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Original Research Article

Chemical Composition and Antifungal Properties of Essential Oil of *Origanum vulgare* Linnaeus (Lamiaceae) against *Sporothrix schenckii* and *Sporothrix brasiliensis*

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Abstract

Purpose: *To evaluate the effect of the essential oil of Origanum vulgare Linnaeus (Lamiaceae) on the growth of Sporothrix schenckii and Sporothrix brasiliensis.*
Methods: *The chemical composition of the essential oil was investigated by gas chromatography/flame ionization detector (GC-FID). The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) were determined by broth micro-dilution method. Scanning electron microscopy (SEM) was also performed to reveal morphological alterations in Sporothrix spp. cells.*
Results: *The major components of the essential oil were γ-terpinene (30.5 %), carvacrol (15.7 %) and 4-terpineol (13.0 %). γ-Terpinene showed potential antifungal activity with MIC ranging from 62.5 to 500.0 µg mL^{-1} for S. schenckii, and 125.0 to 250.0 µg mL^{-1} for S. brasiliensis. SEM micrographs revealed morphological alterations in hyphae and reduction of the adhered conidia numbers.*
Conclusion: *Origanum vulgare Linnaeus essential oil possesses potential antifungal activity, and can, therefore, can be developed as an alternative agent for the treatment of sporotrichosis.*

Keywords: *Antifungal activity, Essential oil, Gas chromatography, Origanum vulgare, Sporotrichosis*

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INTRODUCTION

Sporotrichosis is a subcutaneous mycosis affecting humans and animals, with worldwide distribution, especially in tropical and subtropical areas, constituting an important public health problem [1]. This disease displays a chronic or sub-acute progression and usually affects the skin and lymph vessels near the site of the lesion. In rare cases, there may be secondary transmission to the bones, joints and muscles [2]. It is caused by fungus of the *Sporothrix schenckii* complex, being *Sporothrix sckenckii* and *Sporothrix brasiliensis* the species more frequently related in clinical samples of Brazil [3]. Despite extensive research dedicated to the development of new therapeutic strategies, there are only a limited number of available drugs against fungal infections [4]. Its clinical uses have been limited by the emergence of drug resistance, high risk of toxicity, insufficiencies in their antifungal activity and undesirable side effects [5]. Considering these factors, there is a

need for the discovery of new agents with antifungal potential.

Plants used in traditional medicine usually constitute an important source of new biologically active compounds because their diversity chemical composition [4]. In this context, studies about evaluation of antifungal activities of essential oils have been carried [6] and a number of reports on new antifungal agents from plants have been reported [7].

Origanum vulgare L, popularly known as oregano, is an aromatic herb used in Mediterranean food [8]. Previous studies have confirmed interesting antimicrobial activity of the essential oil from *O. vulgare* against spoilage and pathogenic food-related fungi [9].

The chemical composition of *O. vulgare* essential oil has been investigated [10-12], however few studies evaluate the action of this oil against *Sporothrix* genus. Thus, the aim of this work was to determine the chemical composition of *O. vulgare* essential oil, and evaluate the antifungal activity against *S. schenckii* and *S. brasiliensis*.

EXPERIMENTAL

Essential oil

The essential oil of *Origanum vulgare* L (lot 660411), obtained by hydrodistillation of plant material, was provided by Laszlo Aromatologia LTDA, Brazil.

Chromatographic analysis

The identification and quantification of the volatile compounds were performed on a gas chromatograph (GC), Hewlett Packard 5890 instrument, with flame ionization detector (FID). The chromatographic parameters were: BP-1 (HP) 30 m × 0.32 mm BP1 column; injection (1/50 split) of 1 µL; hydrogen as carrier gas (2 mL min^{-1}); temperature of both the detector and the injector at 220 °C; and a temperature gradient (initial = 60 °C; then an increase of 3 °C min^{-1} until 220 °C) for the column. The identification of the peaks was made by calculating the retention time and comparing these with linear hydrocarbon standards C10 to C18 and literature data [13]. Samples were diluted to 0.5 % (v/v) in chloroform.

Fungal strains

Two clinical strains of *Sporothrix schenckii* (A e B) from human sporotrichosis isolated in 2000 were provided by Departamento de Microbiologia e Imunologia do Instituto de Biociências de Botucatu da Universidade Estadual de São Paulo (UNESP), Brazil. *Sporothrix schenckii* (ATCC MYA 4821, 1099 - 18), *S. schenckii* (ATCC MYA 4820, IPEC 15383), *Sporothrix brasiliensis* (ATCC MYA 4823, 5110) and *S. brasiliensis* (ATCC MYA 4824, IPEC 17943) were provided by Laboratório de Micologia Celular e Proteômica do Instituto de Biologia Roberto Alcântara Gomes da Universidade Estadual do Rio de Janeiro (UERJ), Brazil.

