SUPPLEMENTARY MATERIAL

In vivo protection of the marjoram (*Origanum majorana* Linn.) essential oil in the cutaneous sporotrichosis by *Sporothrix brasiliensis*

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Abstract

Thirty Wistar rats subcutaneously infected by an itraconazole-resistant *Sporothrix brasiliensis* received the oral daily treatment (*n*=10, each) of control (CTL, saline solution), itraconazole (ITZ, 10 mg/kg) and marjoram essential oil (MRJ, 80 mg/kg) for 30 days. Weekly, the clinical evaluation and euthanasia for histopathology and fungal burden were performed. Only animals from MRJ evolved to the remission of the cutaneous lesion with a mild to absent presence of yeasts in footpad, besides decreased the fungal burden in the systemic organs compared to CTL and ITZ ($p<0.05$), preventing the fungal spread, mainly in the liver and spleen. The antifungal activity may have been attributed to the majority composition of terpinen-4-ol (34.09%), γ-terpinene (14.28%) and α-terpinene (9.6%), which the mode of action was at the level of ergosterol complexation. These findings highlighted the antifungal and the systemic protective effects of MRJ, supporting the promising use in the treatment of cutaneous sporotrichosis.

Keywords: *Sporothrix brasiliensis*; sporotrichosis; itraconazole; antifungal resistance; natural product; *Origanum majorana* Linn.

Experimental Section

Essential oil

The essential oil of *Origanum majorana* L. was obtained by the extraction of the dried aerial parts (*Luar Sul Indústria e Comércio de Produção*, Santa Cruz do Sul/RS, Brazil) via distillation by steam dragging in Clevenger equipment for four hours (Brazil, 2011). The oil was dry over in Na_2SO_4 (anhydrous sodium sulfate, p.a.), concentrated in N_2 (nitrogen ultrapure, 99.99% w/v, White Martins) and stored in an amber vial under refrigeration.

Chromatographic analysis

For analysis, it was performed in high-resolution gas chromatography with mass spectrometry (GC-MS) in a Shimadzu QP® 2010 model equipped with split/splitless injector with a Rtx-5MS RESTEK (30 m × 0.25 mm × 0.25 µm) capillary column. The chromatographic conditions were helium carrier gas, fragments obtained by electron impact at energy of 70 eV, flow rate of 1.2 ml/min, 1:10 split, injected volume of 1 µl sample. Programmed oven temperature: the initial temperature was 40 °C, with a heating ramp at 5 °C/min to 280 °C, being stable at that temperature for 10 min, with a total time of 58 min, the injector temperature being 58 °C and interface 200Â °C, the compounds were analyzed based on the NIST08 GC/MS library. The oil was diluted in hexane (analytical grade, ultrapure).

Sorbitol protection and ergosterol effect

The sorbitol protection in the plasmatic membrane and the ergosterol effect on the osmolarity of the wall cell were performed to understand the mechanism of action of the essential oil. The minimal inhibitory concentration (MIC) was evaluated against four *S. brasiliensis* isolates in the absence and presence of sorbitol (0.8 mol/L) and ergosterol at the concentrations of 50 to 200 µg/ml (Dalla-Lana et al., 2018). Anidulafungin (Ecalta®, Pfizer, Kalamazoo, Michigan, USA) was used as positive control for sorbitol assay, whereas amphotericin B (Cristália®, São Paulo, Brazil) was for ergosterol assay. For both assays, the tests were performed in triplicate, and the MIC values were measured along three and seven days at 30 ºC of incubation.

Sporothrix brasiliensis and inoculum preparation

A *Sporothrix brasiliensis* strain (MV1710/S120) isolated from a cat with sporotrichosis in the city of Pelotas/RS (Southern Brazil) was used for the experimental design due to the high MIC value to itraconazole (>16 µg/ml) by the broth microdilution assay using M38-A2 guideline (CLSI, 2008) and performed by us (Waller et al., 2017a). This feline isolate was

previously identified as *S. brasiliensis* based on the by PCR-restriction fragment length polymorphism analysis (Rodrigues et al., 2014) and stored in potato dextrose agar (PDA) in the mycology collection of the *Centro de Diagnóstico e Pesquisa em Micologia Veterinária* of the Veterinary Preventive Department of the Federal University of Pelotas (UFPEL). For inoculum preparation, *S. brasiliensis* was subcultured in PDA and incubated at 27ºC for seven days. Sterile saline solution with a drop of Tween 20 was added to the colonies, which were gently scraped with a scalpel in order to collect the conidia and transferred to a sterile tube. The fungal content was filtered in double-layer sterile gauze, centrifugated (1500 rpm) in 15 min, twice washed in phosphate-buffered saline (PBS), homogenized and standardized in 2×10^5 cells/ml, using sterile saline solution.

