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Pimenta pseudocaryophyllus Gomes Landrum and Elionurus muticus (Spreng) Kunth and their antifungal properties against Sporothrix schenckii and Sporothrix brasiliensis

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***Abstract*— Aim: To evaluate the chemical composition and the effect of the hydroethanolic crude extract and the essential oil of *Pimenta pseudocaryophyllus* and the essential oil of *Elionurus muticus* (leaves for both) on the growth of *Sporothrix* spp. cells. Methods: The chemical composition of essential oils was determined by gas chromatography/mass spectrometry (GC–MS). Total polyphenol and tannins contents of crude extract were determined by the colourimetrics methods. The minimum inhibitory concentration (MIC) was determined by broth micro-dilution method. Scanning electron microscopy (SEM) was also performed to observe the morphological alterations in *Sporothrix* spp. cells. Results: The essential oil of Pimenta pseudocaryophyllus displayed high contents of eugenol (34.38%) and the essential oil Elionurus muticus was primarily composed of monoterpenes (≅90%). The main constituent was citral (72.35%). Total polyphenol, tannin and flavonoid contents of crude extract were 18.77% ± 0.57% (w/w) and 10.63% ± 0.29 (w/w) and 0.5293% ± 0.02 (w/w), respectively. The essential oil of Pimenta pseudocaryophyllus showed potential antifungal activity with MIC values ranging from 260 to 520.9 µg mL-1 for *S. schenckii*, and 260.0 µg mL-1 for S. brasiliensis. The hydroethanolic extract and the essential oil of Elionurus muticus did not show antifungal activity at the evaluated concentrations. SEM revealed morphological alterations in the hyphae and a reduction in the number of adhered conidia. Conclusion: The findings of the present study demonstrated that the essential oil of *P. pseudocaryophyllus* has a fungicidal activity against the primary microorganisms responsible for sporotrichosis in Brazil, probably due to its high content of eugenol*.

Keywords*— *Pimenta pseudocaryophyllus, Elionurus muticus, essential oil, antifungal activity, eugenol, sporotrichosis

I. INTRODUCTION

Plants have been used as a source of traditional medicines throughout the world since ancient times and usually constitute an important source of new biologically active compounds because of their diverse chemical compositions [1,2]. Studies on the evaluation of using the antifungal agents derived from plants have resulted in the increasing replacement of chemical products not only in the pharmaceutical field but also in food, cosmetic and hygiene industries [3,4,5,6]. These naturally occurring compounds are considered as a therapeutic alternative to the use of synthetic antibiotics, entailing a low risk of occurrence of antifungal resistance [5,7,8].

Sporotrichosis is one of the most frequent subcutaneous mycoses in the world [9]. The disease has a sub-acute or a chronic progression and is most often acquired by a traumatic implantation of dimorphic fungi of the S. schenckii complex in the skin [10]. Molecular identification and phylogenetic studies of cryptic species of this fungal complex comprising five species, S. schenckii sensu strict, S. brasiliensis, S. globosa, S. Mexicana, and S. pallida (formerly Sporothrix albicans), were relevant for the comprehension of changes in almost the entire pathogenic cycle of Sporotrichosis ranging from epidemiology, transmission, and biology of the fungus to the clinical and therapeutic implications resulting from this process [11,12,13].

The most frequent clinical manifestation is the subacute or chronic cutaneous-lymphatic form, followed by the fixed cutaneous infection [12,13]. Sporotrichosis primarily affects humans and animals. The zoonotic pathways, as exemplified by animal scratches and bites, particularly from cats, are the most common modes of transmission to humans in hyperendemic areas in Brazil [9,10].

Despite extensive research dedicated to the development of new therapeutic strategies, only a limited number of drugs are available against fungal infections [1]. The clinical uses of the drugs have been limited by the emergence of drug resistance, high risk of toxicity, insufficiencies in their antifungal activity and undesirable side effects [14]. Considering these factors, there is a need for the discovery of new agents with antifungal potential and natural products can be an alternative for this purpose.

