosa*. Families 1 and 2 from Nanning showed higher antibacterial activity than those from Wuxuan. Families 3 and 4 from Wuxuan demonstrated higher activity against *E. coli* than their Nanning counterparts. For *S. typhi* and *S. aureus*, Nanning-grown Family 3 showed higher and lower activity, respectively, compared to Wuxuan-grown Family 3, while Family 4 showed the opposite pattern. Notably, Nanning-grown Family 3 exhibited higher antibacterial activity against *S. typhi* than the streptomycin control.

Table 5 Antibacterial activity of essential oil from four *E. robusta* families in Nanning and Wuxuan

Sample	*E. coli*	*S. typhi*	*S. aureus*	*P. aeruginosa*
Nanning				
Wuxuan				
Streptomycin				
Penicillin				
Normal saline				

Note: indicates antibacterial activity, with more * symbols representing stronger activity; — indicates no observed antibacterial activity.*

2.4.2 Redundancy Analysis of Essential Oil Main Components and Antibacterial Activity

Components exceeding 1% content in leaf essential oils from both locations were selected for redundancy analysis (RDA) with corresponding antibacterial activities, with results shown in Figure 3

. Under a constrained component of 42.64%, RDA1 and RDA2 explained 39.55% of total variation. No systematic differences were observed between Nanning

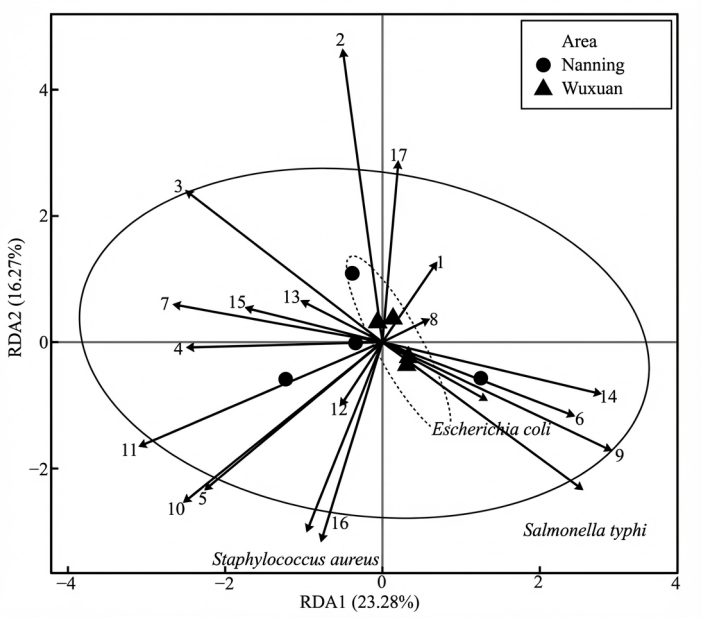

Figure 3: Figure 3

and Wuxuan-grown *E. robusta* leaf essential oils within the 95% confidence ellipse. RDA revealed that antibacterial activity against *E. coli* correlated with 1,8-cineole, *d*-limonene, and α-terpineol content. Activity against *S. typhi* correlated with 1,8-cineole, *d*-limonene, α-terpineol, and 1-hexanol content. Activity against *S. aureus* showed highest correlation with piperitone, plus correlations with 4-terpineol, 1-hexanol, α-terpinene, γ-terpinene, and terpinolene content. RDA results indicated no positive correlation between antibacterial activity and high-content components such as pinene, phellandrene, and *p*-cymene. The constrained component of RDA fitting results was only 42.64%, suggesting antibacterial activity arises from interactions among different compound classes (Tariq et al., 2019).

Component numbers: 1. α-Pinene; 2. β-Pinene; 3. Myrcene; 4. α-Phellandrene; 5. α-Terpinene; 6. d-*Limonene; 7. β-Phellandrene; 8.* p-*Cymene; 9. 1,8-Cineole; 10. γ-Terpinene; 11. Terpinolene; 12. Terpinen-4-ol; 13. Isoborneol; 14. α-Terpineol; 15.* cis-*Sabinol; 16. Piperitone; 17. β-Caryophyllene.*

Figure 3 Redundant Analysis of main essential oil components and antibacterial ability

