IN VITRO SUSCEPTIBILITY OF THE *Sporothrix brasiliensis* TO AQUEOUS EXTRACTS OF THE GREEN TEA (*Camellia sinensis* L. Kuntze)

[*Suscetibilidade in vitro de Sporothrix brasiliensis à atividade inibitória de extratos aquosos de chá-verde (Camellia sinensis L. Kuntze)*]

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ABSTRACT – Sporotrichosis is a zoonosis caused by fungi of the *Sporothrix schenckii* complex, which *Sporothrix brasiliensis* is prevalent in cats and dogs in Brazil. Itraconazole is the antifungal used for treatment, but the emergence of resistance has encouraged the search for therapeutic alternatives in medicinal plants. Among the benefits properties of green tea (*Camellia sinensis* L. Kuntze), it is included the antifungal activity, but there are no data that prove its potential in sporotrichosis. Aimed to evaluate the *in vitro* antifungal activity of green tea against *Sporothrix brasiliensis*. Infusion and decoction of dried leaves were prepared at 10% and tested through broth microdilution test (CLSI M38-A2), that was also performed with itraconazole. Nine clinical isolates of *S. brasiliensis* from cats and dogs in South Brazil and one standard strain of *S. schenckii* were tested. The results were expressed in minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC). All isolates were inhibited by infusion and decoction (MIC_{90} of 3.125 mg mL^{-1} for both extracts), without statistic difference, but the fungicidal activity was weak or no occurred (MFC_{90} > 50 mg mL^{-1} for both). In itraconazole, *S. brasiliensis* were sensitive at MIC_{50} of 2 μg mL^{-1} and *S. schenckii* to MIC of 0.0625 μg mL^{-1}, but the antifungal resistance was observed (MIC_{90} and $MFC_{50/90}$ > 16 μg mL^{-1}). For the first time, the satisfactory inhibitory activity of the infusion and decoction of green tea make its promising in the treatment of sporotrichosis. However, more studies are needed for its use.

Keywords: antifungal resistance; infusion; medicinal plant; sporotrichosis; *Sporothrix schenckii* complex.

RESUMO – Esporotricose é uma micose zoonótica causada por fungos do complexo *Sporothrix schenckii*, o qual *Sporothrix brasiliensis* é prevalente em gatos e cães no Brasil. Itraconazol é o antifúngico utilizado para o tratamento, entretanto, o surgimento de resistência tem encorajado a busca por alternativas terapêuticas em plantas medicinais. Dentre as propriedades benéficas do chá-verde (*Camellia sinensis* L. Kuntze), inclue-se atividade antifúngica, embora não haja publicações que relacionem esse potencial na esporotricose. Esse estudo objetivou avaliar a atividade antifúngica *in vitro* do chá-verde contra *Sporothrix brasiliensis*. Infusão e decocção de folhas secas de chá-verde foram preparadas a 10% e testadas através do teste de microdiluição em caldo (CLSI M38-A2), também realizado com itraconazol. Nove isolados clínicos de *S. brasiliensis* oriundos de gatos e cães com esporotricose na região sul do Rio Grande do Sul, Brasil, e uma cepa padrão de *S. schenckii* foram testadas. Os resultados foram expressos em concentração inibitória mínima (CIM) e concentração fungicida mínima (CFM). Todos os isolados foram inibidos pela infusão e decocção (CIM_{90} de 3.125 mg mL^{-1} para ambos extratos), sem diferença estatística, porém, a atividade fungicida foi fraca ou não ocorreu (CIM_{90} > 50 mg mL^{-1} para ambos). Em itraconazol, *S. brasiliensis* foram sensíveis na CIM_{50} de 2 μg mL^{-1} e *S. schenckii* à CIM de 0.0625 μg mL^{-1}, porém, a resistência antifúngica foi observada (CIM_{90} > 16 μg mL^{-1}). Pela primeira vez, a atividade inibitória satisfatória da infusão e decocção de chá-verde os tornam promissores no tratamento da esporotricose. No entanto, mais estudos são necessários para seu uso.

Palavras-Chave: complexo *Sporothrix schenckii*; esporotricose; infusão; plantas medicinais; resistência antifúngica.

