

Original Research Article

In vitro Antifungal Activity of *Baccharis trimera* Less (DC) Essential Oil against Dermatophytes

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Abstract

Purpose: To identify the main components of the essential oil (EO) of *Baccharis trimera* Less and investigate their *in vitro* antifungal activity against seven fungal strains that cause onychomycosis.

Methods: The chemical composition of EO was determined using gas chromatography, and its minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC), and antifungal activity were compared with those of terbinafine and ketoconazole. Scanning electron microscopy (SEM) was used to investigate morphological changes in the strains of interest.

Results: Twenty compounds, with β -pinene being the major constituent (23.4 %), were identified in EO. EO exhibited fungicide potential, with MFC values in the range of 0.06 to 125 $\mu\text{g mL}^{-1}$, which were lower than those of the reference drugs against *Trichophyton rubrum* CCT 5507 URM1666 and *Microsporum canis* ATCC 32903. MIC range for the compounds was from 0.03 to 125 $\mu\text{g mL}^{-1}$ for five strains of the fungi evaluated. For *Trichophyton mentagrophytes* ATCC 11481 and *Epidermophyton floccosum* CCF-IOC 3757, MIC was $\geq 1000 \mu\text{g mL}^{-1}$. Flattening, distortions, and shrinkage were observed in the SEM images of structures of the five fungal species that were subjected to the action of the EO.

Conclusion: The results indicate that EO has antifungal activity against filamentous fungi and may be developed as an alternative for the treatment of onychomycosis.

Keywords: *Baccharis trimera*, Fungi, Onychomycosis, Dermatophytes, Antifungal

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INTRODUCTION

Mycosis represents an important public health concern and its incidence has been increasing in recent years, being a frequent cause of visits to dermatologists [1]. This increase has numerous causes such as susceptible population, immunocompromised individuals, fact associated with the practice of sports, wearing shoes with poor ventilation, use of public pools, and manicure instruments [2,3].

Among the various types of fungal infections that affect humans, onychomycosis is caused by dermatophytes, yeasts, and non-dermatophyte filamentous fungi, and constitutes a major cause of nail disease worldwide [4-9]. Species most commonly isolated from patients with onychomycosis around the world are the dermatophytes, *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Microsporum canis*, *Microsporum gypseum*, and *Epidermophyton floccosum* [2,5,10,11].

Treatment of onychomycosis is still considered a challenge owing to the anatomical characteristic of the nail substrate, chronicity of the disease, and relapse cases [12]. Many antifungal drugs available in the market are not effective either because of their improper use or due to the development of resistance mechanisms of the fungus [13,14].

In this context, many studies have been carried out in search of new antimicrobial products, including products that make use of medicinal plants [12,15-18], such as *Baccharis trimera* Less (DC) [19,20]. Being a part of the Asteraceae family [21,22], *B. trimera* has numerous pharmacological activities popularly attributed to their leaves, such as anti-inflammatory, digestive [23], antioxidant [24], antiulcer [23,25], and antimicrobial [19,20] properties that can be helpful in the treatment of diabetes [26], rheumatism, and hepatobiliary disorders [27]. Therefore, the aim of this study was to investigate the *in vitro* antifungal activity of the essential oil of *B. trimera* against the main strains of filamentous fungi that cause onychomycosis.

EXPERIMENTAL

B. trimera essential oil

The essential oil of the leaves of *B. trimera* (lot BATHI0111), which was extracted by vapor entrainment, was obtained from Laszlo Aromatology Company LTDA, Brazil.

Gas chromatography

To qualitatively and quantitatively characterize the main chemical constituents of this essential oil, an aliquot (50 μ L) of oil was subjected to high-resolution gas chromatography (HP 5890) equipped with flame ionization detector. A BP-1 (SGE) 25 m \times 0.25 mm column was used, with a temperature gradient of 60 $^{\circ}$ C/min and 3 $^{\circ}$ C/min to 220 $^{\circ}$ C, along with an injector (split of 1/50) at 220 $^{\circ}$ C and detector at 220 $^{\circ}$ C. The carrier gas used was hydrogen (2 mL/min) and the injection volume was 1 μ L. The samples were diluted to 0.5 % in chloroform. Characterization of the peaks was performed by calculating the Kovats retention index and compared with the literature data [28].

