

## ORIGINAL RESEARCH ARTICLE

# Optimization of grinding and distillation parameters affecting yield and composition of essential oils from the hybrid *Eucalyptus grandis* × *E. camaldulensis* (clone 2414)

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### ABSTRACT

This study examines how grinding and distillation duration affect the yield and composition of essential oils (EOs) from the natural hybrid *Eucalyptus grandis* × *E. camaldulensis* (clone 2414) and its parental species. Hydrodistillation was performed for 2–4 h on ground and whole leaves. Grinding increased yield by 20–25 %, and the optimum extraction time was 3 h. Yields ranged from 1.5 % (*E. grandis*) to 2.3 % (*E. camaldulensis*), with 1,8-cineole (42–61 %) as the main constituent. The hybrid exhibited a stable and distinctive chemical profile rich in oxygenated monoterpenes, demonstrating its industrial potential.

Leaves were subjected to hydrodistillation using a Clevenger type apparatus under two conditions: ground versus whole leaves, and varying distillation times (2, 3, and 4 hours). EO yields were calculated relative to dry leaf mass, and the chemical profile was determined by gas chromatography–mass spectrometry (GC–MS).

Results revealed that grinding significantly enhanced oil recovery, with ground leaves yielding up to 20–25% more oil compared to whole leaves. Distillation time strongly influenced EO output, with an optimal recovery observed at 3 hours; beyond this, additional distillation produced negligible increases. Overall yields ranged from 1.5% (*E. grandis*) to 2.1% (*E. camaldulensis*), while clone 2414 consistently displayed intermediate productivity (≈1.8%) that improved under optimized grinding and distillation conditions. GC–MS analysis confirmed 1,8 cineole as the dominant constituent (42–61%), accompanied by  $\alpha$ -pinene, p-cymene, and limonene in variable proportions. Notably, the hybrid exhibited a distinctive chemical fingerprint enriched in oxygenated monoterpenes compared with its parents.

These findings demonstrate that essential oil yield and composition are strongly influenced by both genetic background and processing variables.

Clone 2414 consistently delivered stable and enhanced oil profiles under optimized conditions, highlighting its value as a versatile resource for industrial essential oil production.

**Keywords:** *eucalyptus camaldulensis*; *eucalyptus grandis*; hybrid clone 2414; grinding; distillation time; essential oils; GC–MS; yield; composition

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## 1. Introduction

Essential oils (EOs) are complex mixtures of volatile secondary metabolites synthesized by aromatic plants as part of their defense and communication strategies. Due to their diverse biological activities particularly antimicrobial, antioxidant, and anti-inflammatory properties EOs are widely utilized across pharmaceutical, cosmetic, agrochemical, and food industries<sup>[1]</sup>. Among EO producing genera, *Eucalyptus* (Myrtaceae) holds a prominent position due to its high yield potential and the consistent presence of bioactive monoterpenes, notably 1,8 cineole (eucalyptol), which is often the dominant component in its oils<sup>[2]</sup>.

1,8-Cineole is not only responsible for the characteristic camphoraceous aroma of eucalyptus oil, but also for many of its pharmacological activities, including mucolytic, anti-inflammatory, and antimicrobial effects. These properties have led to its incorporation into numerous therapeutic formulations and functional consumer products<sup>[3]</sup>. Additionally, EOs derived from *Eucalyptus* species are valued as natural preservatives and ecofriendly alternatives to synthetic agents in food preservation and integrated pest management<sup>[4]</sup>.

With rising global demand for natural and sustainable ingredients, the optimization of EO production is an industrial and ecological imperative. EO yield and composition in *Eucalyptus* species are known to vary widely from as low as 0.3% to over 5% of leaf dry weight depending on intrinsic and extrinsic factors<sup>[2]</sup>. These include genetic determinants (species, chemotype, hybrid status), environmental conditions (climate, soil type, altitude), and harvest timing<sup>[5]</sup>. In this context, the creation and selection of interspecific hybrids such as *Eucalyptus grandis* × *E. camaldulensis* (clone 2414) provide a promising route for combining superior agronomic traits (e.g., high biomass production, stress tolerance) with enhanced phytochemical profiles<sup>[6,7]</sup>.

