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Cytotoxicity and anti-*Sporothrix brasiliensis* activity of the *Origanum majorana* Linn. oil

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ABSTRACT

The study aimed to evaluate the anti-*Sporothrix* sp. activity of the essential oil of *Origanum majorana* Linn. (marjoram), its chemical analysis, and its cytotoxic activity. A total of 18 fungal isolates of *Sporothrix brasiliensis* (*n*: 17) from humans, dogs and cats, and a standard strain of *Sporothrix schenckii* (*n*: 1) were tested using the broth microdilution technique (Clinical and Laboratory Standard Institute – CLSI M27-A3) and the results were expressed in minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC). The MIC_{50} and MIC_{90} of itraconazole against *S. brasiliensis* were $2 \mu g/mL$ and $8 \mu g/mL$, respectively, and the MFC_{50} and MFC_{90} were $2 \mu g/mL$ and $>16 \mu g/mL$, respectively, with three *S. brasiliensis* isolates resistant to antifungal. *S. schenckii* was sensitive at MIC of $1 \mu g/mL$ and MFC of $8 \mu g/mL$. For the oil of *O. majorana* L., all isolates were susceptible to MIC of ≤ 2.25–$9 mg/mL$ and MFC of ≤ 2.25–$18 mg/mL$. The MIC_{50} and MIC_{90} were $\leq 2.25 mg/mL$ and $4.5 mg/mL$, respectively, and the $MFC_{50/90}$ values were twice more than the MIC. Twenty-two compounds were identified by gas chromatography with a flame ionization detector (CG-FID) and 1,8-cineole and 4-terpineol were the majority. Through the colorimetric (MTT) assay, the toxicity was observed in 70–80% of VERO cells between 0.078 and 5 mg/mL. For the first time, the study demonstrated the satisfactory *in vitro* anti-*Sporothrix* sp. activity of marjoram oil and further studies are needed to ensure its safe and effective use.

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Introduction

Sporotrichosis is a zoonotic mycosis caused by the fungus of *Sporothrix schenckii* complex, such as *S. schenckii* var. *schenckii*, *S. schenckii* var. *luriei*, *S. brasiliensis*, *S. globosa*, *S. mexicana* and *S. albicans*.[1,2] This disease has a worldwide occurrence, mainly in countries of America, such as Brazil, Mexico, Colombia, Uruguay and Peru, and also in South Africa, India, Japan.[3,4] The infection is acquired through the traumatic inoculation by conidial and can be transmitted through scratch and bite of sick cats.[5,6] *S. brasiliensis* is considered the most virulent among the complex species and with a high prevalence in Brazil, being geographically restricted to this country.[7,8]

Limited lesions to skin and subcutaneous tissue may arise in the host and may have lymphatic involvement with systemic clinical signs.[9] The treatment of choice is performed with the antifungal itraconazole[10–12]; however, the appearance of resistant strains of *Sporothrix* sp. has been observed because of the indiscriminate use of several antifungals in therapies.[2,13,14]

This problem has stimulated the search for new effective chemical compounds, such as those in medicinal plants, but only approximately 30% of antimicrobial medications in the market are derived from natural products and the resources for elaborate drugs from plants are poorly explored.[15] In folk medicine, marjoram (*Origanum majorana* Linn., synonymous of *Majorana hortensis* Linn.) is used to treat asthma, indigestion, cramps, headache, dizziness, depression, and rheumatism, and it has diuretic activity.[16,17] This plant has a strong antioxidant activity attributed to its high content of flavonoids and phenolic acids, which are used for food preservation.[18] Essential oil of *O. majorana* L. has shown antimicrobial properties, such as antiviral, antibacterial and antifungal,[18–22] among others.

