American Journal of Plant Sciences, 2013, 4, 2193-2198
Published Online November 2013 (http://www.scirp.org/journal/ajps)
http://dx.doi.org/10.4236/ajps.2013.411272

Scientific Research

Antimicrobial Activity and Rates of Tannins in *Stryphnodendron adstringens* Mart. Accessions Collected in the Brazilian Cerrado

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Received September 2nd, 2013; revised October 20th, 2013; accepted October 25th, 2013

ABSTRACT

Inner bark extracts of *Stryphnodendron adstringens*, a leguminous tree species known as Barbatimão, are often incorporated to phytotherapic formulations due to their antimicrobial and healing activities. In this study, extracts from *S. adstringens* accessions collected in 12 distinct locations were investigated in order to determine the rates of tannins in inner barks and to validate *S. adstringens* antibacterial and antifungal effectiveness. Yields of tannins were quantified by colorimetric assay following methodology described in the Brazilian Pharmacopoeia and the antimicrobial activity was determined by microdilution technique proposed by the National Committee for Clinical Laboratory Standards using *S. adstringens* hydroalcoholic and aqueous extracts and semi-purified fractions. Investigated extracts did not present significant antibacterial activity though aqueous extracts exhibited antifungal effect against both *Trichophyton rubrum* mutant and clinical strains (MIC 156 µg/mL). A positive correlation between tannin concentration and antifungal activity was observed and the accessions collected in Delfinópolis (MG) were considered elite.

Keywords: Cerrado; Barbatimão; Genetic Diversity; Phytoterapy

1. Introduction

Stryphnodendron adstringens, a leguminous tree species endemic to the Cerrado (savanna biome) is popularly known as barbatimão and its inner bark extracts are traditionally used for ulcer and wound healing [1]. *S. adstringens* extract is rich in condensed tannins which are known to induce tissue repair [2].

Preclinical trials reported the regenerative action of *S. adstringens* inner bark aqueous extracts [3] and clinical trials validated *S. adstringens* healing action on pressure ulcers [4], leading to the development of an effectively commercialized phytopharmaceutical brand named Fitoscar®.

Several studies using *S. adstringens* inner bark extracts have reported their efficacy as anti-inflammatory in subacute and chronic models of inflammation [5], antinociceptive [6], anti-protozoal [7] and antimicrobial [8].

Human infections caused by fungi and bacteria increased drastically in the last years. The treatment of mycosis is based on the therapy used against bacteria and antifungals, which are less prevalent if compared to antibacterial agents. Additionally, the therapeutics used against microorganisms is not always effective, causing resistance or recurrence, producing significant toxicity. The dermatophyte *Trichophyton rubrum* which usually causes well-characterized superficial mycosis acts as invasive in immunodepressed patients [9,10], reported the *in vitro* occurrence of a mutant of the *T. rubrum* (*gri*1) with a knock-out mutation in the ABC genes, that are drug sensitive and can be used to test new compounds with specific fungitoxicity [11].

Considering that the production of secondary metabolites in plants and the consequent therapeutic activity of a medicinal species are directly affected by its genetics,

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biotic and abiotic environmental conditions and by the interaction of all those aspects, the aim of this work was to quantify the concentration of tannins in 12 accessions of *S. adstringens* collected in 3 different states of Brazil and determine the antimicrobial activity of extracts from those accessions in order to identify prime regions for collecting source material for the production of phytopharmaceuticals.

2. Plant Material

Stems from *S. adstringens* accessions were collected in the cities of Paranapanema, Botucatu and Cristais Paulista (SP); Sacramento, Araxá, Delfinópolis, Lagoa Formosa and Luislândia (MG); Cristalina, Caldas Novas, Campo Alegre and São João da Aliança (GO), totalizing 223 individuals. All collection sites were georeferenced using Global Positioning System (GPS) (**Figure 1**). Inner barks of those stems were dried in oven at 45˚C and ground to powder (40 mesh). Flowering stems were herborized and 2 exsiccates of each individual were deposited at the herbarium of the University of Ribeirão Preto (Voucher n˚ 272 - 718).

