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In Vitro Evaluation of the Antimicrobial and Antimycobacterial Activities of *Solanum guaraniticum* A. St.-Hil. Leaves

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ABSTRACT

With the emergence of antimicrobial resistance, it becomes necessary to search for new alternatives for the treatment of infectious diseases. *Solanum guaraniticum* is a shrub known as jurubeba or false jurubeba that has hepatoprotective and antioxidant activities, used in popular medicine for the treatment of various diseases. The aim of this study was to evaluate the *in vitro* antimicrobial and antimycobacterial activities of crude extract, chloroforn, ethyl acetate and butanol fractions from its leaves. Good activities were observed for the ethyl acetate fraction against *Staphylococcus intermedius* and *Listeria monocytogenes* (MIC = 64 µg/mL) and for the crude extract against *Micrococcus luteus* (MIC = 32 µg/mL). In general, the extracts showed moderate activity against Gram-positive bacteria, and were inactive against Gram-negative bacteria and fungi. It was also verified considerable activity against *Mycobacterium smegmatis*, mainly by chloroform fraction (MIC = 156 µg/mL). These results are probably due to the good antioxidant activity and to the presence of high contents of polyphenols, tannins and alkaloids, metabolites known to possess antimicrobial activity. Studies aiming the isolation of compounds are necessary in order to know the main component involved in these activities, since the plant has an antimicrobial potential.

INTRODUCTION

Before the advent of modern medicine, people depended essentially of the plants for the treatment of various diseases, and they still have been an important source for the development of new drugs (Kuete *et al.*, 2009). Plants contain large amounts of chemical compounds which can be explored for preventing microbial infections, through different mechanisms (Cowan, 1999; Savoia, 2012).

Therefore, natural products can be new promising alternatives for the treatment of infectious diseases, taking into account the emergence of antibiotic resistance, and undesirable side effects due to use of synthetic drugs (Cushnie and Lamb, 2011). Tuberculosis (TB) is considered a serious public health problem, taking place among the main infectious diseases (Arruda *et al.*, 2012). *Mycobacterium tuberculosis* is responsible for more human mortality than any other single microbial species (Tekwu *et al.*, 2012).

Currently, the treatment of TB involve along course of combination of antibiotics, leading to poor patient compliance, moreover, the multidrug resistance is also a problem. Thus, the discovery of new antituberculosis agents is urgent and, in this sense, various extracts and isolated compounds from plants have been tested with great results (Gautam *et al.*, 2007; Tawde *et al.*, 2012; Macabeo *et al.*, 2012).

Solanum guaraniticum A. St.-Hil. (syn. *Solanum fastigiatum* var. *acicularium* Dunal) is a shrub popularly known as jurubeba or false jurubeba, occurring in Paraguay, Argentina and Brazil (Soares *et al.*, 2008). According to the Brazilian Pharmacopoeia, the species *Solanum paniculatum* L. is recognized as the true jurubeba, and used in folk medicine as a tonic for fevers, anemia, erysipelas, cholagogue, bitter, and eupeptic to treat gastric and liver dysfunctions (Mesia-Vela *et al.*, 2002, Sabir and Rocha, 2008). Due to the similarities, these two species are used interchangeably by the population. *S. guaraniticum* has hepatoprotective (Sabir and Rocha, 2008) and antioxidant properties, being detected in the extract of its leaves the presence of

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caffeic, chlorogenic and rosmarinic acids, very active phenolic compounds (Zadra *et al.*, 2012). Several *Solanum* species are cited in the literature to possess antimicrobial activity against fungi, bacteria and mycobacteria (Bontempo *et al.*, 2013; Balachandran *et al.*, 2012, Das *et al.*, 2010; Lozoya *et al.*, 1992). *S. paniculatum* had activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Lôbo *et al.*, 2010) but, to the best of our knowledge, no studies concerning the antimicrobial activity of *S. guaraniticum* have been reported. Thus, the aim of this study was to evaluate the *in vitro* antimicrobial and antimycobacterial activities of the crude extract (CE), chloroform ($CHCl_3$), ethyl acetate (AcOEt) and butanol (BuOH) fractions of the *S. guaraniticum* leaves, against Gram-positive, Gram-negative bacteria, fungi and mycobacteria, by broth microdilution method to achieve the minimum inhibitory concentration (MIC), in order to verify their possible potential in the treatment of infectious diseases.