The pathogenic fungi of human and veterinary medical interest that were sensitive to the essential oil of *O. majorana* L. are *Candida* sp., *Aspergillus* sp., *Trichophyton* sp., *Microsporum* sp., *Malassezia* sp. and *Trichosporon* sp.[21,23,24] These results *in vitro* show its promising activity in fungal diseases, including those with zoonotic potential. In sporotrichosis, no studies with *O. majorana* L. have been done, which encouraged the development of this work. This study aimed to evaluate the *in vitro* efficacy of *O. majorana* L. essential oil against clinical isolates of *S. schenckii* complex and to analyze its chemical constituents and cytotoxic activity in mammalian cells.

Materials and methods

Plant material

The essential oil of *O. majorana* Linn., originating from Egypt, was obtained from *Ferquima® Indústria e Comércio Ltda.* (Vargem Grande Paulista, São Paulo, SP, Brazil), for which quality parameters are described in an accompanying technical report (appearance, color, purity, odor, density – 20 °C, refraction index – 20 °C).

Chemical analysis

Chemical analysis was performed using high-resolution gas chromatography with a flame ionization detector (CG-FID) by HP 7820A (Agilent®) equipped with an HP-5 column (30 m × 0.32 mm × 0.25 mm) at an initial temperature of 70 °C with the addition of 3 °C/min up to 240 °C. The injector temperature was 250 °C, and that of the FID detector was 260 °C. The speed of the drag of hydrogen gas was 3 mL/min, and that of the split was 1:30. The solution of essential oil was diluted with 1% chloroform and injected into the chromatograph at a volume of 1 μL. Data were acquired through the EZChrom Elite Compact® program (Agilent).

Cytotoxicity assay

The cytotoxic effects of *O. majorana* L. were estimated using the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay that measures the reduction of the MTT dye to an insoluble and colored formazan. The technique was executed as previously described,[25] and tested in mammalian cells of VERO type, which were grown in RPMI-1640 (Roswell Park Memorial Institute medium, Sigma, Steinheim, Germany) supplemented with L-glutamine, without sodium bicarbonate (pH 7.2) and added of penicillin–streptomycin and fungizone in a humidified atmosphere of 5% CO_2 at 37 °C. Trypsin was added on to the cell monolayer and the cells were resuspended in RPMI-1640, resulting in a suspension with approximately 2×10^5 cells/mL. Aliquots of this cells suspension were placed in individual wells in 96-well microplate except first well as blank. Cells were incubated in RPMI-1640 medium supplemented with 10% fetal calf serum at CO_2 5%, 37 °C by 24 h.

Subsequently, 100 μL of the oil in seven successive concentrations on a logarithmic scale was added in the microplates. Concentrations of 5–0.078 mg/mL of the essential oil diluted in RPMI-1640 were tested in triplicate for 48 h at 37 °C in a humidified atmosphere of 5% CO_2 and for control RPMI-1640 only was used. Then, 50 μL MTT solution (2.5 mg/mL) was added in each well and incubated for 2 h and 30 min at 37 °C and 5% CO_2. After removal of MTT solution, 50 μL of dimethylsulfoxide (DMSO) was added to each well to dissolve the formazan crystals, and gentle shaking for 5 min. The spectrophotometric absorbance of the samples was measured using a microplate reader at wavelength of 540 nm. The appearance of cells was monitored by inverted microscope and the results were expressed as an inhibition percentage relative to the control cells, which was considered 100%.

Fungal isolates

For the antifungal susceptibility test, seventeen clinical isolates of *S. brasiliensis* derived from humans (*n*: 3), dogs (*n*: 6), and cats (*n*: 8) with sporotrichosis were used along with a standard strain of *S. schenckii* (IOC 1226) from human case (*n*: 1), totaling eighteen tested isolates. The fungal isolates were stored in mycology collection of the *Centro de Diagnóstico e Pesquisa em Micologia Veterinária* (Universidade Federal de Pelotas – UFPEL – Pelotas, RS, Brazil) and cooled in an average temperature of 4 °C. The mycology identification of

Table 1 – Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of the *Origanum majorana* Linn. essential oil and itraconazole against *Sporothrix brasiliensis* and *Sporothrix schenckii*.

