

# Chemical composition and antifungal activity of essential oil from *Eucalyptus smithii* against dermatophytes

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## ABSTRACT

**Introduction:** In this study, we evaluated the chemical composition of a commercial sample of essential oil from *Eucalyptus smithii* R.T. Baker and its antifungal activity against *Microsporum canis* ATCC 32903, *Microsporum gypseum* ATCC 14683, *Trichophyton mentagrophytes* ATCC 9533, *T. mentagrophytes* ATCC 11480, *T. mentagrophytes* ATCC 11481, and *Trichophyton rubrum* CCT 5507. **Methods:** Morphological changes in these fungi after treatment with the oil were determined by scanning electron microscopy (SEM). The antifungal activity of the oil was determined on the basis of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values. **Results:** The compound 1,8-cineole was found to be the predominant component (72.2%) of the essential oil. The MIC values of the oil ranged from 62.5 µg·mL<sup>-1</sup> to >1,000 µg·mL<sup>-1</sup>, and the MFC values of the oil ranged from 125 µg·mL<sup>-1</sup> to >1,000 µg·mL<sup>-1</sup>. SEM analysis showed physical damage and morphological alterations in the fungi exposed to this oil. **Conclusions:** We demonstrated the potential of *Eucalyptus smithii* essential oil as a natural therapeutic agent for the treatment of dermatophytosis.

**Keywords:** *Eucalyptus smithii*. Dermatophytes. Medicinal plants. Antifungal activity. Essential oil.

## INTRODUCTION

The essential oils are volatile substances formed by secondary metabolism of plants. They have a complex chemical profile, therefore there are not a specific cellular target, and can have different pharmacological properties. The genus *Eucalyptus* belongs to the Myrtaceae family, which comprises approximately 130 genera and 4,000 species. More than 300 species of *Eucalyptus* contain volatile oils in their leaves<sup>(1)</sup>. *Eucalyptus smithii* R.T. Baker is native to Australia. It belongs to the subgenus *Symphomyrtus*, section *Maidenaria*, and subseries *Viminalinae*<sup>(2)</sup>. It is popularly known as *gully gum* or *gully peppermint*. The essential oils from *Eucalyptus* plants possess biological properties such as antibacterial, antifungal, antitumor, and insecticidal properties, and they are used in spices, cosmetic formulations, and pharmaceuticals worldwide<sup>(3)(4)(5)(6)(7)(8)(9)</sup>.

Several studies have shown the antimicrobial properties of essential oils from various species of *Eucalyptus* against a wide range of microorganisms. The essential oils from *Eucalyptus camaldulensis*, *Eucalyptus globulus*, *Eucalyptus tereticornis*,

*Eucalyptus citriodora*, and *Eucalyptus odorata* have shown strong, moderate, or weak activity against bacteria and fungi, including dermatophytes<sup>(10)(11)(12)(13)(14)</sup>.

Dermatophytes are a group of closely related fungi that cause dermatophytosis, which affects approximately 40% people worldwide and accounts for 30% of all cutaneous mycotic infections. The most common clinical signs are circular patches of alopecia with scales formation. May arise ring injuries with healthy central halo and follicular papules with thin crusts on the outskirts<sup>(15)(16)</sup>. Current treatments for dermatophytosis include synthetic antifungal drugs, which can give rise to adverse reactions and resistant strains if not administered properly<sup>(17)(18)(19)</sup>. Essential oils serve as an alternative treatment for dermatophytosis because most of these oils have some degree of antifungal activity owing to their lipophilic nature<sup>(20)</sup>. The antifungal activities need not be ascribed to the high content of a single chemical compound because synergistic effects among the major and minor components can occur.

Studies on the chemical composition of essential oils from various *Eucalyptus* species have found 1,8-cineole to be the dominant compound. However, these studies have shown large variations in the chemical composition of essential oils from different species and even in those from the same species but from different regions, because these volatile oils are affected by non-genetic factors such as leaf age and seasonal variations<sup>(4)(13)(21)(22)(23)</sup>.