P. pseudocaryophyllus (Gomes) Landrum (Myrtaceae) is the only species of the Pimenta genus native to Brazil [15]. This plant is found in high-altitude regions of the Atlantic forests and the Cerrado regions in Brazil [16,17,18]. It is popularly known as pau-cravo, louro-cravo, louro, craveiro, craveiro-do-mato, chá-de-bugre, and catania [19,20]. In folk medicine, the leaves have been used to produce a refreshing drink with calming, diuretic, and aphrodisiac properties, as well as to treat colds and their complications and digestive and menstrual problems [15,16,19].

The genus Elionurus presents approximately 45 species. It occurs in Africa, Asia, North America and South America. Elionurus muticus (Spreng) Kunth is found in Pampa biome, Brazil; it belongs to the Gramineae family and is known as lemongrass. Its essential oil is rich in citral, which is widely used in the aroma, food, and cosmetic industries in the world [20, 21]. It is notable for containing antiseptic, sudoriferous and febrile properties [20,22]. Puppin et al (2018) [23] showed antifungal activity of the essential oil of E. muticus against the Candida spp strains tested. Results in the literature also describe the antifungal activity of E. muticus essential oil against Candida albicans, C. krusei and Cryptococcus neoformans [ww Chagonda].

Thus, the aims of this study were to carry out a phytochemical investigation and evaluate the antifungal activity of the crude hydroethanolic extract and the essential oil from leaves of P. pseudocaryophyllus and the essential oil from leaves of Elionurus muticus.

II. Methods

A. Plant Material

Leaves of P. pseudocaryophyllus were collected at Serra Gigante, Pico Pasmado, Guaraqueçaba, Paraná, Brazil. The plant specimens were identified by Prof. Dr Alan Yukio Mocochinski, Department of Botany of Federal University of Paraná and a voucher specimen was deposited at the Herbarium of Federal University of Paraná under code UPCB-49.557.

The essential oil from Elionuru muticus leaves was obtained from Lazlo Aromatologia Ltda (Belo Horizonte, Minas Gerais, Brazil), which was extracted by vapor entrainment.

B. Extraction of the Essencial Oil

Samples of P. pseudocaryophyllus dried leaves (100 g) were triturated and submitted to hydrodistillation process, in a Clevenger-type apparatus for 4 h recommended by Brazilian Pharmacopoeia [25]. The essential oil was dried by using anhydrous sodium sulphate, and it was then stored at -10 °C in the dark, until use.

C. Preparation of Hydroalcoholic Crude Extract

Dried and pulverized leaves of P. pseudocaryophyllus (30 g) were extracted with 95% ethanol (1:10, w/v) by dynamic maceration at room temperature (25 °C) for 24 h. The extraction procedure was repeated in triplicate for the same powder. The extracts were combined, filtered, and the obtained filtrates were concentrated under vacuum at 40 ◦C using a rotary evaporator (Buchi, R-215).

D. Analysis of the Essential Oil by CG-MS

Analyses by CG-MS of the essential oils were carried out on a Shimadzu model QP 2010 Plus CG-MS using a non-polar Rtx5-MS fused silica capillary column (30 m × 0.25 mm i.d. x 0.25 µm film thickness) and helium was used as the carrier gas at a flow rate of 1.0 mL min-1. The injector temperature was 240 °C. The column temperature was programmed from 60 °C to 240 °C at 3 °C min-1. Mass spectra were recorded from 40–600 m/z. Individual components were identified by matching their 70 eV mass spectra with those of the spectrometer database using the Wiley 7 and FFNSC 1.2 library using retention indices as a pre-selection routine, as well as by visual comparison of the fragmentation pattern with those reported in the literature [24,26].

E. Determination of Total Phenolic and Tannins Contents

The total phenolic content of the ethanol extract was determined by the literature methods involving Folin–Ciocalteu reagent and gallic acid standard [27]. For determination of total polyphenol content, 0.250 mL of a methanolic solution of 0.6 mg mL-1 (based on total solids content) was mixed with 0.250 mL of the Folin-Ciocalteau reagent, 0.5 mL of 10% Na2CO3, and additional deionized water was added to make a final volume of 5 mL. It was prepared a blank for each concentration, which contained all reagents, except sample. After 30 min, the absorbance was measured in a spectrophotometer (Biospectro SP-220) at a wavelength (λ) of 760 nm. The mean (±SD) results of triplicate analyses were expressed in terms of mg gallic acid equivalents (GAE) g-1.