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INTRODUCTION

Among the zoonotic mycosis, the sporotrichosis is a disease with a large importance in the human and veterinary medicines due to its transmission through scratch or bite of sick cats and this disease is caused by the pathogenic fungi belonging to the *Sporothrix schenckii* complex (Xavier et al., 2004; Marimon et al., 2007). In Brazil, the Southeast and South regions are the higher prevalence, which the clinical reports are very related to the infection by *Sporothrix brasiliensis* (Schubach et al., 2008; Madrid et al., 2012; Rodrigues et al., 2013; Montenegro et al., 2014).

Antifungal drugs are preconized for the treatment of human and animal sporotrichosis, being itraconazole the drug of choice (Madrid et al., 2010; Pereira et al., 2010). However, the emergence of resistant strains of *Sporothrix* spp. have been observed, and this have been occurring because of the indiscriminate use of several antifungals in the therapies (Marimon et al., 2008; Gutierrez-Galhardo et al., 2010; Rodrigues et al., 2014a; Stopiglia et al., 2014). This alarming situation has stimulated the search for new chemical compounds with antifungal property. It is known that the researches in medicinal plants for elaborate drugs are on the rise and only approximately 30% of the antimicrobial medications in the market are derived from natural products (Chattopadhyay et al., 2009).

The use of medicinal plants by the peoples is common, mainly when it is used as teas. According to Cabrera et al. (2006), the teas derived from the plant *Camellia sinensis* L. Kuntze (Theaceae family) is very consumed in the United States, China, Taiwan, Japan and Korea, among others countries. The main varieties of teas from *Camellia sinensis* L. Kuntze are white tea, that is made from very young leaves or buds; the green-tea, that is made from mature unfermented leaves; and Oolong tea and black-tea, that are made from partially and fully fermented leaves, respectively (Jigisha et al., 2012; Gupta et al., 2014).

Many scientific researches about the medicinal properties of green-tea has been reported, such as antioxidant (Camargo et al., 2006; Jigisha et al., 2012; Gupta et al., 2014), anti-inflammatory (Jigisha et al., 2012), cardiovascular (Jigisha et al., 2012), anticarcinogenic (Jigisha et al., 2012; Subramani & Natesh, 2013), as well as antiprotozoan (Thipubon et al., 2015), antiviral (Song et al., 2005; De Oliveira et al., 2013), antibacterial (Almajano et al., 2008; Chan et al., 2011; Farooqui et al., 2015) and antifungal (Almajano et al., 2008; Suwanmanee et al., 2014). In fungal pathogens, *Candida albicans, Trichophyton mentagropytes, T. rubrum, T. tonsurans, Epidermophyton floccosum, Microsporum canis, M. gypseum, Aspergillus niger, Penicillium* spp. also was sensitive to extracts of green-tea (Hirasawa & Takada, 2004; Almajano et al., 2008; Ikeda et al., 2013; Suwanmanee et al., 2015), but the studies in *Sporothrix schenckii* complex were not found.

Due to the necessity to find new therapic alternatives for treatment of sporotrichosis, the promising antimicrobial activity of green-tea encouraged us to realize this study, whose aim was to evaluate the antifungal activity of the aqueous extracts of green-tea (*Camellia sinensis* L. Kuntze) against clinical isolates of *Sporothrix brasiliensis*.

MATERIAL AND METHODS

Plant Material

Dried leaves of *Camellia sinensis* L. Kuntze was purchased from a commercial supplier (Yamamotoyama®, Midri Indústria de Chá Ltda., São Miguel Arcanjo/SP, Brazil, lot 189).

Preparation of the Green Tea

The green tea was prepared in two forms of aqueous extracts: infusion and decoction, according to Snoussi et al. (2014) with adaptations. For infusion, the plant (1 g) was mixed with 100 mL of boiling distilled water for 10 min, with constant shaking. For decoction, the plant (1 g) was mixed with 100 mL of distilled water and placed on a tripod containing a heat source of type Bunsen burner. At the beginning of the boiling process, the mixture was maintained on warming for 10 min with constant shaking. For both preparations, the two extracts of green tea were filtered through Whatman No. 1 filter paper (Whatman® Schleicher & Schuell®, Castelldefels, Spain). Infusion and decoction were kept at room temperature until it is cold and tested in the antifungal assay.