3. Hydroalcoholic Extracts

Pulverized plant material (10 g) was extracted with hydroalcoholic solution (100 mL - 7:3 v/v) by static maceration (15 days). The extract was then evaporated and lyophilized.

4. Aqueous Extracts

The aqueous extracts was prepared by decoction boiling 10 g of inner bark powder, of each *S. adstringens* accession, in 100 mL of distilled water for 3 minutes, following vacuum filtration and lyophilization.

5. Fractionation

The lyophilized aqueous crude extract was chromatographed on a Sephadex LH20 column eluted with ethanol (absolute or 50%), methanol (absolute or 50%) and acetone (70%) gradient solvent system. Obtained fractions (186) were analyzed by thin layer chromatog-

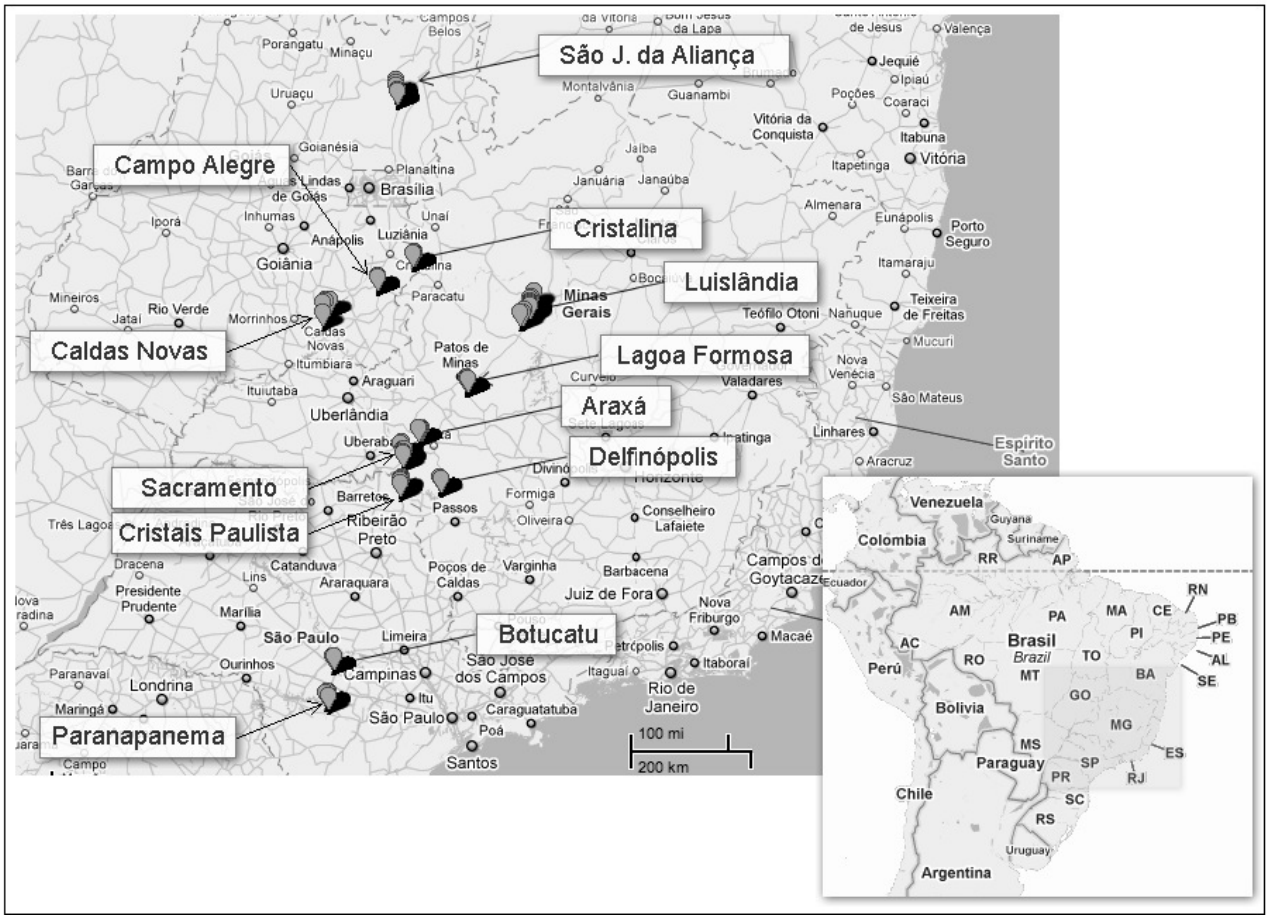

Figure 1. Brazilian Cerrado regions in the States of São Paulo, Minas Gerais and Goiás, sites of collection of *S. adstringens* accessions.

raphy (TLC) and pooled according to their chemical profile in 3 fractions designated I, II and III, being the last the richer in tannin contents.

6. Antifungal Assay

A clinical *Trichophyton rubrum* isolate (ATCC-MYA3108) was obtained from a patient admitted to the UNAERP University Hospital in Ribeirão Preto, SP, Brazil. The mutant strain Δ*Tru*MDR2 was obtained from disruption of the *Tru*MDR2 gene from MYA3108 [12]. Standard techniques for microorganism manipulation and growth were previously described [10]. Susceptibility of the MYA3108 and Δ*Tru*MDR2 mutant strains was tested by assessing the minimal inhibitory concentrations (MIC) values exhibited by different concentrations of aqueous extract and fractions of *S. adstringens* diluted in 10% dimethyl sulfoxide (DMSO) according to the M38-A microdilution technique proposed by the National Committee for Clinical Laboratory Standards (2002) [13]. The final concentration of DMSO, used in the antifungal assay was fixed at a maximum of 0.5%. Microliter trays were incubated at 28˚C and MICs were recorded after 7 days of incubation. The MIC_{100} was defined as the lowest concentration of the extract or fraction that completely inhibited the growth of fungal strains. The assays were carried out in three independent experiments performed in triplicate. Fluconazole (0.07 mg/mL) was used as reference control.