For determination of total tannins content, 5 mL of the methanolic solution of 4 mg mL-1, was added 0.05 g of casein (Sigma-Aldrich, USA) with moving in a closed container for 60 min. The solution obtained was filtered through filter paper and then the filtrate was transferred into a 5 mL volumetric flask. An aliquot of 0.150 mL and additional deionized water were added to make a final volume of 1 mL to obtain a solution of concentration 0.6 mg mL-1 (based on total solids

content). 0.250 mL of this solution was mixed with 0.250 mL of the Folin-Ciocalteau reagent, 0.5 mL of 10% Na2CO3, and additional deionized water was added to make a final volume of 5 mL. It was prepared a blank for each concentration, which contained all reagents, except sample. After 30 min, the absorbance was measured in a spectrophotometer (Biospectro SP-220) at λ=760 nm. Total tannin content was obtained indirectly by the difference between the total polyphenol content and polyphenol not adsorbed by casein. The mean (±SD) results of triplicate analyses were expressed in terms of mg gallic acid equivalents (GAE) g-1.

The same procedure was repeated for all the gallic acid standard solutions (0.5–6.25 µg mL-1) and a standard curve was obtained. The concentration of phenolic compounds was calculated according to the following equation obtained from the standard gallic acid curve:

$$Absorbance = 0.08003396 \times gallic\ acid\ (\mu g) + 0.00662298 \quad (1)$$

F. Determination of Flavonoids Contents

Total flavonoid content was determined by the literature methods involving aluminum chloride and quercetin standard [28]. To 1 mL of solution hydromethanolic (12.5 mg mL-1) was mixed with 240 µL of acetic acid, 4 mL pyridine-methanol solution (20:80, v/v), 1 mL of aluminum chloride solution 8% (w/v), and additional methanol was added to make a final volume of 10 mL. It was prepared a blank for each concentration, which contained all reagents, except sample. After 30 min, the absorbance was measured in a spectrophotometer (Biospectro SP-220) at λ = 420 nm. Total flavonoid contents were calculated as quercetin (Sigma-Aldrich, USA) from an analytical curve and were expressed as the means ± standard deviation (SD). The same procedure was repeated for all the quercetin standard solutions (1–10 µg mL-1) and a standard curve was obtained. The concentration of flavonoids compounds was calculated according to the following equation obtained from the standard quercetin curve:

$$Absorbance = 0.0626006 \times quercetin\ (\mu g) - 0.0120845 \quad (2)$$

G. Fungal Strains

Six filamentous fungal strains were used. Sporothrix schenckii (ATCC MYA 4821, 1099-18), S. Schenckii (ATCC MYA 4820, IPEC 15383), S. 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), Rio de Janeiro, Brazil. Two clinical strains of Sporothrix schenckii (A and 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), São Paulo, Brazil.

H. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

MIC and MFC of samples were determined by broth microdilution method according to the guidelines M38-A2 of the Clinical and Laboratory Standards Institute [29]. The fungal inoculums were prepared from young colonies (7-10 days) from Sporothrix spp filamentous phase, which was re-suspended in tubes containing the 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 the 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 hydroethanolic extract, essential oils and eugenol, in order to obtain concentrations from 7.8 to 1000 µg mL-1 , 8.14 to 1.041,86 µg mL-1 and 8.59 to 1.099,89 µg mL-1 respectively, 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 samples were added to 96-well microplates and incubated at 35 ºC for 72 h. 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 samples, and the second was performed with the medium culture only, without micro-organisms. Ketoconazole and amphotericin B were used as reference drugs. The same procedure was repeated for all the ketoconazole and amphotericin B standard solutions (0.0313- 16 µg mL-1).

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. All tests were performed in triplicate.

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.

I. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was performed to investigate morphological changes in the S. schenckii (ATCC 1099-18) and S. brasiliensis (IPEC 17943) submitted to the treatments of interest. SEM was carried out by a method adapted from Santos (2012) [30]. The fungal suspension was prepared in the same way that obtained for the MIC. An aliquot of 100 µL of the fungal suspension was added to 96-well

microplates and incubated at 27 ºC for 72 h. The suspension was treated with the essential oil, eugenol, ketoconazole and amphotericin B at sub-lethal concentration, ½ MIC value, and then the samples were reincubated at 27 °C for 72 h. After incubation, fungal structures were harvested by centrifugation for 10 min at 5,000 x g and were fixed with 2.5 % glutaraldehyde, 4 % formaldehyde in 0.1 M cacodylate buffer (pH = 7.2) for 24 h at 4 ºC.