Because of the need for new therapeutic agents for the treatment of dermatophytosis, and due to the fact there are reports in the literature of various species of *Eucalyptus*, we decided to investigate the antifungal activity of the essential oil from

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*Eucalyptus smithii* R.T. Baker against major dermatophytosis-causing filamentous fungi such as *Microsporum canis*, *Microsporum gypseum*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum* *in vitro* and determined the resultant micromorphological changes in these fungi by using scanning electron microscopy (SEM). And to the best of our knowledge, this is the first study to test the antifungal activity of essential oil from *E. smithii* against dermatophytes.

## METHODS

### Essential oil from *Eucalyptus smithii*

A commercial sample of essential oil from *E. smithii* (batch EUCCSM105) obtained by steam distillation of plant leaves was acquired from Laszlo Aromatologia Ltda (Belo Horizonte, MG, Brazil). The sample was characterized using a gas chromatograph (HP5890; Hewlett-Packard, Avondale, PA, USA) equipped with a flame ionization detector. The chromatographic parameters were BP-1 (HP) 30m × 0.25mm BP-1 column, 1-μL injection (1/50 split), hydrogen as the carrier gas (2mL·min<sup>-1</sup>), 250°C temperature of both the detector and injector, and a temperature gradient (60°C initially, followed by an increase of 3°C per min until the temperature reached 220°C) for the column. Samples were diluted to 0.5% (v/v) in chloroform. Peaks were identified by calculating the retention time and by comparing these with linear hydrocarbon standards C10-C18 and reports in the literature<sup>(24)</sup>.

### Microorganisms

The test organisms comprised six dermatophyte fungi, namely, *M. canis* ATCC 32903, *M. gypseum* ATCC 14683, *T. mentagrophytes* ATCC 9533, *T. mentagrophytes* ATCC 11480, *T. mentagrophytes* ATCC 11481 (provided by the Instituto Nacional de Controle de Qualidade em Saúde da Fundação Oswaldo Cruz, Brazil), and *T. rubrum* CCT 5507 (kindly gifted by the *Fundação André Tosello*, Brazil).

### Antifungal screening and susceptibility testing

Preliminary antifungal activity was determined using a concentration of 1,000μg·mL<sup>-1</sup> (based on the percentage of the major component) of the essential oil from *E. smithii* against the dermatophyte isolates. The assay was performed as described in an earlier report<sup>(25)</sup> with some modifications. Fragments (2mm) of dermatophytes were inoculated on Sabouraud dextrose agar (SDA) supplemented with the essential oil from *E. smithii* in a sterile 24-well plate for cell culture, and the antifungal activity was examined by determining total growth inhibition.

Minimum inhibitory concentration (MIC) was determined according to the National Committee for the Clinical Laboratory Standards Institute (CLSI) guidelines for filamentous fungi<sup>(26)</sup>, with adaptations described in a previous study<sup>(27)</sup>. Roswell Park Memorial Institute medium (RPMI-1640; Sigma-Aldrich Inc., St. Louis, MO, USA) containing L-glutamine, lacking sodium bicarbonate, and buffered with 0.165mol·L<sup>-1</sup> 3-morpholinopropanesulfonic acid (JT Baker, Griesheim, Germany) at pH 7.0 was used as the basal medium, with or without oil and antifungal agents.

Seven-day-old cultures of dermatophytes maintained in SDA at 28°C were used to obtain conidial inoculum, which was prepared by adding 8mL of 0.9% sterile saline and 20μL of Tween-80: DMSO (1:1 v/v). The densities of the obtained suspension were adjusted using a spectrophotometer (Libra S12; Biochrom, Cambridge, UK) at a 530-nm wavelength to achieve a transmittance of 68-70%. The inoculum suspensions were diluted (1:50v/v) using RPMI test medium to obtain suspensions containing cell numbers ranging from 0.4 × 10<sup>4</sup> to 5.0 × 10<sup>4</sup> colony-forming units·mL<sup>-1</sup>. The essential oil from *E. smithii* was solubilized in the culture medium and was serially diluted to achieve eight concentrations ranging from 7.8 to 1,000μg·mL<sup>-1</sup>. Ketoconazole (Janssen Cilag, Butantã, SP, Brazil) and terbinafine (Galena, Campinas, SP, Brazil) at concentrations of 0.005-0.24μg·mL<sup>-1</sup> and 0.03-16.0μg·mL<sup>-1</sup>, respectively, were used as reference drugs. These antifungal agents were chosen because they have standard testing protocols and because they are the most widely used oral antifungal drugs in clinical practice<sup>(18) (27) (28)</sup>.