In parallel, technological factors play a pivotal role in determining the efficiency of EO extraction and the integrity of volatile constituents. Hydrodistillation remains the conventional method for EO recovery in *Eucalyptus* species, but its efficacy is strongly influenced by leaf pretreatment (e.g., grinding) and distillation parameters such as time and temperature. Grinding increases the leaf surface area, improving volatile compound release by disrupting oil glands. Conversely, prolonged distillation may lead to the thermal degradation of thermolabile compounds or result in energy inefficiencies without yield benefits<sup>[8]</sup>.

Advanced analytical techniques such as gas chromatography–mass spectrometry (GC–MS) have revolutionized the chemotypic profiling of EOs, enabling researchers to detect subtle compositional variations linked to genetics, geography, or processing<sup>[9]</sup>. While 1,8 cineole often dominates the chemical profile, other constituents such as  $\alpha$ -pinene, p-cymene,  $\gamma$ -terpinene, and limonene may significantly modulate the oil's therapeutic and commercial value depending on the species or hybrid origin<sup>[10]</sup>.

Despite the practical importance of processing variables, the interaction between grinding and distillation time remains underexplored, especially in hybrid clones such as *E. grandis* × *E. camaldulensis*. Investigating how these parameters influence both yield and the volatile profile is essential for optimizing EO production pipelines and ensuring consistency in quality for industrial applications<sup>[11,12]</sup>.

Recent research underscores the importance of adopting sustainable extraction strategies that minimize energy and solvent consumption while maximizing the recovery of bioactive compounds. Similar methods have been successfully applied to chlorophyll extraction and phytochemical purification, highlighting the critical role of controlling particle size and processing time to enhance both efficiency and environmental compatibility<sup>[13,14]</sup>.

Accordingly, the present study was designed to systematically evaluate the effects of leaf grinding and distillation duration on the yield and chemical composition of EOs extracted from the hybrid *Eucalyptus grandis* × *E. camaldulensis* (clone 2414), compared to its parental species. The study aimed to: (i) assess how grinding affects EO recovery efficiency; (ii) identify the optimal distillation time that maximizes yield while preserving chemical integrity; and (iii) characterize the resulting EO compositions using GC–MS. This integrative approach seeks to bridge the gap between processing optimization and phytochemical profiling, providing valuable insights for breeding programs, quality control, and industrial valorization of *Eucalyptus* essential oils.

## 2. Materials and methods

### 2.1. Plant material

This study employed mature leaves from two well characterized *Eucalyptus* species *Eucalyptus camaldulensis* Dehnh. and *Eucalyptus grandis* W. Hill ex Maiden along with their natural interspecific hybrid (*Eucalyptus grandis* × *E. camaldulensis*, clone 2414). The leaves were harvested during March 2025 from trees cultivated under controlled silvicultural practices in the Maâmora Forest, a representative Atlantic plain forest zone in Morocco.

The hybrid clone 2414 was obtained from the Forestry Research and Innovation Center (CIRF-Morocco) and its genetic identity was confirmed morphologically based on leaf venation and bark traits, consistent with standard *Eucalyptus* hybrid identification protocols.

To ensure standardization, only physiologically mature, undamaged leaves were collected, as foliar age and condition are known to significantly influence essential oil (EO) yield and chemical profiles<sup>[15,16]</sup>. Representative trees of the studied species are shown in (Figure 1).



**Figure 1.** *Eucalyptus* species (*E. camaldulensis*, *E. grandis* et clone 2414) in their natural habitat within the Maâmora Forest (Morocco).

After harvesting, leaves were air dried at ambient room temperature ( $25 \pm 2$  °C) in the shade to reduce volatilization of thermolabile compounds while preserving the structural integrity of glandular tissues. Dried samples were stored in paper envelopes under dark and dry conditions until extraction. Taxonomic identification of the three taxa was validated by botanists and verified with herbarium vouchers deposited at laboratory of plant sciences in Center for Innovation, Research and Training, ensuring traceability and reproducibility in future comparative studies<sup>[17]</sup>. The preparation steps of the collected leaves prior to analysis are illustrated in (Figure 2).