Fungal structures adhered to poly-L-lysine glass coverslips. Post-fixation was carried out in 1 % osmium tetroxide containing 1.25 % potassium ferrocyanide for 30 min. Then, the fungal structures were washed with 0.1 M cacodylate buffer (pH 7.4) and dehydrated in an ethanol gradient (30 to 100 %) at 15 min intervals for each concentration and dried at room temperature (25 °C). Then, the samples were critical-point-dried in CO2 (Leica EM-CPD030) and coated with gold (Balzers Union FL-9496). The prepared samples were observed under a scanning electron microscope (JEOL, 6390L).

III. Results

A. Chemical Composition of the Essential Oils

The yield of essential oil of *P. pseucocaryophyllus* obtained by hydrodistillation was 0.35% and 22 components were identified by GC-MS accounting for 94.36% of the whole composition. The essential oil was primarily composed of monoterpenes (48.83%), followed by phenylpropanoids (39.08%) and sesquiterpenes (6.45%). The main constituents were eugenol (34.38%), b-pinene (7.79%), α-pinene (7.01%) and p-cimene (6.54%).

The chemical composition of *Elionuru muticus* identified by GC-MS accounting for 91.40% of the whole composition. The essential oil was primarily composed of monoterpenes (≅90%) and sesquiterpenes (2.05%). The main constituents were citral (neral + geranial = 72.35%).

B. Chemical Composition of Hydroethanolic Extract

Total polyphenol, tannin and flavonoid contents of the leaves of *P. pseudocaryophyllus* are shown in Table 1.

TABLE 1: TOTAL PHENOLIC, TANNIN AND FLAVONOID CONTENTS OF CRUDE EXTRACT OF *P. PSEUDOCARYOPHYLLUS* LEAVES.

Sample (plant drug)	Total polyphenolic content *(% w/w)**	Total tannin content (% w/w)*	Total flavonoid content *(% w/w)**
Replicate A	18.43	10.63	0.5404
Replicate B	19.43	11.01	0.5404
Replicate C	18.47	10.43	0.5072
Mean ± SD	18.77 ± 0.57	10.69 ± 0.29	0.5293±0.2

** The results were expressed as percent compared to plant drug (g per 100g of leaves)*

The mean ± SD of three determinations of the polyphenol content expressed as gallic acid in the plant drug was 18.77% ± 0.57% w/w, the mean ± SD of three determinations of the total tannin expressed as gallic acid was 10.63% ± 0.29% w/w and that of the flavonoid content expressed as quercetin in the plant drug was 0.5293% ± 0.02% w/w.

The leaves of *P. pseudocaryophyllus* analysed in this study had a high content of total polyphenols (18.77% ± 0.57% w/w) and significant amounts of tannins (10.69% ± 0.29% w/w). Thus, this result suggests that most of the polyphenol content found in this species was composed of tannins, indicating this class as the major constituent polyphenol present in the species.

C. Antifungal Activity

The MIC and the MFC of the essential oils, eugenol and the hydroethanolic crude extract of *P. pseudocaryophyllus* and the essential oil of *Elionuru muticus*, against the major strains causing sporotrichosis in Brazil, are shown Table 3. The results are expressed as µg mL^{-1} of the sample.

TABLE 2: *IN VITRO* SUSCEPTIBILITY OF FUNGAL STRAIN USING ESSENTIAL OIL AND EUGENOL ADJUSTED TO 100% EUGENOL.

	Essential oil		Eugenol	
Fungal strain	**MIC**	**MIF**	**MIC**	**MIF**
S. schenckii A	179.09	358.19	68.05	272.22
S. schenckii B	89.54	179.09	34.02	136.11
S. schenckii ATCC 1099-18	89.54	179.09	136.11	544.44
S. schenckii IPEC 15383	89.54	179.09	136.11	544.44
S. brasiliensis ATCC 5110	89.54	179.09	136.11	544.44
S. brasiliensis IPEC 17943	89.54	179.09	136.11	272.22

The hydroethanolic extract of P. pseudocaryophyllus and the essential oil of Elionuru muticus did not show antifungal activity at the evaluated concentrations.