Origin of the isolates of *Sporothrix schenckii* complex	*Origanum majorana* L. (mg/mL)						Itraconazole (µg/mL)					
	MIC			MFC			MIC			MFC		
	Range	50%	90%	Range	50%	90%	Range	50%	90%	Range	50%	90%
Sporothrix brasiliensis												
Humans (*n*: 3)	≤2.25–4.5	–	–	≤2.25–4.5	–	–	≤0.03–0.5	–	–	≤0.03 to 2	–	–
Dogs (*n*: 6)	≤2.25–4.5	≤2.25	≤2.25	≤2.25–18	4.5	9	0.5–8	1	2	1 to >16	2	16
Cats (*n*: 8)	≤2.25–9	≤2.25	4.5	≤2.25–9	4.5	9	≤0.03–16	2	16	0.25 to >16	2	>16
Overall (*n*: 17)	≤2.25–9	≤2.25	4.5	≤2.25–18	4.5	9	≤0.03–16	2	8	≤0.03 to >16	2	>16
Sporothrix schenckii												
IOC 1226 (*n*: 1)	≤2.25	–	–	≤2.25	–	–	1	–	–	8	–	–
Overall (*n*: 18)	≤2.25–9	≤2.25	4.5	≤2.25–18	4.5	9	≤0.03–16	1	16	≤0.03 to >16	2	>16

50%, MIC/MFC at which 50% of isolates were inhibited/eliminated; 90%, MIC/MFC at which 90% of isolates were inhibited/eliminated.

S. brasiliensis was analyzed using the polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP), proposed by Rodrigues et al.[26]

Antifungal activity

The antifungal susceptibility tests were performed using the broth microdilution technique, according to the M27-A3 guidelines of the Clinical and Laboratory Standards Institute,[27] adapted for the use of essential oil. Subcultures in Brain-Heart-Infusion agar (BHI, Acumedia, Lansing, MI, USA) at 35 °C for 48 h were performed. A portion of each fungal colony was transferred to individual tubes containing sterile saline solution and adjusted according to the scale of 1.0 McFarland and in the ultraviolet (UV)–visible spectrophotometer (Spectrum Instruments Co., Shanghai, China) in transmittance at 530 nm and at an absorbance of 80–82%. The suspensions were adjusted in saline solution (1:100) and, then, in RPMI-1640 with 3-morpholinopropane-1-sulfonic acid – MOPS – (1:20) in order to obtain the final inoculum concentration of 5×10^4 CFU/mL.

In a microplate with ninety-six wells containing 100 µL of RPMI-1640 with MOPS in each well, 100 µL of essential oil of *O. majorana* L. containing 1% Tween 80 was added to the column corresponding to a higher concentration of the product, and serial dilutions were performed. The oil plant was tested in the concentrations of 72–2.25 mg/mL. Then, 100 µL of the inoculum was added to all wells except in the negative control, where 100 µL of the marjoram essential oil was added. Itraconazole was used as the positive control and was prepared in dimethyl sulfoxide and diluted in RPMI-1640 medium with MOPS in order to obtain the final concentrations of 16–0.0313 µg/mL. The microplates were incubated on a rotatory shaker (Certomat® BS-1, B. Sartorius, Göttingen, Germany) at 35 °C for 72 h, and the minimal inhibitory concentration (MIC) was visually compared with that of the negative control and was defined as the lowest concentration of the tested product inhibiting the visible growth of the fungus (100% inhibition).

For the minimal fungicidal concentration (MFC), 10 µL of aliquots of the wells with no fungal growth was transferred to Petri dishes containing Sabouraud dextrose agar (Acumedia, Lansing, MI, USA) and incubated at 35 °C for 72 h to visualize fungal growth. MFC was determined to be the lowest concentration able to eliminate fungal growth. All experiments were performed in duplicate.