Microbiological screening

Preliminary antifungal assays were performed. For this, fungal fragment (2 mm) was inoculated on potato dextrose agar previously incorporated with essential oil at concentration of 1000 µg mL-1 of the major constituent (γ-terpinene) determined from GC analysis. The inoculated plate was then incubated at 28 ± 2 ºC for 7 days. All analyzes were performed in triplicate.

Determination of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC)

MIC and MFC of essential oil were determined by broth micro dilution method according to the guidelines M38 - A2 of the Clinical and Laboratory Standards Institute [14]. The fungal inoculums were prepared from young colonies (7 - 10 days) from *Sporothrix* spp filamentous phase, which were resuspended in tubes containing sterile saline solution. The suspension formed was analyzed by spectrophotometer (Libra S12, Biochrom, England) using a quartz cuvette, being the transmittance adjusted to 80 - 82 % in fixed wavelength of 530 nm. The fungal suspension was diluted in RPMI 1640 medium buffered with [3-(N-morpholino propane sulphonic acid)] (MOPS) (1:50, v/v).

Serial dilutions of essential oil, in order to obtain concentrations from 7.8 to 1000 µg mL^{-1} of the γ-terpinene, were prepared using RPMI 1640 medium buffered, pH = 7.0, with MOPS. An aliquot of 100 µL of the fungal suspension and 100 µL of the diluted oil were added to 96-well microplates and incubated at 35 ºC for 72 h. Wells containing the RPMI 1640 medium buffered with MOPS, but without micro-organisms, were used as controls. The MIC was defined as the lowest concentration of drug resulting in total inhibition of visual growth compared to the grown in the control wells. Ketoconazole and amphotericin B were used as reference drugs.

The controls text for cell viability and sterility of the culture medium were performed. The first was performed with fungal inoculation in the same medium utilized for dilution of the essential oil, and the second was performed with the medium culture only, without micro-organisms.

To determine MFC, an aliquot of 10 µL from the wells that did not show growth in the MIC procedure were transferred to new 96-well plates, previously prepared with 200 µL of Sabouraud dextrose agar. Plates were incubated at 35 °C for 72 h. The MFC was defined as the lowest concentration that resulted in total inhibition of visible growth.

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was performed to reveal the effects of the essential oil on the fungal morphology. *S. schenckii* (1099-18) and *S. brasiliensis* (5110) treated with essential oil (sub-lethal concentration, ½ MIC) were fixed with 2.5 % glutaraldehyde, 4 % formaldehyde in 0.1 M cacodylate buffer, pH = 7.2, for 24 h at 4 ºC. Cells were adhered to poly-L-lysine glass coverslips. Post-fixation was carried out in 1 % osmium tetroxide in 0.1 M cacodylate buffer containing 1.25 % potassium ferrocyanide and 5 mM $CaCl_2$ for 30 min. Thereafter, the cells were washed with 0.1 M cacodylate buffer and dehydrated in an ethanol gradient (30 at 100 %) in 15 min intervals for each concentration. Then, samples were critical-point-dried in CO_2 (Bal-tec CPD030) and coated with gold (Balzers Union FL-9496). The prepared samples were observed under an SEM (Fei Quanta 250). Cells treated with amphotericin B and ketoconazole at sub-lethal concentration (16 µg mL^{-1}) also were visualized.

RESULTS

Chemical composition of the essential oil

Seventeen compounds were identified in *O. vulgare* essential oil, accounting for 91.6 % of the whole composition (Table 1). The essential oil was mainly composed of monoterpene hydrocarbons (43.2 %) and oxygenated monoterpenes (27.3 %). Within monoterpene hydrocarbons, γ-terpinene (30.5 %) was the major compound detected and within oxygenated monoterpenes, 4-terpineol (13.0 %) was the most abundant. Additionally, carvacrol (15.7 %) represented a substantial fraction.

Antifungal activity of the essential oil

The results of the microbiological screening revealed that the *O. vulgare* essential oil had inhibitory activity against the tested fungal species. The essential oil was assayed for antifungal properties with the broth micro dilution method following the guidelines of CLSI [14]. Results are shown in Table 2.