Fungal Isolates

Nine clinical isolates of *S. brasiliensis* derived from cats and dogs with sporotrichosis from the south of Brazil were used (Table 1, Figure 1), along with a standard strain from human by *S. schenckii* (IOC 1226), totaling ten fungal isolates. All fungal strains used in this study were provided by the mycology collection of the *Centro de Diagnóstico e Pesquisa em Micologia Veterinária* (Universidade Federal de Pelotas, Pelotas/RS, Brazil). The identified species were obtained through polymerase chain reaction with restriction fragment length polymorphism (Rodrigues et al., 2014b) by *Laboratório de Micologia Médica e Molecular* (Universidade Federal de São Paulo/SP, Brazil).

Table 1. Clinical description of the veterinary patients with sporotrichosis by *Sporothrix brasiliensis*.

Specie animal	Breed	Sex	Age	City	Year*
Dog	Mongrel	Male	9 years	Pelotas	2005
Dog	Pitbull	Male	4 years	Pelotas	2010
Dog	Boxer	Female	3 years and 9 months	Pelotas	2010
Cat	Mongrel	Male	5 years	Pelotas	2011
Dog	Rottweiler	Male	3 years	Rio Grande	2012
Cat	Mongrel	Female	Adult**	Rio Grande	2012
Cat	Mongrel	Female	Adult**	Pelotas	2012
Cat	Mongrel	Female	3 years	São Lourenço do Sul	2013
Cat	Mongrel	Male	3 years	Pelotas	2013

*Year of the fungal isolation; **Adult age unespecified.

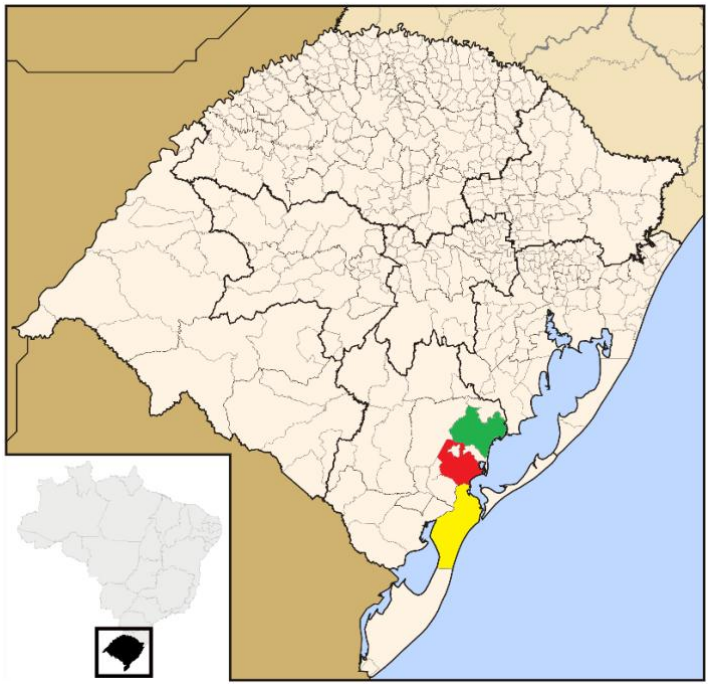

Figure 1. Geographic localization of the cities of the animals with sporotrichosis in the state of Rio Grande do Sul, Brazil: São Lourenço do Sul (green), Pelotas (red) and Rio Grande (yellow).

Antifungal Susceptibility Assay

The antifungal susceptibility tests were performed using the broth microdilution technique, according to the M38-A2 guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2008) with adaptations for using the plant extracts (Stopiglia et al., 2011). The fungal inoculums were prepared from young colonies (7 days at 27ºC) from *Sporothrix* spp. filamentous phase, which were resuspended in tubes containing sterile saline and adjusted according to the scale of 1.0 McFarland and, after, in the ultraviolet (UV)-visible spectrophotometer (Spectrum Instruments Co., Shanghai, Chine) with transmittance adjusted to 80-82% in fixed wavelenght of 530 nm. The suspensions were diluted in RPMI-1640 medium (Roswell Park Memorial Institute medium, Sigma, Steinhiem, Germany) buffered with 2% glucose and MOPS [3-(N-morpholino propanesulfonic acid)] (1:50, v/v).