MATERIALS AND METHODS

Plant material and extractions

S. guaraniticum leaves were collected in Guaporé (Rio Grande do Sul, Brazil), on December 2011. A voucher specimen was identified and archived in the herbarium of Department of Biology at Federal University of Santa Maria, under the registration number SMDB 13158. The plant material was dried in a stove with controlled temperature, powdered in a knife mill and extracted with ethanol (70%) for 7 days with daily agitation. After filtration, the material was concentrated on rotary evaporator to remove the ethanol and obtaining the aqueous extract, which was successively fractionated with solvents of increasing polarity, chloroform, ethyl acetate and n-butanol. The solvent was evaporated to obtain the dried fractions ($CHCl_3$, AcOEt and BuOH, respectively). Part of the aqueous extract was separated and taken to dryness, yielding the crude extract (CE).

Microorganisms tested

CE and fractions were individually tested against *Staphylococcus aureus* ATCC 25923, *Staphylococcus intermedius* (clinical isolate), *Streptococcus agalactiae* (clinical isolate), *Enterococcus faecalis* ATCC 51299, *Micrococcus luteus* ATCC 7468, *Listeria monocytogenes* (clinical isolate), *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 700603, *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 44373, *Cryptococcus neoformans* (clinical isolate) and *Aspergillus fumigatus* (clinical isolate). For the antimycobacterial assay, was used standard strains of *Mycobacterium avium* LR541CDC, *Mycobacterium tuberculosis* H37Rv ATCC 25618 and *Mycobacterium smegmatis* mc^2 155 ATCC 700084.

Antibacterial and antifungal assays

The evaluation of antibacterial and antifungal activities was performed by the broth microdilution method, according to the Clinical and Laboratory Standards Institute CLSI M07-A8 (2009) for bacteria, CLSI M27-A3 (2008) for yeasts, and CLSI M38-A2 (2008) for filamentous fungus. Sterile 96-well microdilution plates were used and the tests were conducted in duplicate. Eight concentrations of crude extract and fractions were assessed (1024, 512, 256, 128, 64, 32, 16 and 8 µg/mL). Simultaneously, a negative control (without the presence of a microorganism) and a positive control (no antimicrobial agent) were carried out. Inoculate for yeasts were prepared from cultures in Sabouraud agar for 48 h at 30°C. A suspension in sterile saline 0.85% was performed, adjusting turbidity until it was equivalent to the 0.5 McFarland, confirmed by spectrophotometrical reading at 530 nm. The final suspension was obtained by dilution 1:50 in sterile saline, followed by further dilution of 1:20 in RPMI culture medium. For bacterial species, inoculate were obtained from cultures in Mueller-Hinton agar for 24 h at 35°C. Similarly, a suspension whose turbidity was adjusted in the spectrophotometer, at 630 nm, was prepared and diluted 1:10 in Mueller-Hinton broth. The filamentous fungus evaluated in this assay was initially incubated on potato dextrose agar for 7 days at 30°C. The colonies were covered with sterile saline, and the resulting conidial suspension was transferred to a sterile tube, where the density was adjusted. Then, the suspension was diluted 1:50 in RPMI. To the wells of microdilution plates, 200 µL of crude extract or fractions were added, which were diluted in the plate resulting in the different concentrations. For yeasts and filamentous fungus were inoculated 100 µL of suspension, followed by incubation at 30°C for 48 h. For bacteria, 10 µL of suspension were inoculated, and incubated at 35°C for 24 h. Finally was performed the visual reading of microbial growth, and the wells with the lowest extract concentration which there was no microbial growth was considered the minimum inhibitory concentration (MIC).