The microdilution plates were incubated at 28°C and were examined after seven days of incubation. Minimum inhibitory concentration was defined as the lowest concentration of the oil at which the tested microorganisms did not show visible growth.

Minimum fungicidal concentration (MFC) was determined using the microdilution method<sup>(20)</sup>. Aliquots of 20μL from the wells that did not show growth in the MIC procedure were transferred to new 96-well plates containing 180μL of SDA. The plates were incubated at 28°C for seven days. The lowest concentration of the oil showing no visible growth in the plates was defined as the MFC.

### Scanning electron microscopy

Samples for SEM were prepared as follows. Untreated control blocks and agar blocks treated with essential oil (1,000μg·mL<sup>-1</sup> concentration) or antifungal agents (ketoconazole and terbinafine; concentrations obtained from susceptibility testing) at 28°C for seven days were fixed with modified Karnovsky's fixative containing 2.5% (v/v) glutaraldehyde and 2.5% (v/v) paraformaldehyde in 0.05M sodium cacodylate buffer (pH 7.2) and incubated for 24h. The samples were then washed three times (each wash for 10 min) with the same buffer and were post-fixed with 1% (w/v) osmium tetroxide for 1h. The post-fixed samples were given three brief washes with distilled water and were dehydrated using increasing concentrations of acetone (25-100% v/v) at every 10-min interval<sup>(29)</sup>. The samples were then assembled in aluminum stubs with double-faced carbon tapes, placed on an aluminum foil sputter coated with gold (2mm) (FL-9496, Balzers, Fürstentum Liechtenstein), and observed by SEM (JSM 6390LV; Jeol, Tokyo, Japan) under 25kw power and a work distance of 17mm.

## RESULTS

Chromatographic analysis of the essential oil identified seven components that accounted for 86.2% of the total oil content. These are represented in **Figure 1** and **Table 1** according to their elution order observed in the chromatographic profile.