Fungal strains

Standard strains of filamentous dermatophytes from the Collection of Tropical Crops (CCT) were provided by Foundation André Tosello,

Campinas, SP, Brazil (*Trichophyton rubrum* CCT-5507 URM 1666) and American Type Culture Collection (ATCC) provided by the National Institute of Quality Control in Health, Oswaldo Cruz Foundation, Rio de Janeiro, RJ, Brazil (*T. mentagrophytes* ATCC 9533, *T. mentagrophytes* ATCC 11480, and *T. mentagrophytes* ATCC 11481, *M. gypseum* ATCC 14683, *M. canis* ATCC 32903 and *E. floccosum* CCF-IOC 3757).

Microbiological screening

Microbiological screening was performed according to the technique described by Souza *et al* [29] by using sterile 24-well plates (Sarstedt, Germany). A 2 mm fungal fragment was inoculated onto a plate containing Sabouraud dextrose agar (SDA) with the essential oil of *B. trimera* (1,000 μ g mL⁻¹ β -pinene). Likewise, a positive control (SDA with fungal fragment in the absence of essential oil) and negative control (SDA with essential oil in the absence of fungal fragment) were prepared. Subsequently, the plates were incubated at 28 $^{\circ}$ C \pm 2 $^{\circ}$ C for 7 days.

Determination of minimum inhibitory concentration (MIC)

The MIC was determined for the essential oil of *B. trimera* according to the protocol M38-A of The National Committee for Clinical Laboratory Standards (CLSI) [30], adapted as required by Almeida *et al* [31]. All analyses were performed in triplicates.

For the preparation of the fungal suspension, fungal cultures obtained after 7 days of incubation in tubes with slanted SDA were used. The cultures were washed thrice by adding 2 mL of 0.85 % sterile saline and 20 μ L of Tween-80 solution/dimethyl sulfoxide analytical grade (1: 1, v/v). The suspension formed [30] was analyzed by employing a spectrophotometer (Libra S12, Biochrom, UK) using quartz cuvette with an optical path of 1.0 cm and the transmittance was adjusted in the range of 68 – 70 % at a fixed wavelength of 530 nm [31]. The suspension was subsequently diluted at a ratio of 1: 50 (v/v) with RPMI-1640 medium (Sigma, USA) buffered with 3 (N-morpholino) propanesulfonic acid (MOPS, JTBaker, Germany). A 100 μ L aliquot of this final solution was transferred to the microtiter plate and used for MIC determination.

The reference drugs terbinafine and ketoconazole were evaluated at concentrations from 0.0234 to 12 μ g mL⁻¹ and 0.0313 to 16 μ g mL⁻¹, respectively, in RPMI-1640 buffered with MOPS [30-31]. Dimethyl sulfoxide analytical

grade (Sigma, Brazil) was used for the solubilization of the reference drugs [30].

The essential oil of *B. trimera* was solubilized in RPMI-1640 buffered with MOPS and evaluated at eight concentrations in the range from 7.8 to 1,000 $\mu\text{g mL}^{-1}$ of β -pinene. Subsequently, 100 μL of each dilution of terbinafine, ketoconazole, and essential oil were transferred to the 96-well microplate. The fungal viability test was performed by adding 200 μL of RPMI-1640 buffered with MOPS containing the fungal inoculum. After these steps, the culture was homogenized for 2 min and incubated at $28 \text{ }^\circ\text{C} \pm 2^\circ\text{C}$ for 7 days.

Minimum fungicidal concentration (MFC)

After the determination of MIC, MFC was determined according to Magagnin *et al* [32]. Aliquots of 10 μL of the medium without fungal growth were transferred to new 96-well microplate containing 200 μL of Sabouraud dextrose broth without any antifungals.

Scanning electron microscopy (SEM)

Untreated fungal fragments and fungal fragments treated with terbinafine, ketoconazole, or essential oil of *B. trimera* were analyzed by SEM. The fungal fragments that were subjected to SEM were prepared according to Martinelli and Santos [33] and Mio *et al* [34] using the Karnovsky solution (2.5 % glutaraldehyde and 2.5 % formaldehyde in 0.05 M sodium cacodylate buffer and 0.001 M calcium chloride; pH of 7.2) for 24 h, a solution of 1 % osmium tetroxide for 1 h in the dark, and solutions of acetone (25 %, 50 %, 70 %, 90 %, and 100 %). Subsequently, the resulting fragment was fixed on aluminum stubs

with the aid of a double-sided carbon tape and brought to the metallizer (Balzers FL-9496/Fürstentum Liechtenstein), where they were subjected to a gold steam bath for 180 s.