Antimycobacterial assay

The strains of mycobacteria were grown onto Löwenstein-Jensen medium and incubated for 3-5 days. Suspensions of these cultures were standardized using the scale 0.5 to Mac Farland, diluted in Middlebrook 7H9 broth supplemented with 10% OADC (oleic acid-albumin-dextrose-catalase) (Difco Laboratories, Detriod, Mich) and 0.2% glycerol (MD7H9) until the concentration of 10^5 CFU/mL. The CE and fractions tested were dissolved in dimethylsulfoxide (DMSO) at a concentration of 50 mg/mL and diluted in MD7H9 until the desired concentrations, beginning the series with 2500 µg/mL. Susceptibility tests were performed by the broth microdilution method, according to CLSI M7-A7 (2006). Mycobacterial inoculums (100 µL) were placed in each well of a microdilution plate, as well as the extracts at corresponding concentrations. The test was performed in triplicate. The plates of *M. smegmatis* were incubated for 2 days, *M. avium* for 5 days and *M. tuberculosis* for 7 days at 37°C. In order to verify the growth of microorganisms, the dye 3-(4,5 dimethyl thiazole-2yl) -2,5 diphenyl tetrazolium bromide (MTT- Sigma, USA) was added to each plate well. Then, the lowest extract concentration can produce inhibition of visible growth of microorganisms was considered as MIC.

RESULTS AND DISCUSSION

The activities of the CE and fractions from the *S. guaraniticum* leaves against bacteria and fungi are shown in Table 1. It was considered that if the extracts displayed a MIC less than 100 µg/mL, the antimicrobial activity was good; from 100 to 500 µg/mL the antimicrobial activity was moderate; from 500 to 1000 µg/mL the antimicrobial activity was weak; over 1000 µg/mL the extract was considered inactive (Holetz *et al*., 2002; Morales *et al.*, 2008). In view of these criteria, it was possible to observe that the extracts were active against Gram-positive bacteria, with variables MICs from 32 to 1024 µg/mL. The AcOEt fraction showed good activity against *Staphylococcus intermedius* and *Listeria monocytogenes*. *Staphylococcus intermedius* is a member of the normal flora of dogs and is also a major opportunistic pathogen responsible for the common canine skin condition pyoderma, and can occasionally cause severe infections of humans (Bannoehr *et al*., 2007). The facultative intracellular bacteria *Listeria monocytogenes* is associated with serious human and animal infections, including abortion and septicemia. It is considered a pathogen of major concern due to high occurrence in foods and high mortality rate associated with listeriosis (Wang *et al*., 2013). Probably, these activities are due to highest content of polyphenols found in this fraction, as these metabolites have antimicrobial activity recognized (Daglia, 2012), and also by good antioxidant capacity by the DPPH method described previously (Zadra *et al.*, 2012). Xiong *et al.* (2013) when performing a screening and identification of the antibacterial bioactive compounds from *Lonicera japonica* leaves found that the phenolic compounds present in the plant extract were responsible for the antibacterial activity shown.

Table. 1: Minimum inhibitory concentrations (MIC) for CE and fractions of *S. guaraniticum* against bacteria and fungi.

Microorganisms	MIC (µg/mL)			
	CE	$CHCl_3$	AcOEt	BuOH
Gram-positive bacteria				
Staphylococcus aureus	256	256	1024	>1024
Staphylococcus intermedius	256	128	64	256
Streptococcus agalactiae	256	128	256	256
Enterococcus faecalis	256	256	256	128
Micrococcus luteus	32	128	512	128
Listeria monocytogenes	128	128	64	512
Gram-negative bacteria				
Pseudomonas aeruginosa	512	>1024	>1024	>1024
Klebsiella pneumoniae	>1024	>1024	>1024	>1024
Escherichia coli	>1024	>1024	>1024	>1024
Yeasts				
Candida albicans	1024	>1024	>1024	>1024
Cryptococcus neoformans	>1024	>1024	>1024	>1024
Filamentous fungi				
Aspergillus fumigatus	>1024	>1024	>1024	>1024

The best activity was found for the CE against *Micrococcus luteus* (MIC = 32 µg/mL), bacteria that causes minor infections especially in patients with suppressed immune system (Bonjar, 2004), and can be attributed to an interaction between the different components of the extract, such as polyphenols, tannins and alkaloids presented (Zadra *et al.*, 2012), whose nature is very complex. In general, the CE and fractions showed moderate activity against other Gram-positive strains, including *Streptococcus agalactiae* and *Enterococcus faecalis*, pathogens associated with important diseases of clinical importance.

