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Synergistic activity from *Hymenaea courbaril* L. and *Stryphnodendron adstringens* (Mart.) Coville against multidrug-resistant bacteria strains

Álan Alex Aleixo[1], Vidyleison Neves Camargos[1], Karina Marjorie Silva Herrera[1], Ana Cláudia dos Santos Pereira Andrade[1], Michelli dos Santos[1], Vívian Correia Miranda[1], Rafaella Souza Carvalho[1], Juliana Teixeira de Magalhães[1], José Carlos de Magalhães[2], Luciana Alves Rodrigues dos Santos Lima[3] and Jaqueline Maria Siqueira Ferreira[1]*

[1]Laboratório de Microbiologia, Universidade Federal de São João del-Rei (UFSJ), Campus Centro Oeste Dona Lindu, Divinópolis, Minas Gerais, Brazil.
[2]Departamento de Engenharia de Bioprocessos e Biotecnologia, Universidade Federal de São João del-Rei (UFSJ), Campus Alto Paraopeba, Ouro Branco, Minas Gerais, Brazil.
[3]Laboratório de Fitoquímica, Universidade Federal de São João del-Rei (UFSJ), Campus Centro Oeste Dona Lindu, Divinópolis, Minas Gerais, Brazil.

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Infections caused by multidrug-resistant bacteria are a problem of public health, turning the search for natural products an alternative to antibiotics of great importance. The aim of this study was to investigate the *in vitro* antimicrobial activities of *Hymenaea courbaril* and *Stryphnodendron adstringens* against bacterial clinical isolates. The crude extracts of both vegetal species in study showed bacteriostatic activity against almost all bacteria evaluated, with minimum inhibitory concentration (MIC) ranging from 125 to 1250 µg/ml. The bacteriostatic activity was observed in all the fractions of *H. courbaril* and *S. adstringens* against at least one bacterial strain, except in the fraction dichloromethane of *S. adstringens*. In regard to bactericidal activity, *H. courbaril* was active only against *E. faecalis*, and *S. adstringens* showed activity against all bacterial strains, except to *Enterococcus faecalis*. The combination of extracts showed potent synergistic antimicrobial activity, with MIC values of 31.25 µg/ml against *Acinetobacter baumannii*, *Escherichia coli* and *Staphylococcus aureus*. *S. adstringens* were considered less cytotoxic compared to *H. courbaril* and the half-maximum cytotoxic concentration (CC_{50}) resulting from the combination of the two plants was 0.0082 ± 3.19 mg/ml. The results showed for the first time the synergic antibacterial activities of *H. courbaril* and *S. adstringens* against resistant bacteria, suggesting their potential use to development of new drugs.

Key words: *Hymenaea courbaril*, *Stryphnodendron adstringens*, antimicrobial activity, synergism, multidrug-resistant bacteria.

INTRODUCTION

The overuse of antibiotics and consequent selective pressure is thought to be the most important factor contributing to the increasing occurrence of resistance to antibiotics, which represents a public health issues worldwide (Ang et al., 2004). Moreover, over last decade, there has been dramatic reduction in the number of

pharmaceutical companies developing new antimicrobial agents (Boucher et al., 2009). In front of the challenge of searching for therapeutic tools that combat bacterial resistance, plants, especially those with ethnopharmacological uses, have been the main sources for the early discovery of new drugs, since the plant biological diversity is a source of a wide range of bioactive molecules, acting by different mechanisms (Chin et al., 2006). Thus, plant extracts can be used as sources of new drugs or antimicrobial compounds, which are of great importance since the emergence of resistant strains makes difficult the treatment of infections (Alviano and Alviano, 2009).

The Fabaceae vegetable family presents more than 490 species of medicinal plants, including *Hymenaea courbaril* and *Stryphnodendron adstringens*, which are used in folk medicine (Gao et al., 2010). *H. courbaril* L. is used in popular medicine as, fluidificant and expectorant, astringent, anti-diarrheal, anti mycotic, and anti-inflammatory (Correia et al., 2008). Martins et al. (2010) described the antibacterial activity of crude ethanol extracts of the bark and pulp of mealy from *H. courbaril* and the best results were obtained with minimum inhibitory concentration (MIC) of 350 µg/ml against clinical isolates of *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*.