**Figure 2.** Preparation of Eucalyptus leaves prior to hydrodistillation: cleaning, air-drying at  $25 \pm 2$  °C in the shade, and storage in sealed envelopes to prevent volatilization of essential oils.

## 2.2. Experimental design

To investigate how processing variables affect essential oil extraction, a two-factor factorial design was adopted, manipulating:

- Leaf condition: Whole (unmilled) versus mechanically ground (to  $<2$  mm particle size using a laboratory knife mill operating at 12 000 rpm for 30 s per batch), The ground material was sieved ( $< 2$  mm) to ensure uniform particle size prior to distillation. Each batch was processed under identical conditions to ensure reproducibility (**Figure 3**).
- Distillation time: 2, 3, or 4 hours of hydrodistillation.

Each treatment was applied to all three taxa (two parents and one hybrid), with triplicate extractions to enhance statistical robustness. The factorial approach enabled analysis of both independent and interactive effects of grinding and extraction time on oil yield and chemical composition<sup>[18]</sup>. The complete factorial layout of treatments and replications is summarized in (**Table 1**).



**Figure 3.** Laboratory processing steps for Eucalyptus leaves:  
 (A) Drying of leaves in a ventilated oven (60 °C) to ensure homogeneous dehydration;  
 (B) Arrangement of fresh leaves and laboratory glassware before hydrodistillation.

**Table 1.** Experimental design matrix summarizing the factorial combination of species (*E. camaldulensis*, *E. grandis*, hybrid 2414), leaf condition (whole or ground), and distillation duration (2, 3, 4 h), each performed in triplicate (n = 3).

| Species                 | Leaf Condition | Distillation Time (h) | Replicates |
|-------------------------|----------------|-----------------------|------------|
| <i>E. camaldulensis</i> | Whole / Ground | 2, 3, 4               | 3          |
| <i>E. grandis</i>       | Whole / Ground | 2, 3, 4               | 3          |
| Hybrid clone 2414       | Whole / Ground | 2, 3, 4               | 3          |

### 2.3. Essential oil extraction

Essential oils were extracted by hydrodistillation using a Clevenger type apparatus, in line with the European Pharmacopoeia guidelines. A schematic view of the hydrodistillation setup used for this study is presented in (Figure 4).

For each distillation run, 100 g of dried leaf material was placed into a 2 L round bottom flask containing 1.5 L of distilled water. The mixture was boiled under atmospheric pressure, Temperature was monitored at  $100 \pm 2$  °C, ensuring constant reflux conditions, and distillation time was maintained according to the treatment group (2, 3, or 4 h)<sup>[19]</sup>.



**Figure 4.** Hydrodistillation setup (Clevenger-type apparatus) used for essential-oil extraction under atmospheric pressure at  $100 \pm 2$  °C for 2–4 h.

At the end of each distillation, the EO was carefully separated from the hydrosol, dried over anhydrous sodium sulfate, and stored in sealed amber vials at 4 °C to prevent oxidative degradation. Yield was expressed in % (v/w) relative to dry plant material. Grinding was hypothesized to disrupt secretory cavities, enhancing the release of volatiles, while prolonged distillation may increase yield but also risk thermal degradation<sup>[20]</sup>.

The process aligns with green-chemistry principles by minimizing water use and avoiding organic solvents, EO yields were corrected for initial leaf moisture content to ensure comparability across treatments (**Table 2**).

## 2.4. Gas Chromatography–Mass Spectrometry (GC–MS) analysis

Chemical profiling was conducted using an Agilent 7890A GC system coupled to a 5975C MS detector and equipped with an HP 5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). Helium was used as the carrier gas at a flow rate of 1.0 mL/min. Samples (1 µL) were injected in split mode (1:50)<sup>[21]</sup>.