Animals and fungal infection

Seven-weeks-old male Wistar rats (*Rattus norvergicus*) were purchased from the Central Vivarium (UFPEL, Brazil) and housed in controlled conditions of humidity, temperature and 12h/12h light-dark cycle, fed with commercial diet and water ad libitum during all the experiment. The procedures used were approved by the *Comissão de Ética em Experimentação Animal* (CEEA, Federal University of Pelotas, no. 23110.001622/2012-55). The animals were sedated and anesthetized (xylazine at 1–5 mg/kg; ketamine at 50–100 mg/kg), intramuscularly (Neves et al., 2013), and, subsequently, were infected with the *S. brasiliensis* inoculum in the left hind footpad (0.2 ml, subcutaneous injection).

Experimental design

Ten days after the infection, 30 animals were allocated in three groups and the oral daily treatment (1 ml) was started for 30 days, which received saline solution with 1% of propylene glycol; itraconazole (Sporanox®, Janssen-Cilag Pharmaceutica, Belgium) at 10 mg/kg; and *Origanum majorana* L. (marjoram) essential oil at 80 mg/kg in saline solution with propylene glycol (1% w/v). For the treatment follow-up, the evolution of the disease was weekly evaluated, and two to three animals were euthanized by intraperitoneal administration of sodium thiopental (150 mg/kg), after sedation and anesthetization with xylazine and ketamine. All procedures followed the good practices for euthanasia in animals, according to the resolution nº 1000 of the Federal Council of Veterinary Medicine (CFMV, 2013).

Experimental design

For the histopathology, the inoculated footpad and the organs liver, spleen, kidney, lymph node and testes were fixed in 10% formaldehyde, included in paraffin. Histological sections were stained with Hematoxylin-Eosin (HE) and Periodic acid-Schiff (PAS). The HE

stained sections were used to classify the tissue lesions and cellular infiltrates and the stained with PAS was to enable better visualization of the fungal structures.

Fungal burden

To estimate the fungal burden, the organs were weighed and submitted to the mechanical homogenization in saline solution, which 100 µl of the homogenate sample was placed onto Mycosel® (Kasvi, Liofilchem®, Italy) and incubated at 25 ºC for 10 days. The colonies were counted to determine the colony-forming units per gram of tissue.

Statistical analysis

For statistical analysis, the analysis of variance of the non-parametric data was performed using the Kruskal-Wallis test, followed by the Dunn's multiple comparison test in the BioEstat® software, version 5.3, which p values <0.05 were considered significant.

List of Tables and Figures (Supplementary Material)

Table S1. Minimal inhibitory concentration (MIC) of the *Origanum majorana* L. (marjoram) essential oil and anidulafungin in the absence and presence of the osmotic protector sorbitol against *Sporothrix brasiliensis* from feline cases.

Table S2. Minimal inhibitory concentration (MIC) of the *Origanum majorana* L. (marjoram) essential oil and amphotericin B in the absence (E–) and presence (50–200 µg/ml) of the exogenous ergosterol against *Sporothrix brasiliensis* from feline cases.