Concerning the Gram-negative bacteria, only the CE showed a weak activity against *Pseudomonas aeruginosa*, the other extracts were inactive against *Klebsiella pneumoniae* and *Escherichia coli*. These results can be explained, at least in part, because beside the efflux pumps, Gram-negative bacteria presents some other characteristic particularities in their outer membrane like the polysaccharides that contributes to cell surface properties, such as membrane permeability and antibiotic susceptibility (Mahlke *et al.*, 2009). All the extracts were inactive against fungi, both as yeasts *Candida albicans* and *Cryptococcus neoformans* and for the filamentous fungi *Aspergillus fumigatus*.

Mycobacteria are Gram-positive, non-motile and obligate aerobic bacteria. Due to the slow growth rate and pathogenicity of *M. tuberculosis*, many groups utilize faster growing and/or non-pathogenic mycobacteria as the test organism, including *M. smegmatis and M. avium* (Copp, 2003; Kuete *et al.*, 2012; Boligon *et al.*, 2012). In this study, the antimicrobial activity of the CE and fractions of *S. guaraniticum* was evaluated (Table 2), observing moderate activity for the CE, $CHCl_3$ and BuOH fractions against *M. smegmatis*, especially the $CHCl_3$ fraction, which showed the lowest MIC value (156 µg/mL). This activity is probably due to the highest contents of tannins and flavonoids present in the fraction, which also showed the best antioxidant activity assessed by inhibition of lipid and protein oxidation, demonstrating radical scavenging properties (Zadra *et al*., 2012).

It is well established that the antioxidant activity and phenolic compounds of plant extracts is related to its antimicrobial activity (Katalinic *et al.*, 2013; Koysomboon *et al.*, 2006; Alves-Silva *et al*., 2013). No significant activity was observed against *M. tuberculosis* and *M. avium*. Similar results were described by Cruz *et al.* (2012) regarding the activity of *Ficus luschnathiana*, whose butanolic fraction showed MIC value of 156.25 µg/mL for *M. smegmatis*, and was considered a promising antimycobacterial activity.

Table 2. Minimum inhibitory concentrations (MIC) for CE and fractions of *S. guaraniticum* against mycobacteria.

Microorganisms	MIC (µg/mL)			
	CE	$CHCl_3$	AcOEt	BuOH
Mycobacterium tuberculosis	>2500	>2500	>2500	>2500
Mycobacterium avium	1250	1250	2500	>2500
Mycobacterium smegmatis	312	156	2500	312

The solubility property of plant metabolites extracted with solvents of different polarity appears to contribute to the outcome of the antimicrobial assays employed (Othman *et al.*, 2011). Higuchi *et al.* (2011) carried a bioassay-guided fractionation of the chloroform fraction from *Byrsonima fagifolia* leaves, resulting in the isolation of terpenoids with promising

activity against *M. tuberculosis*. In another similar study, the compound methyl caffeate, isolated from the methanolic extract of *Solanum torvum* fruits, presented prominent antimycobacterial activity (Balachandran *et al.*, 2012).

CONCLUSIONS

The results show that the CE and fractions from *S. guaraniticum* leaves possess good activity against *Staphylococcus intermedius*, *Listeria monocytogenes* (AcOEt fraction) and *Micrococcus luteus* (CE), and in most cases, moderate activity against Gram-positive bacteria. Although the CE has shown weak activity against *Pseudomonas aeruginosa*, it can be considered that the extracts were practically inactive against Gram-negative bacteria and inactive for fungi. Moderate activity against *Mycobacterium smegmatis* were observed for CE, BuOH, and especially for the $CHCl_3$ fraction, which can be attributed to its good antioxidant activity and the highest content of flavonoids, tannins and alkaloids previously described. These findings suggest that *S. guaraniticum* leaves have antimicrobial and antimycobacterial potential and may come to be used primarily to treat diseases associated with Gram-positive bacteria, requiring further studies regarding the isolation of compounds in order to better understand which substances are responsible for these activities.

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