3.1 Discussion

(1) Essential oil components and yield of *E. robusta* families

This study identified 37 components in *E. robusta* essential oils, accounting for 92.00%–97.24% of total composition. Seventeen compounds exceeded 1% content across families, with eight shared components: α-pinene, β-pinene, α-phellandrene, *d*-limonene, *p*-cymene, γ-terpinene, 4-terpineol, and α-terpineol. Major constituents (pinene, phellandrene, *p*-cymene, 1,8-cineole, terpineol, and *d*-limonene) comprised 80.772%–86.774% of volatile compounds, with oils dominated by pinene, phellandrene, and *p*-cymene. α-Pinene content reached 41.629% in Wuxuan Family 1. Monoterpenoids constituted 73.608%–84.535% of essential oils, with monoterpene hydrocarbons comprising 46.283%–77.874%. Previous studies by Ye (2007) and Meng et al. (2020) identified α-pinene as the main component of *E. robusta* essential oil, with high monoterpene content reported (Patrícia et al., 2007; Atmani-Merabet et al., 2018). Consistently, this study found monoterpenoids and oxygenated monoterpenes as primary constituents.

Chemotype classification studies of *E. robusta* essential oil have identified α-phellandrene, *p*-cymene, and β-pinene chemotypes in different regions, while Chinese *E. robusta* essential oils are dominated by α-pinene and 1,8-cineole (Filomeno et al., 2016; Atmani-Merabet et al., 2018). This study also found abundant phellandrene and *p*-cymene, a phenomenon more commonly reported in international research. Differences in major component content (pinene, phellandrene, *p*-cymene, and 1,8-cineole) among families suggest different chemotypes. Plants can be classified into different chemotypes based on how genes and environmental factors affect major essential oil compounds (Benomari et al., 2023). From a chemical composition perspective, Wuxuan Family 4 belongs to the 1,8-cineole chemotype, while Family 3 is directly classified as 1,8-cineole chemotype. Essential oil yields in this study ranged from 0.313% to 0.793%, consistent with yields reported by Chen et al. (2018) and Meng et al. (2020), confirming that *E. robusta* leaf essential oil yields are generally low. Family 1 showed the highest yields in both Nanning (0.717%) and Wuxuan (0.793%), while Families 3 and 4 (both 1,8-cineole chemotypes) exhibited lower yields than the other two families at both locations.

(2) Variation in essential oil components among *E. robusta* families

Among the four *E. robusta* families at both locations, Family 3 showed higher 1,8-cineole content in Nanning, while Family 4 showed higher content in Wuxuan. Families 1, 3, and 4 from Wuxuan exhibited higher pinene content, while Family 2 showed the opposite trend. Families 1, 2, and 4 from Nanning had higher phellandrene content than Wuxuan, while Family 3 showed the reverse pattern. The essential oil composition was unstable and showed no regular pattern, likely because the introduced *E. robusta* families belong to different essential oil chemotypes that respond differently to environmental changes (Karimi & Meiners, 2021). Although climate conditions were similar between sampling sites, weather and temperature differed on the sampling day. The higher pinene and 1,8-cineole content observed in Families 1, 3, and 4 from Wuxuan may reflect recent differences in water availability and temperature between locations,

consistent with findings that drought-stressed *Eucalyptus camaldulensis* leaves showed higher pinene and 1,8-cineole content compared to daily-watered treatments (Leicach et al., 2010). This study demonstrates that different families exhibit different responses to environmental changes.

Essential oils are metabolic products, and correlated compounds in the same plant may share common metabolic pathways (Sá Filho et al., 2022). Correlation analysis of different *E. robusta* families revealed that major components including α-phellandrene, 1,8-cineole, *d*-limonene, α-terpineol, and *p*-cymene were correlated, suggesting shared metabolic pathways for essential oil synthesis and conversion across families.