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

To evaluate the influence of eugenol on the antifungal activity, we set the results in terms of mg mL^{-1} of the essential oil to µg mL^{-1} of eugenol, which was calculated based on the percentage of eugenol found by GC-MS (essential oil: 34.38% of eugenol = 716.40 µg eugenol), obtaining the results shown in Table 3.

TABLE 3: *IN VITRO* SUSCEPTIBILTY FROM DIFFERENT FUNGAL STRAINS.

	Itraconazole		Amphotericin B		Ketoconazole	
Fungal strain	MIC	MFC	MIC	MFC	MIC	MFC
S. schenckii A	>128	-	1.0	1.0	2.0	4.0
S. schenckii B	>128	-	2.0	2.0	2.0	4.0
S. schenckii ATCC 1099-18	>128	-	2.0	2.0	4.0	8.0
S. schenckii IPEC 15383	>128	-	1.0	1.0	4.0	8.0
S. brasiliensis ATCC 5110	>128	-	2.0	2.0	4.0	8.0
S. brasiliensis IPEC 17943	>128	-	2.0	2.0	2.0	4.0

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

D. SEM

The analysis of electron micrographs allowed us to morphologically identify the fungal structures and perceive the morphological changes in the fragments of *S. brasiliensis* strains, which were subjected to the essential oil of *P. pseudocaryophyllus*. The abnormalities identified are shown in Fig. 1 and 2.

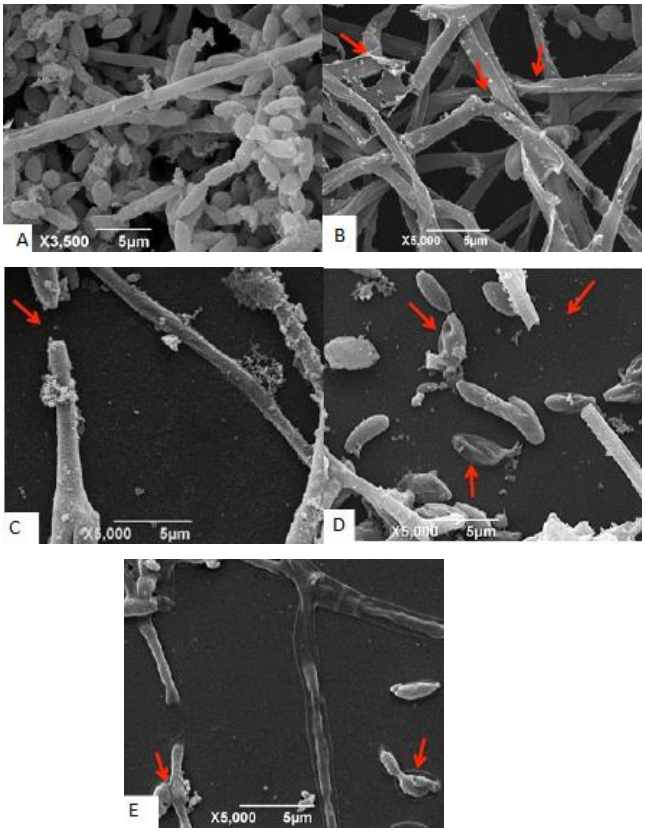

Fig. 1: *Electron micrographs of S. brasiliensis* IPEC 17943 *exposed or not to reference drugs and experimental drugs: A) S. brasiliensis* IPEC 17943 *not subjected to pharmacological treatment; B) S. brasiliensis* IPEC 17943 *subjected to treatment with essential oil; C) S. brasiliensis* IPEC 17943 *subjected to treatment with itraconazole; D) S. brasiliensis* IPEC 17943 *subjected to treatment with amphotericin B; E) S. brasiliensis* IPEC 17943 *subjected to treatment with eugenol.*