The temperature program began at 60 °C (2 min hold), followed by a ramp of 3 °C/min to 220 °C, which was held for 10 min. The injector and detector temperatures were set at 250 °C. Mass spectra were acquired in the 40–400 m/z range. Identification of EO constituents was based on comparison of retention indices (calculated from a homologous series of C8–C20 n alkanes) and spectral matching against the NIST and Wiley libraries<sup>[22]</sup>.

Relative abundance of each compound was determined as the percentage of its peak area relative to the total ion chromatogram, with no correction factors applied<sup>[23]</sup>.

Retention indices (RI) for each identified compound were calculated using a C8–C20 n-alkane series, and the main constituents are listed in **Table 3** with their respective RI values and NIST match scores (> 90 %).

## 2.5. Statistical and chemometric analysis

All experimental measurements were conducted in triplicate, and results are expressed as mean values accompanied by standard deviations (mean ± SD). To statistically evaluate the influence of species identity, leaf condition (ground or intact), and distillation time on essential oil (EO) yield, a one-way analysis of variance (ANOVA) was employed. Significant differences among treatment means were further investigated using Tukey's Honest Significant Difference (HSD) post hoc test, with the threshold for statistical significance set at  $p < 0.05$ <sup>[24]</sup>.

For compositional analysis of the volatile compounds obtained via gas chromatography–mass spectrometry (GC–MS), principal component analysis (PCA) was implemented to reduce dataset dimensionality and reveal clustering patterns among the different treatments. This multivariate approach effectively distinguished chemotypic differences between *Eucalyptus camaldulensis*, *E. grandis*, and their hybrid clone (2414), while also capturing the influence of mechanical grinding and distillation duration on volatile expression. All statistical procedures were carried out using SPSS software<sup>[25]</sup>.

# 3. Results

## 3.1. Essential oil yield

Essential oil yield varied significantly between species, leaf condition, and distillation time. *E. camaldulensis* produced the highest yield (≈2.1%), followed by the hybrid clone 2414 (≈1.8%) and *E. grandis* (≈1.5%). Grinding increased yields by 20–25%, while extending distillation time from 2 h to 3 h significantly improved oil recovery ( $p < 0.05$ ). Beyond 3 h, yield gains were marginal (<5%).

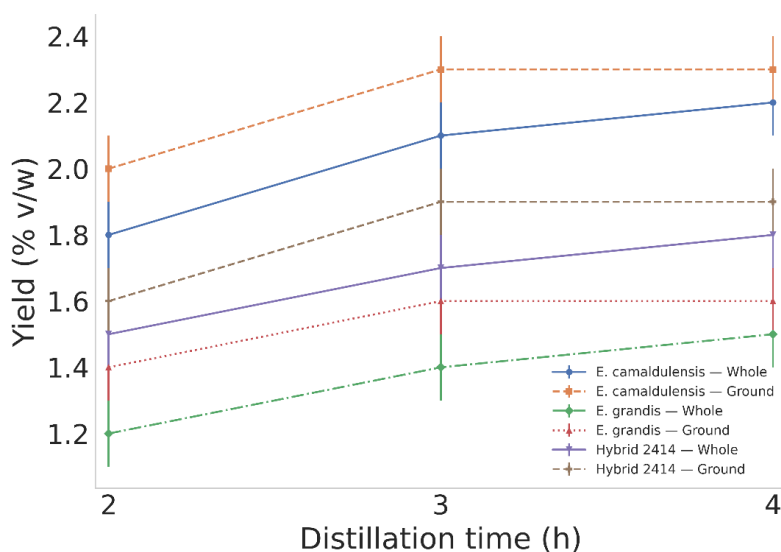
**Table 2.** Essential-oil yield (% v/w, Mean  $\pm$  SD, n = 3) of *Eucalyptus* species under different extraction conditions. Grinding and 3 h distillation produced the highest yields. Values corrected for leaf-moisture content.