7. Antibacterial Assay

Staphylococcus aureus (ATCC 6538) and *Escherichia coli (*ATCC 25922) strains were used as test organisms. Antimicrobial activity against the ATCC strain was evaluated according to the microdilution method in a BHI medium [14]. Briefly, each experimental well containing different concentrations of crude extract and fractions diluted in 10% DMSO was inoculated with the bacterial suspension in a final concentration of 105 CFU/mL. DMSO final concentration for antifungal assay was fixed at a maximum of 0.5%. Microdilution trays were incubated at 37˚C and the MIC100, as defined above, was recorded after 24 hours of incubation. MIC_{100} was defined as the lowest concentration of the extract or fraction that completely inhibited the growth of bacterial strains. The assays were carried out in three independent experiments performed in triplicate. Ampicillin and chloramphenicol (10 mg/mL) were used as reference controls.

8. Tannin Evaluation

Tannin concentration was determined by modified colorimetric assay, absorbance wavelength at 750 nm, as described in the Brazilian Pharmacopoeia [15] for *S. adstringens* cortex. Powdered bark (0.75 g) was placed in a round-bottomed flask (250 mL) containing 100 mL of distilled water and heated for 30 min at 90˚C - 100˚C temperature. After cooling under running water the extract was transferred to volumetric flask (250 mL) and the volume was completed with distilled water. After decantation, the solution was filtered through filter paper for three times.

9. Statistical Analysis

Experiments were conducted in a Completely Randomized Design (CRD), and the statistical analysis of obtained data was carried out using the software SISVAR, Federal University of Lavras, MG, Brazil, applying the F test to verify differences between treatments and the Scott-Knot test ($p > 0.05$) for comparison of treatment means. Pearson's correlation was used to determine the correlation between concentration of tannins and minimal inhibitory concentrations (MIC).