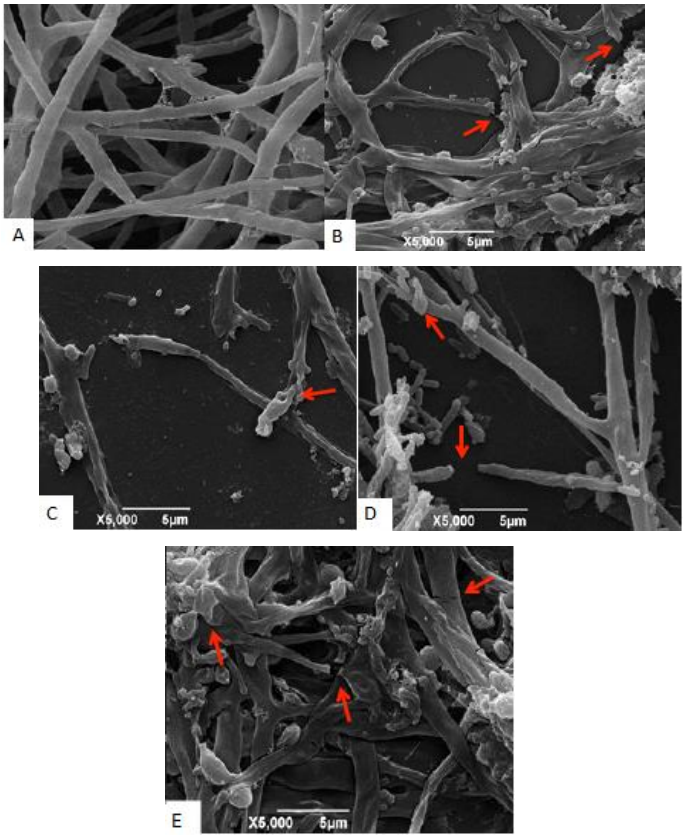

Fig. 2: *Electron micrographs of S. schenckii ATCC 1099-18 exposed or not to reference drugs and experimental drugs: A) S. schenckii ATCC 1099-18 not subjected to pharmacological treatment; B) S. schenckii ATCC 1099-18 subjected to treatment with essential oil; C) S. schenckii ATCC 1099-18 subjected to treatment with itraconazole; D) S. schenckii ATCC 1099-18 subjected to treatment with amphotericin B; E) S. schenckii ATCC 1099-18 subjected to treatment with eugenol.*

IV. DISCUSSION

Since ancient times, folk medicine and agro-food science have benefitted from the use of essential oils to combat different diseases, as well as to preserve food. Global market for medicinal herbs has been growing rapidly and significant economic gains are being realized. The essential oils of both species exhibit high volatility, clear coloration and low viscosity. Focus on the discussion will be given for the *P. pseudocaryophyllus*, since only it was active for the tested fungi. The composition of *P. pseudocaryophyllus* essential oil has been described for many authors, and the phenylpropanoid eugenol has been described as its major constituent, especially in studies of specimens collected in regions near Guaraqueçaba – Paraná, Brazil, where the

studied specimen was collected, which, due to geographical proximity, are exposed to similar edaphoclimatic conditions [31,32].

The literature also reports that citral and (E)-methyl isoeugenol are the major constituents of *P. pseudocaryophyllus* [33,34] but (E)-caryophyllene and (E)-asarone have been listed for the first time as the major constituents in the samples of this species. Furthermore, chavibetol and methyl eugenol have been cited as the primary constituents in the essential oils of this species in samples collected from the Ribeira Valley, in the southeastern region of Brazil [19]. The differences in the chemical composition may be due to the differences in the environmental conditions, geographical origins, genetic variability, vegetative plant phases and the extraction and quantification methods.

According with Paula et al. [24] the chemical variability observed in the leaf essential oils of *P. pseudocaryophyllus* of 12 specimens natural from three different locations in the central Brazilian Cerrado, evaluated by GC–MS, clearly indicates that genetic factors contribute to the chemical polymorphism observed in this plant species. It is crucial to consider the chemical variations in the essential oils caused by genetic, physiological or environmental factors when domesticating and improving the species of medicinal interest. Therefore, it is necessary to characterize and identify the existence of chemotypes, especially when referring to plant material used in chemical, pharmacological and agronomic studies aimed at producing herbal medicines, once the pharmacological activities of the same species can differ due to differences in the essential oil composition [24].