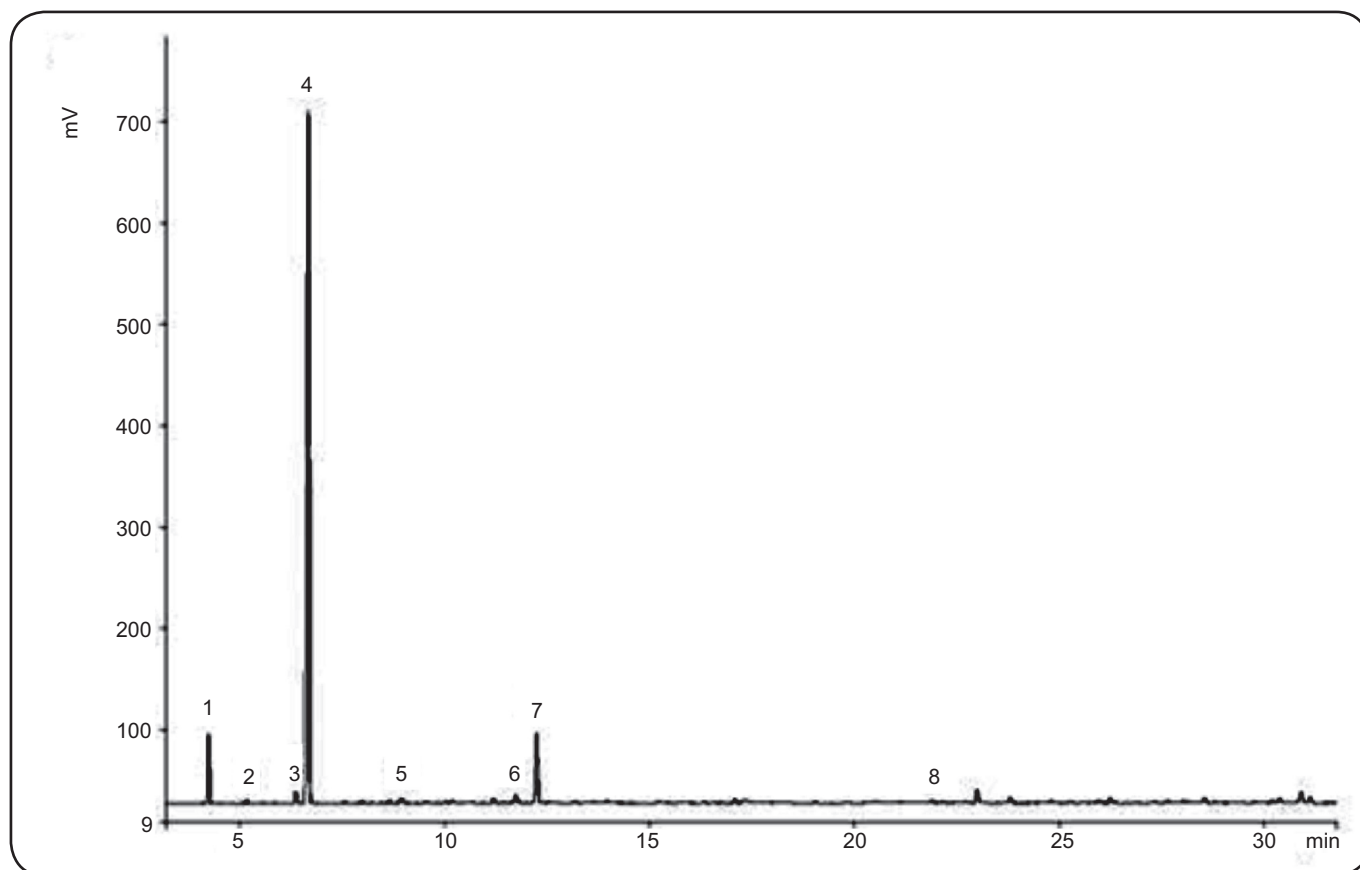


FIGURE 1 - Chromatographic profile of the essential oil of *Eucalyptus smithii*. Peaks lower than 0.1% were not documented. (1)  $\alpha$ -pinene, (2)  $\beta$ -pinene, (3)  $\rho$ -cymene, (4) 1,8-cineole, (5) linalool, (6) terpinen-4-ol, (7)  $\alpha$ -terpineol, and (8)  $\beta$ -caryophyllene. mV: millivolt; min: minutes.

TABLE 1 - Chemical composition of the essential oil from *Eucalyptus smithii* obtained by high-resolution gas chromatography.

| Peak         | Kovats index (calculated) | Kovats index (Adams, 2009) | Constituent            | Percentage  |
|--------------|---------------------------|----------------------------|------------------------|-------------|
| 1            | 948                       | 939                        | $\alpha$ -pinene       | 3.5         |
| 2            | 1,016                     | 1,026                      | $\rho$ -cymene         | 1.1         |
| 3            | 1,013                     | 1,033                      | 1,8-cineole            | 72.2        |
| 4            | 1,078                     | 1,098                      | linalool               | 0.7         |
| 5            | 1,143                     | 1,177                      | terpinen-4ol           | 0.8         |
| 6            | 1,156                     | 1,189                      | $\alpha$ -terpineol    | 7.5         |
| 7            | 1,399                     | 1,418                      | $\beta$ -caryophyllene | 0.4         |
| <b>Total</b> | -                         | -                          | -                      | <b>86.2</b> |

Peaks under 0.1% were not considered.