A serial dilution of the aqueous extracts was performed using RPMI-1640 medium buffered with MOPS to obtain a final concentration between 1.56 to 50 mg mL^{-1}. In a 96-well microplates, an aliquot of 100 µL of the fungal suspension and 100 µL of the diluted tea were added. Reference itraconazole for veterinary use (Cepav Pharma® Ltda, São Paulo, SP, Brazil, 2.5 mg) was prepared in dimethyl sulfoxide (DMSO), according to the CLSI guidelines. For negative control, wells containing 100 µL of RPMI-1640 buffered with MOPS and 100 µL of the tea/itraconazole was performed; for positive control, 100 µL of the fungal inoculums was added. The microplates were incubated at 27ºC

for 72 h. The tests were performed in duplicate and the results were expressed as minimal inhibitory concentration (MIC), that was determined to be the lowest concentration able to inhibit the fungal growth visually. To obtain the minimal fungicidal concentration (MFC), 10 µL of aliquots of the wells with no fungal growth were transferred to Petri dishes containing sabouraud dextrose agar (Acumedia, Lansing, Michigan, United States) and incubated at 27ºC for 72 hours to visualize fungal growth. The MFC was determined to be the lowest concentration able to eliminate fungal growth.

Data 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 $p < 0.05$ considered significant.

RESULTS AND DISCUSSION

According to Table 2, a good inhibitory activity was observed in the aqueous extracts of green tea against all *S. brasiliensis* tested, including *S. schenckii*. The infusion and the decoction of green tea showed a MIC_{90} of 3.125 mg mL^{-1} against overall of clinical isolates. The fungicidal activity was not observed for most of the *S. brasiliensis* tested (MFC_{90} greater than 50 mg mL^{-1} for the both extracts) and only 30% (03/10) of them were sensitive to the infusion (MFC of 50 mg mL^{-1}). The good inhibitory activity of the green tea also was demonstrated against fungal pathogenic. Suwanmanee et al. (2014) showed the sensibility of *T. mentagrophytes* and *M. canis* to the crude extract from leaves of *Camellia sinensis* L. Kuntze between the concentrations of 18.67 to 53.33 mg mL^{-1}, which were superior than ours findings, demonstrating that *S. brasiliensis* and *S. schenckii* were more sensitive than the dermatophytes. In another study, the seed extracts of *Camellia sinensis* L. Kuntze inhibited the growth of *C. albicans* and *Cryptococcus neoformans* in the disk diffusion test at concentration of 100 mg mL^{-1} (Yoon et al., 2005). In relation to our study, a low concentration of the infusion and decoction of green tea inhibited the clinical isolates of *S. brasiliensis*, which seemed to be more sensitive than the other fungal genera reported in the literature, even the *in vitro* tests were not the same due to the lack of standardization in the tests with plant extracts. The preparation of the extracts and the *in vitro* methodology were different and this difficult the comparison of the results. Although the antifungal tests of green tea are concentrated in yeasts and dermatophytes (Hirasawa & Takada, 2004; Almajano et al., 2008; Ikeda et al., 2013; Suwanmanee et al., 2014), no studies were found in the ethiological agents of sporotrichosis, being this paper the first to demonstrate the *in vitro* anti-*Sporothrix* spp. activity of the aqueous extracts of green tea.

Table 2. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of the preparations of *Camellia sinensis* L. Kuntze (green tea) against clinical isolates of *Sporothrix brasiliensis*.

Sporothrix schenckii complex (No. samples)		Infusion of Green Tea (mg mL^{-1})		Decoction of Green Tea (mg mL^{-1})		Itraconazole (µg mL^{-1})	
		MIC	MFC	MIC	MFC	MIC	MFC
Sporothrix brasiliensis (9)	Range	≤1.56 – 3.125	50 – > 50	≤1.56 – 6.25	> 50	0.5 – > 16	> 16
	50%	≤1.56	> 50	3.125	> 50	2	> 16
	90%	3.125	> 50	3.125	> 50	> 16	> 16
Sporothrix schenckii IOC 1226 (1)	Range	≤1.56	50	3.125	> 50	0.0625	> 16
Overall (10)	Range	≤1.56 – 3.125	50 – > 50	≤1.56 – 6.25	> 50	0.0625 – > 16	> 16
	50%	≤1.56	> 50	3.125	> 50	1	> 16
	90%	3.125	> 50	3.125	> 50	> 16	> 16

50% and 90% refers to the percentage for inhibition/elimination the fungal growth.