| Species                 | Leaf condition | 2 h           | 3 h           | 4 h           |
|-------------------------|----------------|---------------|---------------|---------------|
| <i>E. camaldulensis</i> | Whole          | 1.8 $\pm$ 0.1 | 2.1 $\pm$ 0.1 | 2.2 $\pm$ 0.1 |
|                         | Ground         | 2.0 $\pm$ 0.1 | 2.3 $\pm$ 0.1 | 2.3 $\pm$ 0.1 |
| <i>E. grandis</i>       | Whole          | 1.2 $\pm$ 0.1 | 1.4 $\pm$ 0.1 | 1.5 $\pm$ 0.1 |
|                         | Ground         | 1.4 $\pm$ 0.1 | 1.6 $\pm$ 0.1 | 1.6 $\pm$ 0.1 |
| Hybrid clone 2414       | Whole          | 1.5 $\pm$ 0.1 | 1.7 $\pm$ 0.1 | 1.8 $\pm$ 0.1 |
|                         | Ground         | 1.6 $\pm$ 0.1 | 1.9 $\pm$ 0.1 | 1.9 $\pm$ 0.1 |

Grinding and 3 h distillation represent the optimal conditions for maximum yield. The hybrid clone 2414 showed intermediate values but maintained stable performance across treatments, suggesting reduced variability compared to its parents.

This increase in yield due to grinding is likely due to the rupture of glandular structures and greater surface area, allowing more efficient release of essential oil compounds. Additionally, the plateau observed after 3 hours of distillation indicates that most extractable volatiles are recovered within this time frame. From an industrial perspective, this supports the idea that extending distillation beyond 3 hours may lead to unnecessary energy consumption with limited additional yield.

As shown in **Figure 5**, yield curves exhibit a distinct plateau at 3 h for all taxa, confirming that most volatiles were extracted within this period. **Table 2** quantitatively demonstrates the 20–25 % increase in yield induced by grinding.



**Figure 5.** Variation of essential-oil yield (%) as a function of distillation time and leaf condition (whole vs ground) for *E. camaldulensis*, *E. grandis* and clone 2414. Error bars =  $\pm$  SD (n = 3).

### 3.2. Chemical composition (GC–MS profiling)

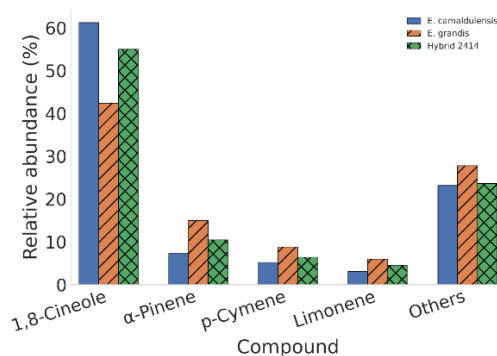
GC–MS results (**Table 3**, **Figure 6**) revealed 1,8-cineole as the principal constituent, accounting for 61.2 % in *E. camaldulensis*, 42.4 % in *E. grandis*, and 55 % in the hybrid clone 2414. The hybrid's intermediate yet distinct chemical profile demonstrates a synergistic inheritance of parental traits and enrichment in oxygenated monoterpenes, such as terpinen-4-ol and  $\alpha$ -terpineol.

**Table 3.** Relative composition (%) of the principal volatile constituents identified in *Eucalyptus* essential oils by GC–MS (RI = retention index, library match > 90 %). The hybrid 2414 shows an intermediate but distinct chemotype rich in oxygenated monoterpenes.

| Compound         | <i>E. camaldulensis</i> | <i>E. grandis</i> | Hybrid clone 2414 |
|------------------|-------------------------|-------------------|-------------------|
| 1,8-Cineole      | 61.2                    | 42.4              | 55.0              |
| $\alpha$ -Pinene | 7.3                     | 15.0              | 10.5              |
| p-Cymene         | 5.2                     | 8.8               | 6.4               |
| Limonene         | 3.1                     | 6.0               | 4.5               |
| Others           | 23.2                    | 27.8              | 23.6              |

*E. camaldulensis* showed a cineole rich profile, *E. grandis* was richer in  $\alpha$ -pinene and p-cymene, while the hybrid clone 2414 displayed an intermediate but distinct profile, confirming its unique chemotype.