The fungal samples were subjected to scanning electron microscope (JSM-5310, JEOL) under the conditions of 25-Kv power and working distance of 17 mm. The analyzes were performed at the Electron Microscopy Center Scanning the Federal University of Rio de Janeiro and the Department of Physics of the Pontifical Catholic University of Rio de Janeiro.

RESULTS

The essential oil of *B. trimera* subjected to gas chromatography showed monoterpenes carquejyl acetate and β -pinene as the major constituents (Figure 1 and Table 1), with β -pinene exhibiting the highest percentage (23.4 %). Peaks under 0.1 % were not considered.

Preliminary assay of the fungal fragments revealed that *T. mentagrophytes* ATCC 9533; *T. mentagrophytes* ATCC 11480, *T. rubrum* CCT 5507 URM 1666, *M. canis* ATCC 32903, and *M. gypseum* ATCC 14683 showed no visible fungal growth when exposed to *B. trimera* oil (1,000 $\mu\text{g mL}^{-1}$ β -pinene). However, *T. mentagrophytes* ATCC 11481 and *E. floccosum* CCF-IOC 3757 presented some growth. Thus, it was possible to observe the respective antifungal activity of the essential oil against five strains tested. The MIC values for the essential oil of *B. trimera* and the reference drugs (terbinafine and ketoconazole) are shown in Table 2.

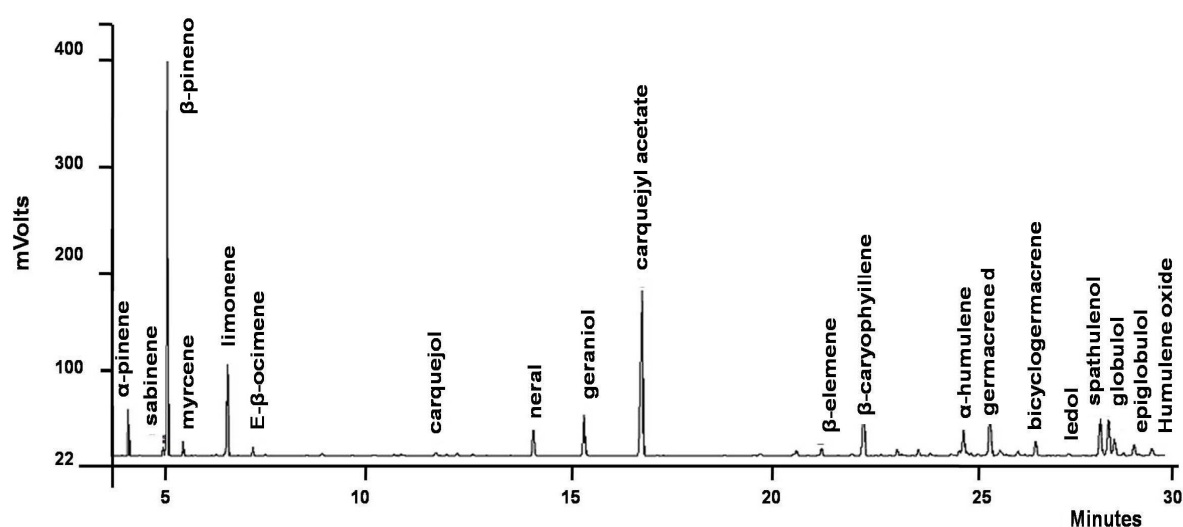


Figure 1: Chromatographic profile of the essential oil of *B. trimera*.

Table 1: Chemical profile of the essential oil of *B. trimera*

No.	Compound	%	Kovat's index
1	α -pinene	2.2	928
2	Sabinene	0.5	967
3	β -pinene	23.4	973
4	Myrcene	0.8	986
5	Limonene	5.9	1021
6	E- β -ocimene	0.6	1039
7	Carquejol	0.4	1150
8	Neral	2.3	1231
9	Geranial	4.1	1258
10	carquejyl acetate	19.0	1295
11	β -elemene	0.8	1388
12	β -caryophyllene	6.4	1412
13	α -humulene	3.1	1477
14	germacrene D	5.0	1494
15	bicyclogermacrene	1.6	1523
16	ledol	4.6	1563
17	spathulenol	4.4	1569
18	globulol	2.3	1575
19	epiglobulol	1.3	1586
20	humulene oxide	1.0	1596
Total		89.7	

Table 2: *In vitro* susceptibility of the filamentous dermatophytes to the essential oil of *B. trimera*, terbinafine and ketoconazole