Figure S1. Chromatogram of the *Origanum majorana* L. (marjoram) essential oil and their respective molecular masses (m/z), retention times (min) and area (%): unknown (**1** – 5.92 min; 0.37%); α-thujeno (**2** – m/z 136.238; 6.36 min; 1.68%); α-pinene (**3** – m/z 136.238; 6.54 min; 0.74%); α-phellandrene (**4** – m/z 136.238; 7.59 min; 5.5%); β-pinene (**5** – m/z 136.238; 8.05 min; 1.57%); α-terpinene (**6** –m/z 136.238; 8.78 min; 9.6%); o-cymene (**7** – m/z 134.222; 8.98 min; 1.74%); β-phellandrene (**8** – m/z 136.238; 9.11 min; 3.03%); γ-terpinene (**9** – m/z 136.238; 9.99 min; 14.28%); trans-sabinene hydrate (**10** – m/z 154.253; 10.10 min; 1.66%); δ-2-carene (**11** – m/z 272.476; 10.82 min; 3.27%); cis-sabinene hydrate (**12** – m/z 138.254; 11.11 min; 6.81%); unknown (**13** – 11.77 min; 2.77%); unknown (**14** – 12.29 min; 1.99%); terpinen-4-ol (**15** – m/z 154.253; 13.49 min; 34.09%); α-terpineol (**16** – m/z 154.253; 13.81 min; 5.86%); unknown (**17** – 14.26 min; 0.95%); unknown (**18** – 15.55 min; 0.57%); 4-terpinenyl acetate (**19** – m/z 196.29; 16.81 min; 0.51%); isocaryophyllene (**20** – m/z 204.357; 19.99 min; 1.38%); elixene (**21** – m/z 204.357; 21.92 min; 0.88%); spathulenol (**22** – m/z 220.356; 23.87 min; 0.48%); unknown (**23** – 24.01 min; 0.27%).

Figure S2. Effect of different concentrations of exogenous ergosterol (50–200 µg/ml) on the minimum inhibitory concentration (MIC) of amphotericin B (□) and *Origanum majorana* L. essential oil (●) against an itraconazole-resistant *Sporothrix brasiliensis* (S120) used in the experimental cutaneous sporotrichosis of this study.

Figure S3. Fungal burden in spleen, liver, lymph node, kidney and testicle (CFU/tissue) from rats subcutaneously infected by an itraconazole-resistant *Sporothrix brasiliensis* and treated at the following experimental groups: saline solution as control (CTL), itraconazole (ITZ) and *Origanum majorana* L. (marjoram) essential oil (MRJ). Data were analyzed at the days 7, 14, 21 and 30 of treatment; median values were compared by Kruskal-Wallis test, followed by Dunn's test ($p<0.05$).

Table S1. Minimal inhibitory concentration (MIC) of the *Origanum majorana* L. (marjoram) essential oil and anidulafungin in the absence and presence of the osmotic protector sorbitol against *Sporothrix brasiliensis* from feline cases.

Sporothrix brasiliensis*	**MIC *Origanum majorana* L. essential oil (mg/ml)**				**MIC Anidulafungin (µg/ml)**			
	3 days		**7 days**		**3 days**		**7 days**	
	S (–)	**S (+)**	**S (–)**	**S (+)**	**S (–)**	**S (+)**	**S (–)**	**S (+)**
Itraconazole-sensitive								
S68	0.28	0.28	0.28	0.28	32	256	32	512
S103	0.07	0.07	0.07	0.07	32	256	32	512
Itraconazole-resistant								
S72	0.07	0.07	0.07	0.07	32	256	32	512
S120**	0.07	0.07	0.07	0.07	32	256	32	512

S (–), absence of sorbitol; S (+), presence of sorbitol; * All *S. brasiliensis* came from feline cases in the city of Pelotas/RS (Southern Brazil); **S120 was used for the experimental infection in the murine model.

Table S2. Minimal inhibitory concentration (MIC) of the *Origanum majorana* L. (marjoram) essential oil and amphotericin B in the absence (E–) and presence (50–200 µg/ml) of the exogenous ergosterol against *Sporothrix brasiliensis* from feline cases.

Sporothrix brasiliensis*	**MIC *Origanum majorana* L. essential oil (mg/ml)**										**MIC Amphotericin B (µg/ml)**									
	3 days					**7 days**					**3 days**					**7 days**				
	E–	**50**	**100**	**150**	**200**	**E–**	**50**	**100**	**150**	**200**	**E–**	**50**	**100**	**150**	**200**	**E–**	**50**	**100**	**150**	**200**
Itraconazole-sensitive																				
S68	0.28	0.28	0.28	0.28	0.28	0.28	0.28	1.12	2.24	>35.84	1	16	>256	>256	>256	1	>256	>256	>256	>256
S103	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.56	0.56	>8.96	1	16	>256	>256	>256	1	>256	>256	>256	>256
Itraconazole-resistant																				
S72	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.56	0.56	>8.96	1	16	>256	>256	>256	1	>256	>256	>256	>256
S120**	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.56	0.56	>8.96	1	16	>256	>256	>256	1	>256	>256	>256	>256