The *S. adstringens* (Mart) Coville is employed in folk culture in the form of a decoction or infusion as an astringent, anti-diarrheal, antimicrobial and hypoglycemic agent for the treatment of gynecological problems and healing wounds (Ishida et al., 2009). Studies showed antimicrobial activity in the extract obtained from the bark of this plant against *S. aureus, E. coli*, *P. aeruginosa* and *S. epidermidis* (Audi et al., 2004; Souza et al., 2007). There are some data on antimicrobial activity and synergy between extracts of *H. courbaril* and *S. adstringens* since the promising potential use of medicinal plants in treatment of diseases. So, the current investigation carried out the antimicrobial activity from ethanol extract and fractions obtained from barks of *H. courbaril* and *S. adstringens* and their synergism was evaluated against six bacteria of clinical interest, to prospective new antibacterial therapy.

MATERIALS AND METHODS

Plant collection

The barks from *H. courbaril* (BHCB 159,399) and *S. adstringens* (BHCB 159,400) were collected in the city of São Sebastião do Oeste, Minas Gerais, Brazil, in August, 2011. The voucher specimens were deposited at the Herbário do Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil.

Plant extract and fractions preparation and phytochemical screening

The plants material were dried at 40°C and triturated. The material (1000 g) was extracted by cold maceration in 4.0 L of ethanol P.A (Vetec, Brazil) for a period of 10 days at room temperature (25 ± 2°C) for 10 days. After it was filtrated with Whatman filter paper and concentrated in a rotary evaporator (IKA equipment, model RV10) at 40°C under reduced pressure to yield ethanol extract. The dried crude extract was obtained after lyophilization. Part of this extract (5.0 g) was dissolved in ethanol/water (7:3) and then partitioned successively with hexane (C_6H_{14}), dichloromethane (CH_2Cl_2) and ethyl acetate (AcOEt) (100 ml, 3 times with each solvent), resulting in hexane (F1), dichloromethane (F2), ethyl acetate (F3), and hydroethanol (F4) fractions, respectively (Araújo et al., 2013). The extracts and fractions were maintained in the dark and refrigerated at 4°C. They were solubilized in dimethylsulfoxide (DMSO) 2% v/v. The extract and fractions were screened qualitatively for the presence of different classes of natural products such as alkaloids, steroids, triterpenoids, coumarins and flavonoids by thin-layer chromatography (TLC) (Wagner et al., 1996). The analysis was performed on Merck silica gel 60 F254 aluminum plates. Other tests described by Matos (2000) were carried out to determine the presence of tannins and saponins.

Microorganisms and stock conditions

Six clinical isolates provided by Hospital São João de Deus, Divinópolis, Minas Gerais, Brazil, were used in antibacterial tests: *Acinetobacter baumannii* 7810, *Klebsiella pneumoniae* 7845, *P. aeruginosa* 530, *E. coli* 3004, *S. aureus* 8066 and *E. faecalis* 3110. The origin of strains was performed from urine, except for *A. baumannii* and *S. aureus,* obtained from tracheal secretions and exudates of injury, respectively. The resistance profile was performed by the automated system of identification and antibiogram (VITEK2 compact, bioMérieux): aminoglycosides, β-lactams, fluorquinolones, polymyxins, carbapenems, fosfomycin, nitrofurans, glycylcyclines and sulfonamides. Bacteria were stored in nutrient broth with 10% glycerol at freezer -80°C and subsequently activated in nutrient broth at 37°C for 24 h for use in assays. This study was approved by Ethics Committee of Hospital São João de Deus, Divinópolis, Minas Gerais, Brazil (Protocol: 186/2011).

Minimum inhibitory concentrations (MIC) and minimal lethal concentration (MLC) assays

The MICs were determined using the broth microdilution method, with modifications from standards recommended according to the Clinical and Laboratory Standards Institute (CLSI, 2003). The crude extracts and fractions were diluted in DMSO at concentrations 1250, 1000, 750, 500, 250, 125, 62.5, 31.25, 15.62 and 7.81 µg/ml. Bacterial were cultured on Mueller-Hinton agar and following bacterial growth, a standardized bacterial suspension equivalent to 0.5 McFarland was used. Subsequently, 50 µl of this solution were diluted in Mueller-Hinton broth (MHB) to a concentration of approximately 5×10^5 CFU/ml. An inoculum of 125 µl was added to

*Corresponding author. E-mail: jackmaria4@gmail.com, jaque@ufsj.edu.br. Tel: +55 37 8813-1232. Fax: +55 37 3221-1352.