Table 1: Chemical composition of volatiles in *Origanum vulgare* essential oil

Compound	Content (%)	Kovat's index calculated*
Monoterpene hydrocarbons		
β-pinene	0.4	973
Myrcene	0.2	986
α-terpinene	0.8	1017
p-cymene	2.5	1024
trans-ocimene	1.3	1049
cis-ocimene	7.0	1056
γ-terpinene	30.5	1081
Oxygenated monoterpenes		
1,8-cineole	0.5	1031
cis-sabinene hydrate	2.8	1085
trans-sabinene hydrate	1.0	1101
4-terpineol	13.0	1158
α-terpineol	2.9	1170
Geraniol	7.1	1223
Sesquiterpene hydrocarbons		
β-caryophyllene	2.5	1297
germacrene-D	1.9	1471
Phenols		
Carvacrol	15.7	1241
Oxygenated sesquiterpenes		
Spathulenol	1.5	1545
Total	91.6	

**Relative to C_{10}-C_{18} n-alkanes on the BP1-column*

Table 2: Anti-microbial activity of *Origanum vulgare* essential oil and reference drugs

Fungal strain	*Origanum vulgare* essential oil	γ-terpinene		Amphotericin B		Ketoconazole	
	MIC	*MIC*	*MFC*	*MIC*	*MFC*	*MIC*	*MFC*
S. schenckii A	216.8	62.5	125.0	2.0	2.0	1.0	4.0
S. schenckii B	216.8	62.5	125.0	2.0	2.0	1.0	2.0
S. schenckii ATCC 1099-18	433.7	125.0	125.0	4.0	4.0	2.0	2.0
S. schenckii IPEC 15383	1735.0	500.0	500.0	4.0	4.0	2.0	4.0
S. brasiliensis ATCC 5110	433.7	125.0	250.0	2.0	2.0	2.0	8.0
S. brasiliensis IPEC 17943	867.5	250.0	500.0	2.0	2.0	4.0	4.0

MIC: minimum inhibitory concentration. MFC: minimum fungicidal concentration. All concentrations are expressed in μg mL^{-1}

SEM

The Figure 1 (A – H) shows the images obtained by SEM.

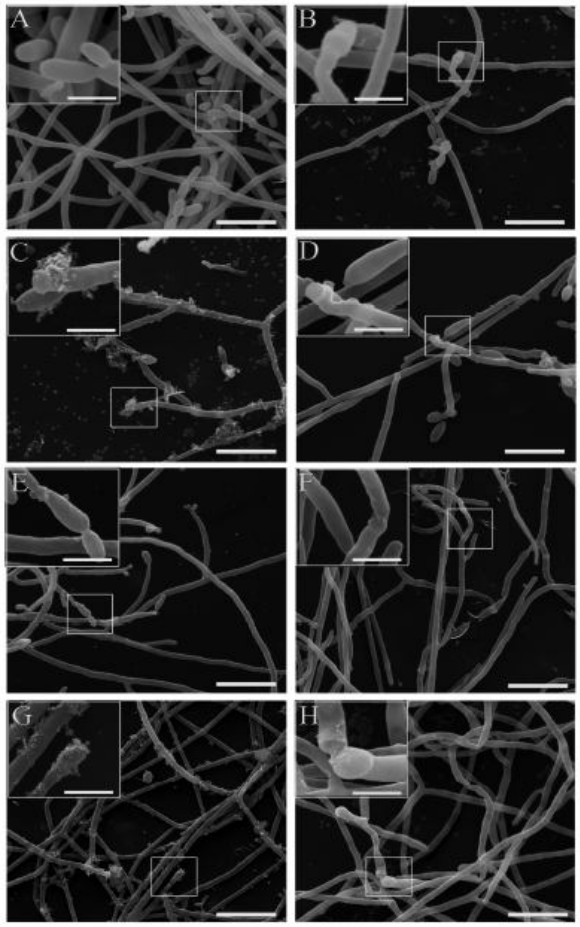

Figure 1: Scanning electron microscopy of filaments of the fungi *Sporothrix brasiliensis* 5110 untreated (A) and treated with the essential oil of *Origanum vulgare* (B), Amphotericin B (C) and Ketoconazole (D); and *Sporothrix schenckii* 1099-18 untreated (E) and treated with the essential oil of *Origanum vulgare* (F), Amphotericin B (G) and Ketoconazole (H). Bars: 10 μm and 2.5 μm (insets)

DISCUSSION

The composition of *O. vulgare* essential oil from different geographical origins has been characterized by several authors, with carvacrol and thymol as the major components [6,15,16]. Other components have also been reported as important essential oil components, such as p-cymene, γ-terpinene, caryophyllene, spathulenol and germacrene-D [12,13]. The differences in the chemical composition may be due to differences of environmental conditions, geographic origins, genetic variability, vegetative plant phases and extraction and quantification methods [11,15]. In addition, the proportion of thymol and γ-terpinene in the essential oil of *O. vulgare* can differ during the flowering and non-flowering stages of the plant. The increase of one of these constituents is accompanied by a decrease of the other and vice-versa [17], what can explain the absence of thymol in this current study.