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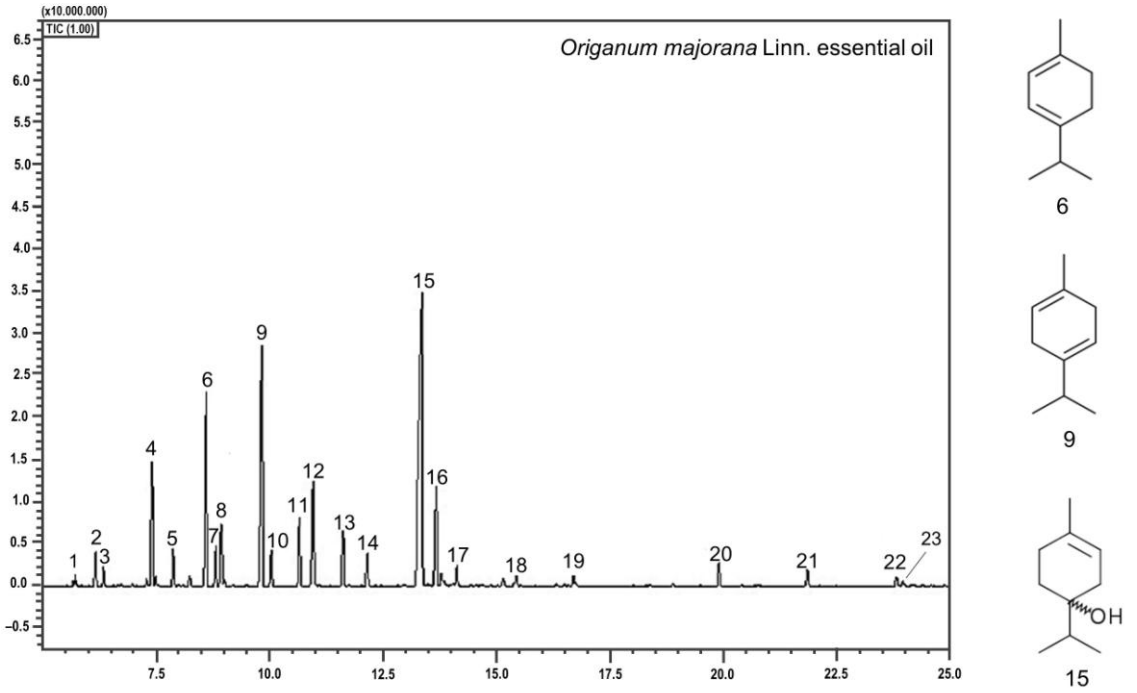

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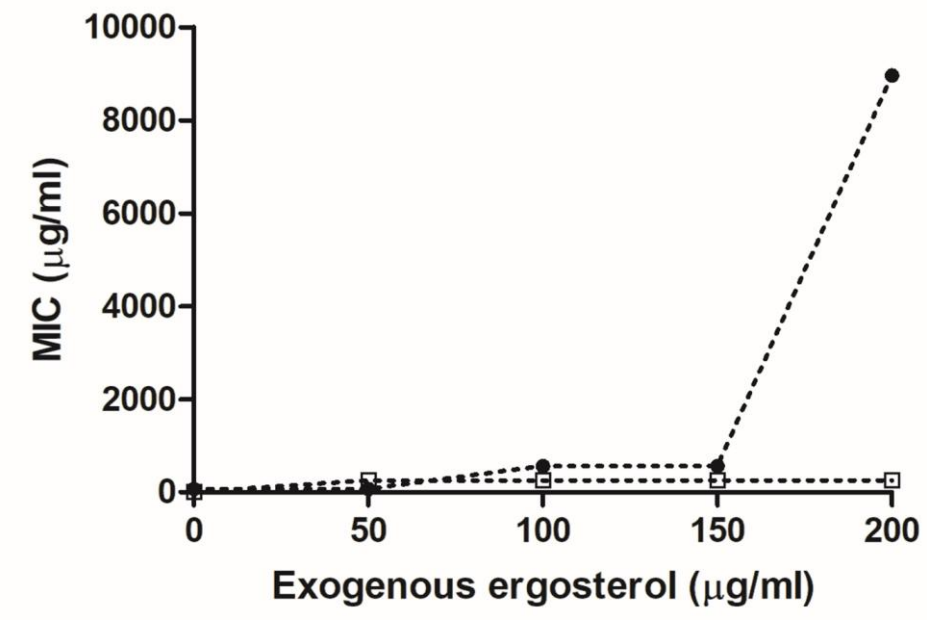