TABLE 2 - Antifungal activity\* of essential oil from *Eucalyptus smithii* and reference drugs.

| Microorganism                                 | Substances                |     |              |      |             |      |
|---|---------------------------|-----|--------------|------|-------------|------|
|   | <i>Eucalyptus smithii</i> |     | ketoconazole |      | terbinafine |      |
|   | MIC                       | MFC | MIC          | MFC  | MIC         | MFC  |
| <i>Microsporum canis</i> ATCC 32903           | 500                       | 500 | 0.06         | 0.06 | 0.06        | 0.06 |
| <i>Microsporum gypseum</i> ATCC14683          | 1,000                     | -   | 8.00         | 16.0 | 0.12        | 0.12 |
| <i>Trichophyton mentagrophytes</i> ATCC 9533  | 250                       | 250 | 4.0          | 4.0  | 0.12        | 0.12 |
| <i>Trichophyton mentagrophytes</i> ATCC 11480 | -                         | -   | 2.0          | 2.0  | 0.03        | 0.03 |
| <i>Trichophyton mentagrophytes</i> ATCC 11481 | 125                       | 125 | 0.25         | 0.25 | 0.03        | 0.03 |
| <i>Trichophyton rubrum</i> CCT 5507           | 62.5                      | 125 | 1.0          | 4.0  | 0.06        | 0.06 |

MIC: minimum inhibitory concentration; MFC: minimum fungicide concentration; -: not detected at any of the tested concentrations (7.8 to 1,000 $\mu\text{g}\cdot\text{mL}^{-1}$  of oil).

\*Results expressed as  $\mu\text{g}\cdot\text{mL}^{-1}$ .

The results of this study also showed that the commercial sample of the essential oil from *E. smithii* (batch EUCCSM105) could inhibit the growth of the tested fungal strains (Table 2).

Lastly, SEM observations confirmed the physical damage and morphological alteration that could compromise fungal cell viability (Figure 2).

## DISCUSSION

Essential oils are very complex natural mixtures and are characterized by two or three major components having fairly high concentrations. Monoterpenes, which perform several biological functions, are the most dominant compounds in essential oils<sup>(30)</sup>. In the present study, monoterpenes, represented essentially by  $\alpha$ -pinene (monocyclic hydrocarbon);  $\rho$ -cymene (monocyclic aromatic); 1,8-cineole (ether); and linalool, terpinen-4-ol, and  $\alpha$ -terpineol (alcohols), were found to be the major compounds<sup>(8)(13)(22)</sup>.  $\beta$ -Caryophyllene, another compound that was identified, is a bicyclic sesquiterpene. Elassi et al.<sup>(7)</sup> showed that the major compounds present in the essential oils of 10 *Eucalyptus* species had expected similarities because they were obtained from plants belonging to the same genus, followed by  $\alpha$ -pinene,  $\rho$ -cymene, borneol, cryptone, spathulenol, viridiflorol and limonene. Ogunwande et al.<sup>(22)</sup> showed that  $\beta$ -pinene (39.4%) and  $\alpha$ -pinene (30.4%) were the dominant constituents in oil samples obtained from *E. tereticornis* Smith and *Eucalyptus grandis* W. Hill, respectively, with no detectable 1,8-cineole. 1,8-cineole, the predominant compound observed in the present study, has relatively strong antimicrobial properties against many important pathogens<sup>(5)(8)(30)</sup>, besides low toxicity observed in *in vitro* tests<sup>(13)</sup>. However, the strong antimicrobial effect of essential oils is mostly attributed to additive effects, and minor components appear to play a significant role. In addition, studies have shown that the antimicrobial activity of essential oils differs among oils obtained from different *Eucalyptus* species<sup>(4)(5)</sup>.