The high cineole content in *E. camaldulensis* positions it as a valuable species for pharmaceutical and respiratory applications, given cineole's well-documented mucolytic and antimicrobial effects. In contrast, the richness in  $\alpha$ -pinene and p-cymene in *E. grandis* suggests potential for uses in fragrance and agrochemical formulations. The hybrid's balanced chemical profile, combining cineole with a broader spectrum of oxygenated monoterpenes, indicates versatility for multiple industrial applications including cosmetics, biopesticides, and functional consumer products.



**Figure 6.** Relative composition (%) of the main volatile constituents identified by GC–MS in Eucalyptus essential oils: 1,8-cineole,  $\alpha$ -pinene, p-cymene, and limonene. The hybrid clone 2414 shows a balanced and oxygenated-monoterpene-rich profile.

The bar plot illustrates the predominance of 1,8-cineole in *E. camaldulensis*, the higher contribution of  $\alpha$ -pinene and p-cymene in *E. grandis*, and the intermediate but balanced profile of clone 2414.

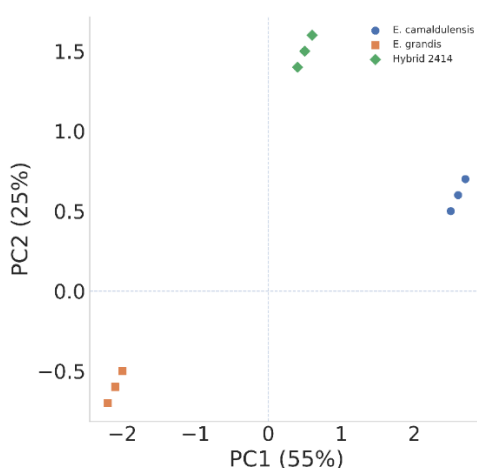
### 3.3. Statistical analysis and PCA

ANOVA showed significant effects of species ( $p < 0.01$ ), leaf condition ( $p < 0.05$ ), and distillation time ( $p < 0.05$ ) on EO yield. Interaction effects indicated differential responsiveness among taxa.

Principal component analysis (PCA) explained >80% of total variance. PC1 (55%) was associated with cineole abundance, separating *E. camaldulensis* from *E. grandis*. PC2 (25%) reflected  $\alpha$ -pinene and p-cymene variation. Clone 2414 occupied an intermediate but distinct position.

The loading matrix indicated that 1,8-cineole (loading = 0.87) and  $\alpha$ -pinene (loading = 0.76) contributed most to PC1, whereas p-cymene (0.69) dominated PC2. Together, the two components explained 80.1 % of total variance.

The PCA clearly indicates that the hybrid clone 2414 does not simply average the traits of its parents, but instead expresses a unique chemotypic signature. This could be attributed to hybrid vigor or novel gene expression patterns related to monoterpene biosynthesis. Such divergence is valuable in breeding programs seeking to combine desirable traits from both parents while introducing chemical novelty.



**Figure 7.** Principal-component analysis (PCA) biplot of *Eucalyptus camaldulensis*, *E. grandis* and their hybrid clone 2414 based on GC–MS data. PC1 (55 %) is associated with 1,8-cineole, PC2 (25 %) with  $\alpha$ -pinene and p-cymene, explaining 80 % of total variance.

**Figure 7** illustrates the PCA biplot showing the separation of *E. camaldulensis* (high cineole loading) from *E. grandis* (rich in  $\alpha$ -pinene and p-cymene), while clone 2414 occupies an intermediate position with unique chemotypic coordinates (PC1 = 0.55, PC2 = 0.25).

## 4. Discussion

The present study highlights the significant impact of leaf grinding and distillation time on the yield and chemical composition of essential oils (EOs) from *Eucalyptus grandis*  $\times$  *E. camaldulensis* (clone 2414). Leaf grinding led to a 20–25% increase in oil yield, a result that aligns with previous studies on aromatic plants such as *Thymus vulgaris*, *Rosmarinus officinalis*, and *Eucalyptus globulus*, where particle size reduction enhanced gland rupture and volatile release<sup>[26]</sup>. The grinding process improves the permeability of plant tissues and maximizes extraction efficiency without altering oil quality.