Statistical analysis

The analysis of variance and comparison of geometric means were performed according to Tukey test using the statistical software BioEstat®, 5.3 version, and value $p < 0.05$ was considered significant.

Results

Antifungal activity

According to the results of the anti-*Sporothrix* sp. activity of the essential oil of *O. majorana* L. and itraconazole (Table 1), no significant difference existed between the results in the MIC values; however, the MFC values differed statistically ($p = 0.05$) between itraconazole and essential oil, in which *O. majorana* L. presented better results. All tested isolates were susceptible to the essential oil of marjoram with fungistatic activity being observed at concentrations of ≤2.25–9 mg/mL, while fungicidal activity occurred between ≤2.25 and 18 mg/mL. The concentrations of the oil up to 9 mg/mL showed antifungal activity against all *S. brasiliensis*, the MIC_{50} and MIC_{90} being observed at the concentrations ≤2.25 mg/mL and 4.5 mg/mL, respectively. At the minimal concentration tested of the oil, 66.7% (2/3) of the humans isolates, 83.3% (5/6) of the isolates from dogs and 50% (4/8) from cats were sensitive (MIC ≤2.25 mg/mL), as well as the standard strain of *S. schenckii*. In the fungicidal activity, the MFC_{50} and MFC_{90} values of overall *S. brasiliensis* were 4.5 mg/mL and 9 mg/mL, respectively, and were twice the $MIC_{50/90}$ values, whereas the standard strain was more sensitive (MFC ≤2.25 mg/mL). In relation to the itraconazole, all isolates were sensitive to the fungistatic activity between the MIC values ≤0.03 and 16 µg/mL, which MIC_{50} was 1 µg/mL and MIC_{90} was 16 µg/mL. However, the fungicidal

Table 2 – Chemical constituents identified in the essential oil of *Origanum majorana* L. and respective concentrations through high resolution gas chromatography in a flame ionization detector (CG-FID).

Constituents	Retention rate	Area	Concentration (%)
α-Thujene	932	281,814	1.1
α-Pinene	932	494,138	2.0
Camphene	941	96,074	0.4
Sabinene	946	1,669,329	6.7
β-Pinene	975	470,225	1.9
Myrcene	984	408,894	1.6
α-Phellandrene	1003	226,808	0.9
α-Terpinene	1015	1,152,102	4.6
p-Cymene	1019	1,755,163	7.0
Limonene	1023	1,316,921	5.3
1,8-Cineole	1027	5,222,929	20.9
β-Ocimene	1056	34,661	0.1
γ-Terpinene	1089	2,130,943	8.5
Trans sabinene hydrate	1094	564,967	2.3
Linalool	1099	1,096,294	4.4
Camphor	1142	48,346	0.2
Terpinen-4-ol	1175	5,100,062	20.4
α-Terpineol	1188	1,162,894	4.7
Linalyl acetate	1261	438,735	1.8
Bornila acetate	1285	212,333	0.8
β-Caryophyllene	1421	546,553	2.2
Humulene	1453	41,993	0.2
Others		517,426	2.1

activity was observed between ≤ 0.03 and $>16\,\mu g/mL$, where MFC_{50} was $2\,\mu g/mL$, but the MFC_{90} was $>16\,\mu g/mL$, indicating that 17.6% (3/17) of the *S. brasiliensis* were resistant to the maximal concentration tested of this antifungal.

Chemical composition

Chemical analysis found twenty-two compounds, and 1,8-cineole was the majority component, followed by 4-terpineol, γ-terpinene, p-cymene, sabinene, and others (Table 2).

Cytotoxic effects

In the observed values of MIC_{50} and MIC_{90} for total isolates, the cytotoxicity of *O. majorana* L. occurred between 77.5% and 78.91%. The toxicity activity in mammalian cells was directly proportional to tested concentrations and decreased to 73.45% at a concentration of 0.078 mg/mL (Fig. 1).