The literature also describes results similar to those found in this study. Paula et al. [24] found high percentages of total polyphenols in *P. pseudocaryophyllus* leaves collected in São Gonçalo do Abaeté – Minas Gerais, Brazil (9.77% w/w) and in Brasília – Distrito Federal, Brazil (10.52% w/w) and considerable amounts of total tannins (15.41% w/w, São Gonçalo do Abaeté – Minas Gerais and 14.84% w/w, respectively, Brasília - Distrito Federal). The values found for flavonoid content (0.5293% w/w) were slightly below those reported in the literature, as Paula et al. [24] described a content of 1.07% w/w and 1.37% w/w for the species collected in São Gonçalo do Abaeté - Minas Gerais, Brazil and Brasília - Distrito Federal, Brazil, respectively. The description for total polyphenols, tannins and flavonoids, combined with the literature data, is an important step as quality control parameters can be established for the species *P. pseudocaryophyllus.*

To evaluate antifungal activity of natural products Holetz et al. [35] suggested a parameter based on the value of MIC value: ≤100 µg mL^{-1} as considered good, between 100 and 500 µg mL^{-1} considered moderate, and between 500 and 1000 µg mL^{-1} considered weak. Based on the described above and at Table 3, the essential oil showed moderate antifungal activity (MIC = 260.46 µg mL^{-1}) against the clinical lineages B, *S. schenckii* IPEC 15383, ATCC 5110 *S. brasiliensis*, *S. schenckii* ATCC 1099-18 and *S. brasiliensis* IPEC 17943; already in relation to clinical lineage A, the essential oil showed a weak activity (MIC = 520.90 µg mL^{-1}). The essential oil showed fungicidal activity against all the strains at concentrations ranging from 520.90 to 1041.86 µg mL^{-1}. Eugenol had a good antifungal activity against the clinical strains A (MIC = 68.73 µg mL^{-1}) and B (MIC = 34.36 µg mL^{-1}) and moderate antifungal activity front to other strains used in the study (MIC = 137.48 µg mL^{-1}). Eugenol showed fungicidal activity against all the strains at concentrations ranging from 137.48 to 549.94 µg mL^{-1}.

The ethanol extract and the essential oil of *Elionuru muticus*, evaluated in the concentration range 7.8-1.000,00 µg mL^{-1}, showed no antifungal activity, compared to the tested strains. This result suggests that eugenol is the component responsible in large part for the antifungal activity found in the essential oils in view of the essential oil present 34.38% of eugenol. To evaluate the influence of eugenol on the antifungal activity, we set the results in terms of mg mL^{-1} of the essential oil to µg mL^{-1} of eugenol, which was calculated based on the percentage of eugenol found by GC-MS (essential oil: 34.38% of eugenol = 716.40 µg eugenol), as shown in Table 3. When the results were adjusted to 100% eugenol, the better antifungal activity of the essential oil (MIC and MFC) was observed for all the standard strains compared to that with eugenol. For the clinical strains A and B, the best activity was verified for eugenol. These results reflect the antifungal activity exhibited by the essential oil, which is largely due to eugenol. However, the best activity was found for the essential oil relative to eugenol, reflecting the existence of synergism between the components of the essential oil, as identified by GC-MS, 22 compounds of which monoterpenes, oxygenated monoterpenes and sesquiterpenes, which may have interacted synergistically with eugenol, contributed to a greater antifungal activity presented by the essential oil.

Eugenol is a phenolic substance present in the essential oils of some plants, the most relevant being *Eugenia caryophyllus*, the "Carnation-Da-India"; *Dicipelium cariophyllatum*, "the carnation of Maranhão or cloves" and Croton zenhtneri, the "cinnamon-de-wedge" [36,37]. Eugenol has various pharmacological actions described in the literature, such as antimicrobial, anti-inflammatory, antioxidant, modulator of immune responses, local anaesthetic and antinociceptive [38,39,40,41].

The antimicrobial activity of eugenol has been attributed to its phenolic structure at higher concentrations that causes the degeneration of proteins of the cell membranes of microorganisms, thereby resulting in damage to the cell membrane [34,38].