10. Results and Discussion

MIC values determined for *S. adstringens* extracts indicated their significant antifungal activity. The MIC value obtained for aqueous crude extract was 56 µg/mL against both strains of *T. rubrum*. The fraction I presented higher antifungal activity for ATCC MYA3108 mutant strain and for Δ*Tru*MDR2 wild strain (312 µg/mL and 1250 µg/mL respectively).

Obtained results indicate a synergic action among compounds present in *S. adstringens* crude extract that can enhance antifungal activity (**Table 1**). [16], reported similar results when testing the efficacy of *S. adstringens* extracts against different strains of the same microorganism.

The synergic effect of secondary metabolites found in plant crude extracts has been continuously demonstrated. [17] screening *Plinia glomerata*, a plant species as rich in phenolic compounds as *S. adstringens*, reported that *P. glomerata* crude extract showed more significant anti-

Table 1. MIC values (µg/mL) determined for aqueous extract and fractions from *S. adstringens* plants collected in Delfinópolis-MG against *T. rubrum* strains.

Material	*T. rubrum* strains	
	Mutant	MYA3108
Crude extract	156	156
Fraction I	312	1250
Fraction II	62500	62500
Fraction III	1250	1250
Fluconazole	70	70

fungal activity than the purified fractions or the pure compounds isolated from that plant.

With the exception of the accessions collected in Delfinópolis every other presented different MIC values for each individual and dissimilarities on tannin concentration were observed inter and intra plant populations. Considering the concentration of tannin the *S. adstringens* accessions were divided into 2 groups: the first group comprised individuals from Botucatu, Sacramento, Araxá, Lagoa Formosa, Cristalina, Caldas Novas and São João da Aliança which presented lower tannin rates (21.76% to 28.34%) and the individuals from Paranapanema, Cristais Paulista, Delfinópolis, Luislândia and Campo Alegre composed the second group which exhibited higher percentages of tannins (30.44% to 37.09%). Accordingly, all the investigated accessions presented superior rates of tannins considering the 8% minimum recommended by the Brazilian Pharmacopoeia [15].

The variation found in the accumulation of tannins in *S. adstringens* accessions may be related to both genetic diversity and edaphic characteristics. Soil collected in the natural habitat of the investigated accessions of *S. adstringens* was structurally different and according to [18] soil fertility directly influences the production of tannins in *S. adstringens*, being that populations native to less fertile soil accumulate higher amount of tannins.

S. adstringens collected in Delfinópolis (MG) presented higher rates of tannins and significant inhibition against *T. rubrum* strains (**Table 2**). Moreover it was observed that the greater the production of tannin, the lower was the volume of extract necessary for inhibiting mutant strain (r^2 = 0.339; **Figure 2**) and wild strain (r^2 = 0.259; **Figure 3**), those results confirm that tannins play a role on *S. adstringens* antifungal activity and that other compounds like flavonols and polymeric tannins isolated by [6] may comprise a synergic action that promote antifungal activity.

[16] reported three hypothesis that might explain the antimicrobial mechanism of tannins: inhibition of enzyme activity by complexation with substrates of bacteria and fungi; direct action of tannins on the microorganism metabolism, through the inhibition of oxidative phosphorylation; a mechanism involving the complexation of tannins with metabolic ions, decreasing the availability of essential ions to the metabolism of the microorganisms.

Regarding the antimicrobial activity of *S. adstringens*, the lyophilized crud extracts fro all investigated accessions showed no antibacterial activity against *S. aureus* (ATCC 6538) and *E. coli* (ATCC 25922) presenting MIC values > 10.000 µg/mL. Similar results were reported by [19], investigating hydroalcoholic extract from a single *S. adstringens* plant against *E. coli* strain. However, as there are reports on the bactericidal effect of *S. adstringens* hydroalcoholic extract (96:4 v/v) against *Staphylococcus aureus* (ATCC 12692), *Streptococcus mitis* and *Lactobacillus casei* [16] it would be necessary additional investigations to validate *S. adstringens* bactericidal activity.