Fungal strain	Essential oil of <i>B. trimera</i>		Terbinafine		Ketoconazole	
	MIC	MFC	MIC	MFC	MIC	MFC
<i>T. mentagrophytes</i> ATCC 9533	31.25	62.50	3	3	2	4
<i>T. mentagrophytes</i> ATCC 11480	125	125	0.03	0.03	1	1
<i>T. mentagrophytes</i> ATCC 11481	>1,000	NE	0.03	0.03	0.25	0.25
<i>T. rubrum</i> CCT 5507 URM 1666	0.03	0.06	0.19	0.19	1	4
<i>M. canis</i> ATCC 32903	0.24	0.98	0.19	0.19	0.06	8.01
<i>M. gypseum</i> ATCC 14683	125	125	0.12	0.12	8	16
<i>E. floccosum</i> CCF-IOC 3757	>1,000	NE	>480	NE	>640	NE

MIC: Minimum inhibitory concentration; MFC: Minimum fungicidal concentration. *Results expressed as $\mu\text{g mL}^{-1}$; NE: not established (for strains with MIC values above the determined working range)

The electron micrographs of the fungal strains (exposed or not exposed to the reference drugs and essential oil of interest) are presented in Figure 2.

The analysis of electron micrographs allowed us to morphologically identify the fungal structures and perceive the morphological changes in the fragments of *T. rubrum* CCT 5507 URM 1666, *T. mentagrophytes* ATCC 9533, *M. gypseum* ATCC 14683, and *M. canis* ATCC 32903, which were subjected to the essential oil of *B. trimera*. The abnormalities identified suggested the "flattening" of the fungal structures caused by this essential oil.

DISCUSSION

The constituents considered as chemical markers of the essential oil of this species were identified as carquejyl acetate and carquejol;

however, these were present in small percentages [19,20]. Nevertheless, the percentage of the chemical constituents of the essential oil analyzed is different from that reported in other works [19,35]. It should be noted that there are factors related to the essential oil can cause changes in its chemical composition. These include the time of collection and drying of the plant material, soil types, genetic factors, climate, and extractive processes [36].

In *B. trimera* oil, monoterpenes were identified, and among them, β -pinene and carquejila acetate were found at high levels. This class of substances present in the Asteraceae family is related to antifungal activity [37], because they may increase the concentration of lipid peroxides such as hydroxyl, alkoxy, and alkoperoxy radicals and lead to cell death [36].