Suzuki et al. (2014) [43] evaluated the effectiveness of the essential oil of *P. pseudocaryophyllus* in inhibiting the growth of the

primary bacteria responsible for the bad odour from perspiration (*Staphylococcus epidermidis*, *Proteus hauseri*, *Micrococcus* and *Corynebacterium xerosis yunnanensis*). In this study, the essential oil of *P. pseudocaryophyllus*, which presented eugenol (88.6%) as the major constituent exhibited bacteriostatic activity against all bacteria tested, except for *S. epidermidis*, with MIC values ranging from 500 to 1000 µg mL^{-1}.

For reference drugs, the evaluation of the fungal susceptibility may be performed based on MICs. Values ≤1 µg mL^{-1} for amphotericin B and the azole antifungal agent indicate sensitivity; values >2 µg mL^{-1} have been associated with failure of treatment; while values ≥4 µg mL^{-1} indicate the resistance of fungal strains [29,31].

The results shown in Table 3 indicate that only two of the studied strains studied (*S. schenckii* and *S. schenckii* IPEC 15383) were susceptible to amphotericin B and none were susceptible to ketoconazole. In addition, three strains were considered resistant to ketoconazole with MIC values of 4.0 µg mL^{-1}, which were *S. schenckii* ATCC 1099-18, *S. schenckii* IPEC 15383 and *S. brasiliensis* ATCC 5110. By evaluating the *in vitro* susceptibility profile of three standard strains of the fungus *S. brasiliensis* against amphotericin B, Santos (2012) [26] found that two of the three strains exhibited resistance to the drug, with MIC values of 4.0 to 16.0 µg mL^{-1}. Only one strain was considered sensitive (MIC = 1.0 µg mL^{-1}).

The images obtained by SEM were from the yeasts strains *S. schenckii* 1099-18 ATCC 17943 and *S. brasiliensis* IPEC not exposed to any type of treatment (untreated group) and exposed to the essential oil, eugenol and reference drugs (itraconazole and amphotericin B) show structural and morphological changes in the treated groups when compared with the untreated group. The untreated groups showed hyphae with regular surface and stretched and elongated conidia with a smooth surface. The fungal structures under the action of the antifungal agents showed deformities in structure (cracks and breaks in hyphae, contorted hyphae and conidia, thin hyphae and cell extravasation), with probable cellular destruction. When *S. brasiliensis* was treated with essential oil (Fig. 1-B), the hyphae were observed to be tuned and broken, with contorted parts and cell extravasation, however, when the strain *S. schenckii* was treated with essential oil (Fig. 2-B), the changes were pronounced, and broken hyphae were observed, with the presence of roughness, numerous contorted parts and hyphal clusters.

S. brasiliensis when treated with eugenol (Fig. 1-D), the conidia and the hyphae were observed with grooves in the membrane. Similarly, when *S. schenckii* was exposed to eugenol (Fig. 2-E), broken hyphae with the presence of grooves or contorted parts were observed. After being subjected to treatment with the reference drug itraconazole, S. brasiliensis strain (Fig. 1-C) showed broken hyphae. When *S. schenckii* was treated with itraconazole, thin hyphae with the presence of roughness were observed (Fig. 2-C).

As shown in Fig. 1-D and 2-D, the strains *S. brasiliensis* and *S. schenckii* exposed to amphotericin B, exhibited broken hyphae, and the presence of roughness in the hyphae and conidia.

The SEM micrographs revealed a reduction in the number of conidia in both the fungi treated with the essential oil and the fungi treated with ketoconazole and amphotericin B. Furthermore, it was observed that the essential oil caused morphological alterations in the fungal structures similar to or in greater intensity than those caused by the drugs amphotericin B and ketoconazole.

V. Conclusion

This study demonstrates that quantification of secondary metabolites in plant drugs corroborated with what has been described for the species.

The findings of the present study demonstrated that the essential oil of *P. pseudocaryophyllus* has a fungicidal activity against the primary microorganisms responsible for sporotrichosis in Brazil, probably due to its high content of eugenol. Therefore, *P. pseudocaryophyllus* (essential oil) has a potential to be developed as new and safe antimicrobial agents. Furthermore, the potential toxicity of this essential oil should be evaluated *in vivo*.

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Conflicts of Interest

The authors declare that the research reported here was conducted in the absence of any commercial or financial relationships that could constitute potential conflicts of interest.

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