Surprisingly, in this work, the low values of MIC and MFC were obtained for clinical strains, what can be explained by genetic and physiological differences when compared with standard strains. According to Santos *et al* [18], the antifungal activity of essential oils is considered good in the case of MIC < 100 μg mL^{-1}, moderated for MIC between 100 and 500 μg mL^{-1}, and weak in the case from 500 to 1000 μg mL^{-1}. The strains: *S. schenckii* A and B (MIC = 216.8 μg mL^{-1} for both), *S. schenckii* (1099 - 18, MIC = 433.7 μg mL^{-1}), and *S. brasiliensis* (5110, MIC = 433.7 μg mL^{-1}) were moderately inhibited. The results obtained for clinical strains were supported by Cleff *et al* [16], where GC analysis also showed high concentration of γ-terpinene, 4-terpineol, besides thymol, and the essential oil showed antifungal activity against seven clinical isolates of *S. schenckii* (all values obtained for MIC was 250 μg mL^{-1}). On the contrary, the action of the essential oil against *S. schenckii* (IPEC 15383) and *S. brasiliensis* (IPEC 17943) was classified as weak.

The standard strains of *S. schenckii* (ATCC 1099 - 18 and IPEC 15383) and *S. brasiliensis* (IPEC 17943) were less susceptible to amphotericin B and ketoconazole (MIC = 4 μg mL^{-1}) than others strains, that exhibited MIC equal to 1 or 2 μg mL^{-1} (Table 2). There are no MIC breakpoints establishing for *S. schenckii* complex species, however, for some filamentous fungi, MIC values ≤ 1 μg mL^{-1} may be considered indicative of susceptibility, MIC = 2 μg mL^{-1} is considered as intermediate susceptible and MIC ≥ 4 μg mL^{-1} is indicative of resistance [14].

According to MFC values, the γ-terpinene showed a fungicidal activity profile, with MFC values between 125 and 500 μg mL^{-1}, being that γ-terpinene have been shown to possess antifungal properties [19]. For antifungals, MFC is considered fungicidal when this value is equal to or less to four times the MIC value [20]. The concentration of carvacrol (15.7 %) also can to contribute for this fungicidal action, once previous reports have identified carvacrol and thymol as the main compounds associated with the antifungal activity of *O. vulgare* essential oil [6,16], though of thymol has not been detected. So, our results showed that the antifungal activity of *O. vulgare* essential oil can be associated with high concentration of γ-terpinene, besides carvacrol.

The images obtained by SEM indicate that the control showed the presence of hyphae with morphology elongated and rounded, besides budding cells. *S. brasiliensis* treated with *O. vulgare* essential oil showed hyphae with altered morphology, with the presence of thin hyphae exhibited breaking process and few conidia. When treatment was performed with ketoconazole and amphotericin B, morphological changes were less pronounced, but the presence of broken and roughness hyphae was observed. *S. schenckii* treated with essential oil showed flattened hyphae, revealing damage to fungal structure, besides of the presence of thinner cells. The treatment with amphotericin B revealed less rounded hyphae, thinner and with few conidia adhered. In the presence of ketoconazole, the cells showed up twisted and with kinks. In support of our results, Santos [21] observed changes in the length and width of *S. schenckii* and *S. brasiliensis* cells in the yeast form, when exposed to drugs azole and amphotericin B.

The SEM micrographs revealed that occurred reduction of the conidia numbers both treated fungi with essential oil as fungi treated with ketoconazole and amphotericin B. Furthermore, it was observed that essential oil caused morphological alterations in the fungal structures similar or greater intensity when compared to drugs amphotericin B and ketoconazole.

CONCLUSION

This study demonstrates that γ-terpinene is the major compound present in the *O. vulgare* essential oil analysed. Although thymol is not present in the essential oil, comparison of the antifungal activity and the chemical composition of the oil suggests that other compounds, such as γ-terpinene, in addition to carvacrol, may contribute to the oil's antifungal properties. However, the essential oil tested in the present study showed weak to moderate activity, and therefore its potential use in clinical practice is limited.

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