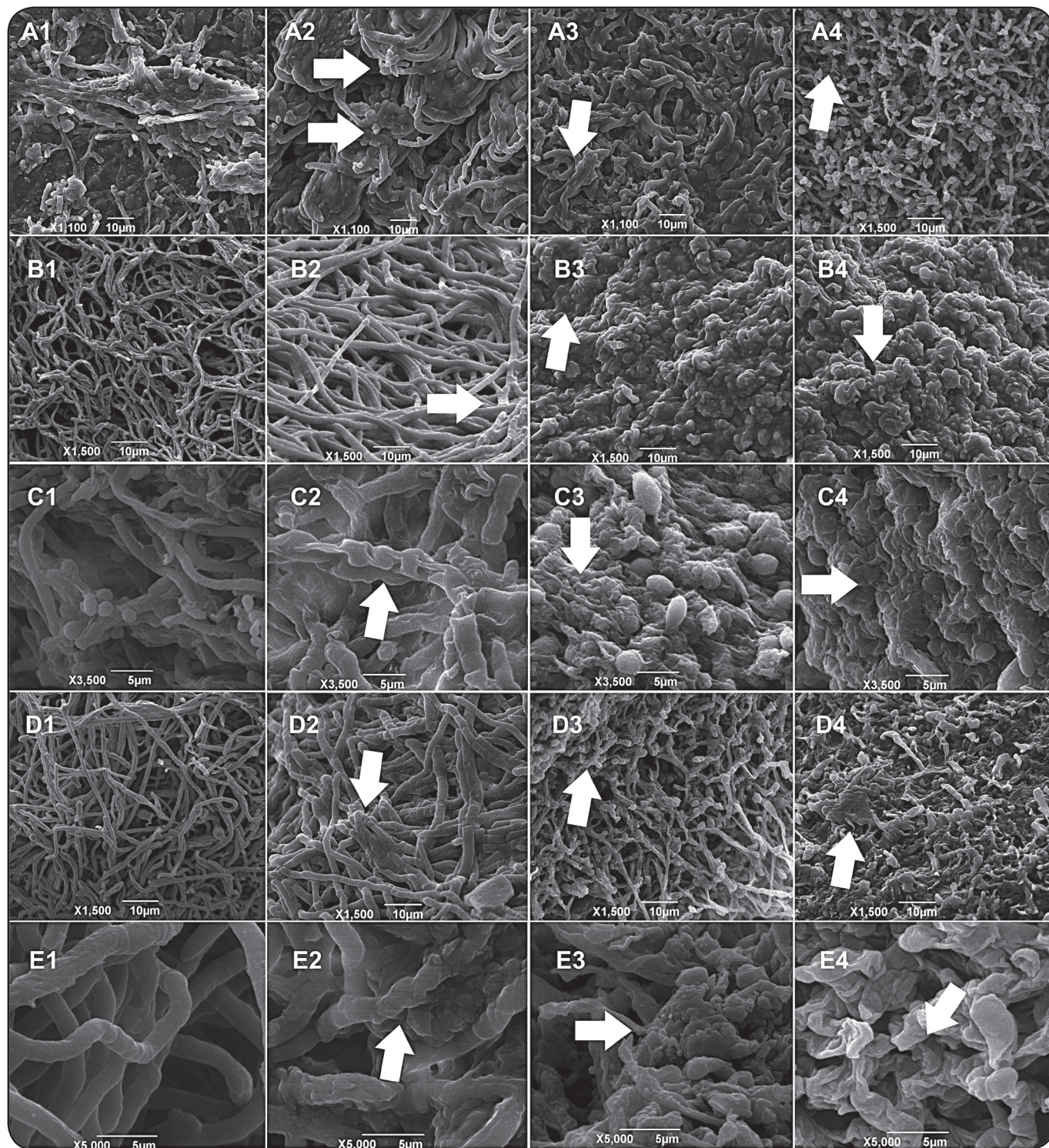
Most studies on the characterization of essential oils of *Eucalyptus* indicate that the samples vary characteristically

over the sampling period. This wide variation in the chemical composition of the oils can be attributed to environmental factors and different ages of the leaves from which the oils are extracted<sup>(4)(13)(21)(22)(23)</sup>. In the present study, the antifungal activity of the essential oil showed considerable variations against different fungal species tested in this study, with the best activity being against *T. rubrum*. It is noteworthy that the antifungal activity of the essential oil from *E. smithii* was tested against three strains of *T. mentagrophytes* and that the oil was inactive only against one strain, namely, *T. mentagrophytes* ATCC 11480. The variation in the effect against these three fungal strains might be mainly because of their origin. For example, *T. mentagrophytes* ATCC 11480 is of animal origin, while *T. mentagrophytes* ATCC 9538 and *T. mentagrophytes* ATCC 11481 are of human origin, which may result in different levels of resistance to the essential oil.