The drug of choice for the treatment of sporotrichosis showed a MIC of 0.0625 µg mL^{-1} against the standard strain of *S. schenckii* from human, and 77.8% (07/09) of the clinical isolates of *S. brasiliensis* from cats and dogs were sensitive to the MIC from 0.5 to 2 µg mL^{-1}, which MIC_{50} was 2 µg mL^{-1} for *S. brasiliensis*. However, according to the values established for the criterion of sensibility to itraconazole (CLSI, 2008), the MIC values greater than 8 µg mL^{-1} should be considered resistant and this observation occurred in 22.2% (02/09) of the tested *S. brasiliensis*. This actual situation about antifungal resistance has also been reported as alarming for the therapeutic control of the sporotrichosis (Marimon et al., 2008; Montenegro et al., 2014; Rodrigues et al., 2014a). In turn, all clinical isolates of *S. schenckii* complex were inhibited by the infusion and decoction of

green tea (MIC_{90} of 3.125 mg mL^{-1} for boths extracts). These findings are in agreement with the literature, because the antimicrobial activity of the extract infusion of green tea was also demonstrated against multidrug resistant *Salmonella* spp. (Farooqui et al., 2015) and methicillin-resistant *Staphylococcus aureus* (Chan et al., 2011). Besides, other promising extracts from plants have been studied, such as the crude methanolic extract of *Pteurocaulon* genus of the Asteraceae family, that showed fungistatic activity between 1.56 and 50 mg mL^{-1}, including *S. schenckii* resistant to itraconazole (Stopiglia et al., 2011).

Both the extracts of green tea and the itraconazole showed a inhibitory activity against the isolates of the *S. schenckii* complex, but no fungicidal activity was observed in the tested products against all *S. brasiliensis*, indicating that they were only inhibited to growth. Only the strain of *S. schenckii* were sensitive to the fungicidal activity of the infusion of the green tea (MFC of 50 mg mL^{-1}). In relation to the type of preparation of the green tea, the both extracts showed a fungistatic activity without statistic difference (p>0.05), demonstrating that the method of preparation did not interfere in the activity against *S. brasiliensis* and *S. schenckii*. Although there are no possibility to make a chromatographic analysis of the extracts, it is known that the polyphenolic compounds are prevalent in the extracts from the leaves of the tested green tea. According to Zhao et al. (2011), the tested samples of green tea were rich in the catechin compounds, mainly epigallocatechin 3-*O*-gallate (EGCG), (-)-epigallocatechin, epicatechin, (+)-catechin and (−)-gallocatechin 3-*O*-gallate, which also showed activity when tested individually against *Candida* sp., *T. rubrum*, *M. canis* and *M. gypseum* (Hirasawa & Takada, 2004; Park et al., 2006, 2011; Han, 2007). The sensibility of the *S. brasiliensis* and *S. schenckii* tested in the mycelial phase to the aqueous extracts can be explained by the mode of action of EGCG compound. In *T. mentagrophytes*, Toyoshima et al. (1994) demonstrated that this compound changes the morphological characteristics of the conidia, provoking deformation and swelling, and thereby, the germination and hyphal growth. A scanning and transmission electron microscopy needs be performed in *Sporothrix* spp. isolates to evaluate the mechanism of action of the green tea in them.

However, the promising use of the plants in the treatment of diseases should be studied with respect to its possible toxicity in humans and animals, which has been showed a lower citotoxicity (Suwanmanee et al., 2014) and a safe use in *in vivo* studies (Hsu et al., 2011). These benefits properties should be considered and the good inhibitory activity *in vitro* of the extracts against *S. brasiliensis* and *S. schenckii* makes these promising products a strong candidate for furthers studies in the treatment of sporotrichosis. More researches needs be performed with a greater number of fungal isolates, including an experimental sporotrichosis, as well as the chemical and cytotoxic analyzes.

CONCLUSION

The results in the current work indicated that the infusion and decoction of green tea showed a good inhibitory activity against *S. brasiliensis* and *S. schenckii* in the MIC values between ≤ 1.56 and 6.25 mg mL^{-1}, including in resistant isolates to itraconazole. The findings evidence the aqueous extracts as potential candidates for the use in the treatment of sporotrichosis. Further studies should be performed for its safe use in animals.

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