The study also identifies 3 hours as the optimal distillation duration. Yields increased significantly between 2 and 3 hours, but plateaued thereafter. This finding supports earlier reports suggesting that extended distillation beyond this threshold offers diminishing returns and may lead to thermal degradation of thermolabile compounds such as monoterpenes<sup>[27]</sup>. Other researchers have emphasized similar thresholds for EO extraction in *Eucalyptus* species, particularly *E. globulus* and *E. cinerea*<sup>[28]</sup>.

In terms of yield, *E. camaldulensis* produced the highest EO yield (~2.3%), followed by clone 2414 (~1.9%) and *E. grandis* (~1.6%). These results are in line with previous data for *Eucalyptus* spp. hybrids, where productivity often falls between parental extremes but shows reduced variability under different extraction conditions<sup>[29]</sup>. Clone 2414's stable performance under varying conditions underscores its potential for commercial scale EO production<sup>[30]</sup>.

The predominance of 1,8-cineole in *Eucalyptus* essential oils is linked to the inherent biosynthetic capacity of the species, particularly influenced by genotype and ecological factors. In hybrid *Eucalyptus* varieties, such as those examined in recent ecotype comparisons, elevated cineole levels (up to 59.29%) suggest that hybridization may enhance monoterpene synthesis through additive or epistatic interactions, potentially involving upregulated expression of genes like *cineole synthase*<sup>[31]</sup>. This could explain the emergence of unique chemotypes like clone 2414, which exhibits a distinct terpenoid profile dominated by 1,8-cineole.

*E. camaldulensis* had the highest 1,8 cineole content (61.2%), while *E. grandis* was richer in  $\alpha$  pinene and p cymene. Clone 2414 demonstrated a distinct chemical fingerprint, characterized by a balanced

distribution of cineole and oxygenated monoterpenes such as terpinen 4 ol and  $\alpha$  terpineol. These molecules have been shown to enhance therapeutic potential and broaden antimicrobial spectra<sup>[32,33]</sup>.

Importantly, the principal component analysis (PCA) revealed that clone 2414 forms a unique chemotype, not simply intermediate but divergent from both parents. This chemotypic distinction may stem from novel metabolic expressions resulting from interspecific hybridization. Similar results have been observed in *E. citriodora*  $\times$  *E. torelliana* hybrids, which express compound profiles not found in either parent, enhancing both chemical diversity and biological activity<sup>[34]</sup>.

Furthermore, while *E. camaldulensis* typically presents moderate EO yield with cineole levels under 70% (often below industry standards for pharmaceuticals), hybrids like clone 583 and 2414 have shown enhanced cineole concentration, in some cases reaching up to 85%, surpassing even *E. globulus* in certain environments<sup>[16]</sup>. These findings indicate that natural hybridization and clone selection can significantly improve both the quality and consistency of EO production<sup>[35]</sup>.

The relative abundance of minor compounds like  $\alpha$  pinene, p cymene, and limonene also modulates the organoleptic properties, biological potency, and industrial suitability of these oils. Several studies emphasize the role of minor terpenes in enhancing antimicrobial synergy, making hybrids like 2414 especially valuable in multi-functional applications, from pharmaceuticals to biopesticides<sup>[36,37]</sup>.

In summary, this research demonstrates the dual importance of genetic background and processing conditions in determining the yield and composition of eucalyptus essential oils. Clone 2414 stands out as a robust and promising candidate for EO valorization, combining high cineole content, moderate yield, chemical richness, and processing stability. These traits make it particularly well suited for scalable industrial applications where product consistency, therapeutic efficacy, and extraction efficiency are essential.

Under optimized sustainable conditions (grinding + 3 h distillation), clone 2414 yields stable and cineole-rich oils suitable for pharmaceutical and eco-industrial applications. Its intermediate yet resilient performance confirms the hybrid's potential for scalable essential-oil production in Mediterranean climates.

## Conflict of interest

The authors declare no conflict of interest.

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