Discussion

Antifungal activity

The drug of choice in the treatment of sporotrichosis was effective against several *S. brasiliensis*, being similar to the findings of Marimon et al.,[2] that showed *in vitro* activity between 0.5 and $2\,\mu g/mL$ against twenty-three isolates of *S. brasiliensis*, with MIC_{50} and MIC_{90} of $0.5\,\mu g/mL$ and $1\,\mu g/mL$, respectively. In our work, the MIC_{50} and MIC_{90} found were $2\,\mu g/mL$ and $8\,\mu g/mL$, demonstrating that the fungal isolates of *S. brasiliensis* originating from humans and animals in the south of Brazil were twice more resistant.
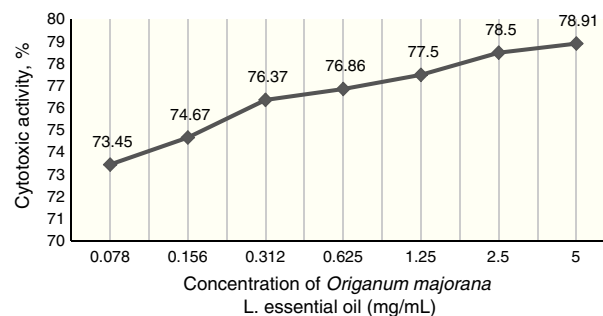

Fig. 1 – Cytotoxic percentage (%) of the essential oil of *Origanum majorana* L. in VERO cells tested through MTT assay in the concentration of 0.078–5 mg/mL.

In isolates from humans, no resistance was observed, and according Kauffman et al.[28] and Yamada et al.,[29] the resistance was lower compared to that of animals, mainly cats, due to greater care during human therapy in relation to their own health. In relation to feline sporotrichosis, it is known to have a high incidence in the Southeast and Southern regions of Brazil.[8,11,30,31] MIC_{50} of itraconazole against *S. brasiliensis* isolated from cats were $2\,\mu g/mL$, but the MIC_{90} was $16\,\mu g/mL$, demonstrating its lower susceptibility to antifungal. In the overall *S. brasiliensis* tested, one clinical isolate from a dog and two isolates from cats were resistant to the fungicidal activity (MFC $>16\,\mu g/mL$).

This observation may be a reflection of previous factors that favor the emergence of antifungal resistance. It is noticed that the antifungal administered in animals may not always be the same used in humans and its efficacy may be affected due to pharmacokinetics parameters and kind of formulation, among others,[32] and a difference in the pharmaceutical technology between human and veterinary drugs may occur,[33] which may influence the therapeutic antifungal response. Besides, the irregularities in veterinary therapeutic management by animal owners are common due to the difficulties in the oral administration and high cost of antifungals and this often culminates in the abandonment of therapy, particularly when there is improvement of skin lesions.[34]

The resistance of *Sporothrix* spp. to itraconazole has been evidenced by Rodrigues et al.,[14] and this problem reflects in an alert because sporotrichosis is important in the public health. Interestingly, the same resistant isolates were susceptible to essential oil in concentrations equal to or less than 9 mg/mL, thus suggesting that this plant can be used for further studies in the treatment of sporotrichosis. In accordance with our study, Souza et al.[23] showed the promising activity of the commercial essential oil of *O. majorana* L. at $160\,\mu L/mL$ against pathogenic fungi, including in strains of *Candida albicans*, *C. tropicalis*, *Cryptococcus neoformans*, *T. mentagrophytes*, *M. gypseum* and *A. flavus*, which were resistant to conventional antifungal, as amphotericin B, 5-fluorocytosine and fluconazole. This observation reflects in the promising use of this essential oil in antifungal therapies.