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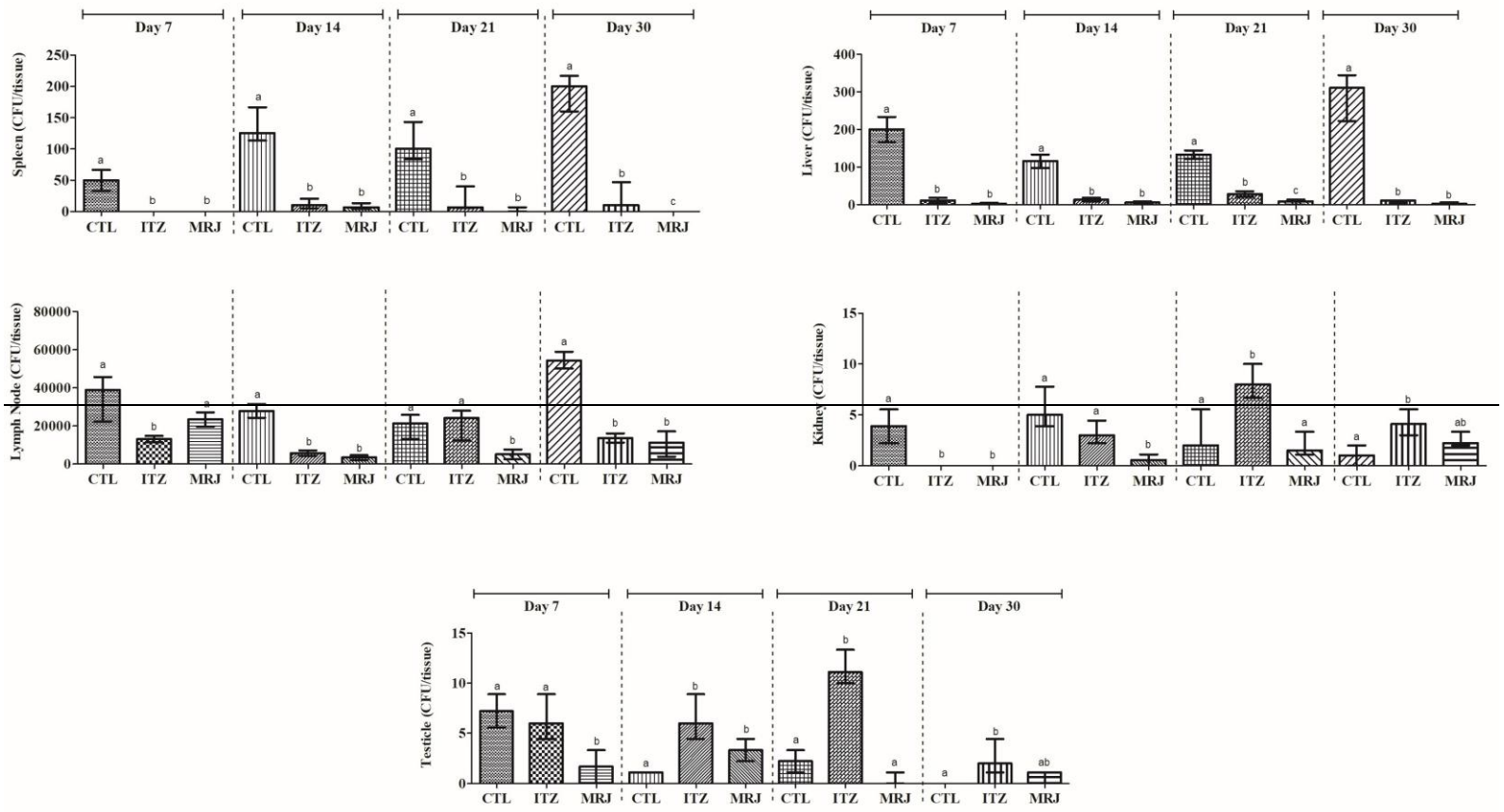

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