(3) Essential oil components and antibacterial activity of *E. robusta*

This study found that *E. robusta* leaf essential oil exhibited antibacterial activity against *E. coli*, *S. typhi*, and *S. aureus*, but no activity against *P. aeruginosa*. However, previous studies reported antibacterial activity against clinically resistant *P. aeruginosa*. This discrepancy is unlikely due to minor component variations. Ameur et al. (2021) reported *E. robusta* essential oils containing moderate amounts of *trans*-pinocarveol, citronellal, borneol, and octahydrotetramethylnaphthalenemethanol exhibited antibacterial activity against *P. aeruginosa*. Similarly, essential oils containing moderate amounts of myrtenal, cuminaldehyde, and spathulenol also showed activity against *P. aeruginosa* (Cimanga et al., 2002). The missing compounds in this study all belong to alcohol and aldehyde classes, suggesting that *E. robusta* essential oil components may require synergistic interaction with these alcohols and aldehydes to exhibit antibacterial activity against *P. aeruginosa*.

Only Nanning-grown Family 3 essential oil showed antibacterial activity against *S. typhi* exceeding antibiotic levels, with 1,8-cineole content reaching 16.759%. Redundancy analysis of essential oil components and antibacterial activity revealed that antibacterial activity against *E. coli* and *S. typhi* correlated with higher 1,8-cineole content. Antibacterial activity showed no positive correlation with high-content components such as pinene, phellandrene, and *p*-cymene. The constrained component of RDA fitting was only 42.64%, indicating antibacterial activity arises from interactions among different compound classes (Tariq et al., 2019). Some studies have reported that 1,8-cineole exhibits strong antibacterial activity against *E. coli* and *S. aureus*, affecting biofilm formation and inhibiting protein synthesis in *E. coli*. Combined with other terpenes such as camphene, α-pinene, and limonene, it shows synergistic effects against *S. aureus*, *E. coli*, and *P. aeruginosa* (Hendry et al., 2009; Miguel et al., 2018; Ameur et al., 2021; Wang, 2023). Consistently, this study found antibacterial activity correlated with 1,8-cineole content, with activity manifested through interactions among essential oil components.

Regarding major terpene hydrocarbon constituents, previous research found that *p*-cymene exhibits strong affinity for bacterial cell membranes, disrupting membrane integrity and causing cytoplasmic membrane swelling (Burt et

al., 2007), and affecting protein synthesis in *E. coli* cells (Chouhan et al., 2017). However, fractionation antibacterial tests of essential oil components revealed that single components such as pinene, *p*-cymene, limonene, and myrcene showed no or only weak antibacterial activity, while terpenes appear more significant for disrupting fungal cell walls (Hyldgaard et al., 2012). Individual testing of phenols, aldehydes, ketones, alcohols, and esters from essential oils demonstrated significant antibacterial properties, such as 4-terpineol showing strong activity against *S. aureus* (Tariq et al., 2019). Monoterpenoid antibacterial activity primarily targets bacterial biofilms, likely because oxygen-containing functional groups in terpenoid compounds possess greater bacterial biofilm disruption capacity than hydrocarbons (Rao et al., 2019). Similarly, this study found that for Gram-negative bacteria *E. coli* and *S. typhi*, higher antibacterial activity was observed when essential oils contained higher levels of oxygenated monoterpenes such as 1,8-cineole and α-terpineol. The enhanced antibacterial activity exhibited by essential oils correlated with oxygenated monoterpene content. Therefore, increasing oxygenated monoterpene content is more significant for improving *E. robusta* essential oil antibacterial activity than increasing other terpene components.

3.2 Conclusion

Eucalyptus robusta leaf essential oils contain 73.695%–84.535% monoterpenoid compounds, with pinene, phellandrene, and *p*-cymene as major constituents. α-Pinene content reached 41.629% in Wuxuan Family 1. α-Pinene, β-pinene, α-phellandrene, *d*-limonene, *p*-cymene, γ-terpinene, 4-terpineol, and α-terpineol were common components across families. Although different *E. robusta* families responded differently to environmental changes, α-phellandrene, 1,8-cineole, *d*-limonene, α-terpineol, and *p*-cymene showed correlations among families. *Eucalyptus robusta* leaf essential oil exhibited considerable antibacterial activity against *E. coli*, *S. aureus*, and *S. typhi*, with Nanning-grown Family 3 showing particularly high activity against *S. typhi*. The lack of observed antibacterial activity against *P. aeruginosa* may be due to the absence of synergistic effects from certain moderate-quantity alcohol and aldehyde compounds. The content of oxygenated monoterpenes such as 1,8-cineole and terpineol is important for enhancing antibacterial activity. However, families producing the 1,8-cineole chemotype showed low oil yields, requiring further breeding efforts.

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