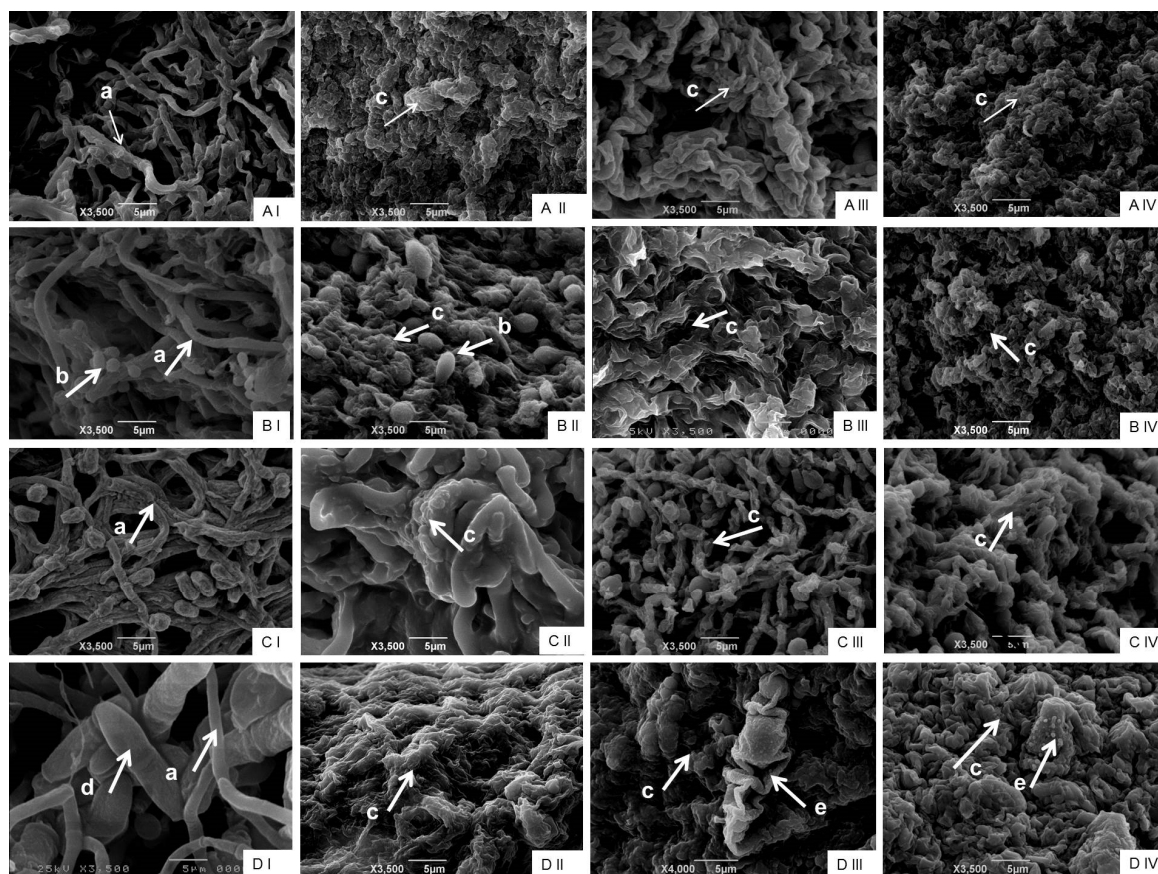


Figure 2: Electron micrographs of the fragments of *T. rubrum* CCT 5507 URM 1666 (A), *T. mentagrophytes* ATCC 9533 (B), *M. canis* ATCC 32903 (C), and *M. gypseum* ATCC 14683 (D). The magnification of the images corresponds to 3,500x, with the exception of images D III, whose magnification is 4,000x. Fungal fragments: not exposed to any substance of interest (I), exposed to ketoconazole (II), exposed to terbinafine (III), and exposed to the essential oil of *B. trimera* (IV). "a" indicates hyphae and no changes in tubular appearance. "b" denotes hyphae/mycelium puckered and/or compacted. "c" indicates hyphae/mycelium forming a compact mass and/or contorted and rough. "d" denotes macroconidia. "e" indicates macroconidia and rough

Therefore, further experiments were developed according to the percentage of β -pinene because it is the major constituent for this *B. trimera* oil and has been reported to possess antifungal activities [35]. It is important to emphasize that there is a possibility of synergism between different molecules of the essential oil, which could be responsible for the pharmacological activity [38]. Sharma and Tripathi [39] indicated that essential oil acts on the hyphae presumably causing leakage of the cytoplasm, less stiffness and integrity of the cell wall, and collapse and death of the mycelium. In general, essential oil, being hydrophobic, could penetrate the mitochondrial membrane and cause cell damage, allowing easier release of vital cell components, besides interfering with the enzymatic and cellular respiration system [36].

It should be noted that the essential oil of *B. trimera* ($\text{MFC} = 0.06 \mu\text{g mL}^{-1}$) was more potent than the drugs terbinafine and ketoconazole ($\text{MFC} = 0.19$ and $4.00 \mu\text{g mL}^{-1}$, respectively)

against *T. rubrum* and also exhibited lower value of MFC ($0.98 \mu\text{g mL}^{-1}$) than ketoconazole ($8.01 \mu\text{g mL}^{-1}$) against *M. canis*. In other words, the antifungal activity of the essential oil against the two fungal strains, *T. rubrum* and *M. canis*, was higher, when compared with that of ketoconazole. These data are in accordance with those reported by Rodriguez *et al* [40], who stated that plants of the genus *Baccharis* L. are used in Argentina and other South American countries for the treatment of fungal diseases.

Although the MIC values of the essential oil for some fungal strains were higher than those of terbinafine and ketoconazole, with the increase in recurrence, development of fungal resistance, and high number of adverse reactions caused by the current treatment available, the use of this vegetable substance may be an alternative way to treat onychomycosis and/or act as an adjuvant therapeutic [2].

The electron micrographs of the untreated *T. mentagrophytes* presented tubular form with branches, similar to that described by Park *et al* [41], indicating the effect of terpenes on this strain. After being exposed to terbinafine, ketoconazole, and essential oil of *B. trimera*, distortions and shrinkages in the hyphal morphology were observed. Comparison of the electron micrographs of the strains exposed to essential oil with those not exposed to any substance of interest revealed the potential damage of the fungal structures, exhibiting results similar to those obtained with the reference drugs. This finding demonstrated the effectiveness of this oil against the fungal strains analyzed.

CONCLUSION

The essential oil of *B. trimera* has antifungal effect against two of the three genera of filamentous fungi. Further studies on the safety and toxicity of the oil is required.

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