Elassi et al.<sup>(13)</sup> evaluated the antibacterial, antifungal, and antiviral activities of essential oils from eight *Eucalyptus* species. Among these eight species, essential oils from seven *Eucalyptus* species were tested against three dermatophytes (*Trichophyton rubrum*, *Trichophyton soudanense*, and *M. canis*). The activities of these essential oils varied significantly with species and strains. The essential oil from *E. odorata* showed the best better antifungal activity against all the tested fungal strains. This essential oil contained the highest mean percentage of cryptone (20.9%  $\pm$  1.3%) and the lowest mean percentage of 1,8-cineole (4.5%  $\pm$  1.6%). Therefore, the authors concluded that there was no correlation between the amount of 1,8-cineole and high antifungal activity. However, essential oils from *Eucalyptus sideroxylon* and *Eucalyptus bicostata*, containing the highest mean percentage of 1,8-cineole and moderate amount of  $\alpha$ -pinene, were less toxic and could inhibit the growth of *M. canis*.

The biological activity of essential oils varies according to the presence of test compounds in the oil and the dose. Moreover, structure-activity relationships of certain phytochemicals are found to be important for the biological activity of these essential oils. A study by Kim and Park<sup>(14)</sup> showed that aldehydes





**FIGURE 2 - A.** SEM images of *Microsporium canis* ATCC 32903. Magnification: A1-3: 1,100×; A4: 1,500×. **B.** SEM images of *Microsporium gypseum* ATCC14683; magnification: 1,500×. **C.** SEM images of *Trichophyton mentagrophytes* ATCC 9538; magnification: 3,500×. **D.** SEM images of *Trichophyton mentagrophytes* ATCC 11481; magnification: 1,500×. **E.** SEM images of *Trichophyton rubrum* CCT 5507; magnification: 5,000×. E1: fungal cells not exposed to substances of interest; E2: fungal cells exposed to *Eucalyptus smithii* essential oil; arrows indicate clusters of hyphae with little vesicles on the surface, compressed and furrowed hyphae, and swollen appearance and alteration in the surface structure; E3: Fungal cells exposed to ketoconazole, and E4: fungal cells exposed to terbinafine: arrows indicate the appearance of dried and wrinkled hyphae. Scale bars: A, B, D = 10µm; C, E = 5µm. SEM: scanning electron microscopy.



(neral and geranial) and alcohols (citronellol and geraniol) were more effective antifungal agents against *Aspergillus* species than hydrocarbons. This study also showed that the position of the hydroxyl group was associated with the antifungal activity.

In the present study, the MIC and MFC results were complemented by micromorphological results obtained using SEM. Scanning electron microscopy showed that treatment of the fungal strains with the essential oil had a detrimental effect on their morphology.

Electron micrographs of all tested fungi showed differences in their structures compared with those of fungi not treated with the *E. smithii* essential oil, ketoconazole, or terbinafine. Exposure to essential oil and drugs caused similar alterations in all fungi, only with different intensities: the fungal cells exposed to the drugs appeared more dehydrated and therefore more compact than exposure to oil. The most common changes were the compression and clustering of hyphae without the original tubular shape, and appearance of dried and wrinkled hyphae. A review of the investigated literature suggests that this is caused by the extravasation of cell cytoplasm. This is partly associated with the lipophilic nature of essential oils, which leads to their accumulation in membranes and subsequent energy depletion<sup>(5)(31)(32)</sup>. Thus, we surmise that the present essential oil from *E. smithii* can modify the permeability of fungal cells, leading to various structural and biochemical changes and, eventually, death.

The findings of the present study indicate that the essential oil from *E. smithii* (batch EUCCSM105) could be useful for inhibiting the growth of some dermatophytes and has potential as a natural therapeutic agent for the treatment of dermatophytosis. One must take into account that the knowledge of *Eucalyptus*'s pharmacological activity, antifungal in particular, has been consolidated and increased with every new study carried out with the genus.

However, further research is necessary to elucidate its mechanism of action and to develop formulations for improving its activity and stability for use in humans and animals. Moreover, large variations in the chemical compositions of essential oils from various *Eucalyptus* species and the subsequent effects this can have on biological activities imply the need for more efficient quality control of the raw materials.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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