11. Conclusions

In this work the aqueous extract exhibited significant activity and it was observed a positive correlation between tannin production and antimicrobial action.

Plants collected in Delfinópolis, MG, presented higher rates of tannin and also greater inhibition against *T. rubrum*

Table 2. Rates of tannins and MIC values (µg\mL) obtained for *S. adstringens* aqueous extracts against *T. rubrum* strains.

Collection sites	% Tannin	MIC *T. rubrum* fungal strains	
		Mutant	MYA3108
Araxá (MG)	21.81b	351.56a	546.87c
Botucatu (SP)	28.34b	421.87a	499.99b
Caldas Novas (MG)	27.34b	390.62a	468.75b
Campo Alegre (GO)	30.80a	304.68a	429.68b
Cristais Paulista (SP)	30.44a	440.62a	625.00b
Cristalina (GO)	21.76b	359.37a	484.37b
Delfinópolis (MG)	32.85a	249.98a	273.42a
Lagoa Formosa (MG)	26.72b	888.88c	343.75a
Luislandia (MG)	37.09a	381.94a	425.34b
Paranapanema (SP)	32.20a	296.87a	578.12c
Sacramento (MG)	23.87b	507.81a	359.65a
São João da Aliança (GO)	23.80b	687.50b	765.62d

Means followed by the same letter do not statistically differ (Scott-Knott P > 0.05). Fluconazole (70 µg/mL) was used as reference control.

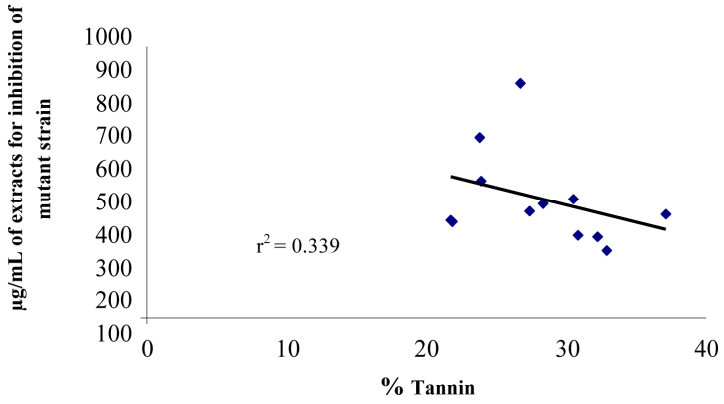

Figure 2. Correlation between tannin concentration and MIC values for *S. adstringens* extracts against ΔTruMDR2 *T. rubrum* mutant strain.

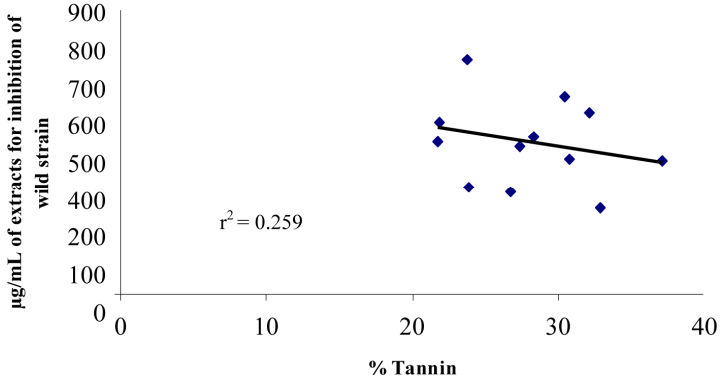

Figure 3. Correlation between tannin concentration and MIC values for *S. adstringens* extracts against MYA3108 *T. rubrum* strain.

strains, indicating the superiority of genotypes native to that region. Obtained results evidence that *S. adstringens* accessions from that population could be selected for genetic improvement programs and used as high-quality source material for the production of phytopharmaceuticals.

12. Acknowledgements

We thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial support (Process: 2008/00762-0; 2008/10499-4) and to Professor Nilce M. Martinez-Rossi for kindly providing the *T. rubrum* mutant strain.

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