No valid criteria exist for the minimal antifungal concentrations *in vitro* tests with plant extracts,[35] but *O. majorana* L. oil showed satisfactory activity with MIC_{50} and MIC_{90} of $\leq 2.25\,mg/mL$ and $4.5\,mg/mL$, respectively, including in

resistant isolates to itraconazole (*n*: 3), that were sensitive to the plant at MIC of ≤2.25 mg/mL for one isolate from dog and one from cat, and at MIC of 9 mg/mL for one isolate from cat. No studies were found on the activity of *O. majorana* L. in *S. schenckii* complex, and for the first time, the *in vitro* sensibility of *S. brasiliensis* to this plant was studied.

Similar to our study, the resistance of *S. schenckii* and *S. brasiliensis* to itraconazole also was reported as sensitive to plant extracts from the *Pterocaulon* genus[35] and *Camellia sinensis* L.,[36] respectively, indicating the promising use of the several plants in the sporotrichosis. Furthermore, resistance of bacteria and yeasts to antimicrobial drugs was sensitive to the essential oils of *Rosmarinus officinalis* L.[37] and *Origanum vulgare* L.[38,39] showing that, as found in this study with *O. majorana* L., other medicinal plants also are potential candidates as new antimicrobial agents. Given this fact, it is necessary to explore the studies for their promising use in the treatment of sporotrichosis.

Chemical composition

The chemical compounds were similar to those scientifically described.[18,21,24,40] However, our data differed from the findings by Marino et al.,[41] in which thymol and carvacrol were prevalent. The presence of phenolic compounds in plants is highly correlated to antimicrobial properties,[40,42] but the identified compounds were predominated by terpenoids, which were related to antimicrobial activity when tested alone.[43] The plant causes an increase in the permeability of the cytoplasmatic fungal membrane, destroying the physical structure.[44] However, Souza et al.[23] suggested that the essential oil of *O. majorana* L. acts as an inhibitor of the microorganism's cell wall, although other mechanisms for expressing their anti-*Sporothrix* sp. activity may be involved.

Cytotoxic effects

O. majorana L. oil presented a cytotoxicity between 73.45% and 78.91% in the tested concentrations, which were high, but the gradual reduction of the cytotoxicity to the lower tested concentration should be noted. In overall isolates, 66.67% (12/18) of tested *Sporothrix* spp. were sensitive to the MIC ≤2.25 mg/mL, indicating that the lower concentrations may present inhibitory activity with lower cytotoxicity. Although the maximal non-toxic concentration of *O. majorana* L. essential oil in VERO cells was 3.2 μg/mL,[22] this value was four times lower than the minimal tested concentration. However, it is noticed that the use of the *O. majorana* L. oil against sporotrichosis need to be studied at its effective antifungal concentrations and, also, our study was performed *in vitro*.

According to Nogueira and Andrade,[45] the mammalian cells in the MTT test are more susceptible in comparison to the *in vivo* test due to the direct exposure of the product, whereas to the *in vivo* the product is influenced by the route of administration under consideration, that can suffer internal actions by oral administration, as well as influence by topical absorption, and may present lower toxic potential. The dose and frequency of administration can influence the degree of toxicity of medicinal plants,[46] and low doses can cause an allergic reaction in sensitive patients as well as skin irritations.[47,48] Studies on the toxic effects of this plant *in vivo* should be conducted in order to evaluate the influence of its dose and the route of administration in patients with sporotrichosis.

Conclusion

The *in vitro* activity of *O. majorana* L. essential oil against *S. brasiliensis* and *S. schenckii*, including in resistant isolates to itraconazole, encourages greater studies on antifungal potential of this promising plant, that had 1,8-cineole as majority compound among chemical constituents identified. However, the cytotoxic activity of *O. majorana* L. oil was observed between 70% and 80% of mammalian cells in all concentrations tested. Further studies are needed to ensure its safe and effective use.

Conflict of interest

The authors declare having no conflict of interest in this study.

Acknowledgments

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