Table 1. Phytochemical study of crude extract (CE) and hexane (F1), dichloromethane (F2), ethyl acetate (F3) and hydroethanol (F4) fractions from *H. courbaril* and *S. adstringens.*

Metabolites	*H. courbaril*					*S. adstringens*				
	CE	F1	F2	F3	F4	CE	F1	F2	F3	F4
Steroids/Triterpenoids	+/-	+/-	+++	-	+++	-	-	+/-	-	+/-
Flavonoids	+++	-	-	-	-	+++	++	++	+++	++
Coumarins	+/-	+/-	+++	++	+/-	+/-	+/-	+/-	+/-	++
Saponins	-	-	-	-	-	-	-	-	-	-
Alkaloids	+++	+/-	+++	+++	+++	++	-	+/-	++	+/-
Tanins	+/-	+/-	+/-	-	+/-	++	+/-	+/-	-	+++

(-) absence, (+/-) minimal presence, (+), (++) and (+++) grading presence

25 µl of each sample concentration plant in 100 µl of MHB in 96-well microplates. After incubation for 24 h, turbidity of the broth in the wells was observed. MIC was defined as the lowest concentration of the extract at which no visible growth could be detected. All assays were performed in triplicate and repeated three times in independent experiments. Sterile 2% DMSO was used as negative vehicle control and a Streptomycin/Penicillin solution (Sigma-Aldrich, USA) as positive control of inhibition. Following incubation of MICs plates, the minimal lethal concentration (MLC) were determined by removal of 25 µl from wells without visible turbidity and transferred to Mueller Hinton agar by a Spread-Plate method. The lowest concentration that resulted in absence of bacterial growth was determined as the MLC.

Synergy testing by microdilution checkerboard

The synergistic effects were assessed by the checkerboard test as previously described by Lee et al. (2012), with adaptations. Samples of crude extract of the species studied were serially diluted in concentrations ranging from 1.95 to 125 µg/ml. Subsequently, solutions of the same concentration were combined in a 1:1 ratio to evaluate the antimicrobial effect resulting from the interaction of *H. courbaril* and *S. adstringens*. The fractional inhibitory concentration index (FIC index) is the sum of the FICs of each of the drugs, which in turn is defined as the MIC of each drug when it is used in combination divided by the MIC of the drug when it is used alone. All experiments were independently repeated three times. Values of FIC index less than or equal to 0.50 were considered to be indicative of a synergic effect. Values ranging from 0.51 to 1.00 indicated an additive effect, values from 1.01 to 2.00 were considered as indifferent and values above 2.00 indicated an antagonist effect.

Cell culture and cytotoxicity analysis by the MTT assay

Vero cells (ATCC CCL-81) were cultured in Dulbecco's modified eagle medium (DMEM) with 2% of fetal bovine serum (FBS), at 37°C, 5% of CO_2 atmosphere, until reach 95% of confluence. Cytotoxicity of crude extracts and fractions (1000 to 0.025 µg/ml) was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Merck solution 2 mg/ml in phosphate buffered saline (PBS) (Twentyman and Luscombe, 1987). Each sample was assayed in three replicates.

Statistical analyses

All tests were made in triplicate in three independent experiments. When appropriate, mean ± standard deviation were used to describe the results. The half-maximum cytotoxic concentration (CC_{50}) was determined by non-linear regression using GraphPad Prism, 5.0 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Phytochemical screening

The phytochemical analysis of *H. courbaril* revealed the presence of alkaloids, coumarins, flavonoids, steroids/triterpenoids and tannins in ethanol crude extract (Table 1). Alkaloids and coumarins are present in all fractions. Steroids/triterpenoids and tannins also were found in hexane, dichloromethane and hydroethanol fractions. Saponins were absent in the samples. On the other hand, the phytochemical analysis of *S. adstringens* revealed the presence of alkaloids, coumarins, flavonoids and tannins in ethanol crude extract (Table 1). Coumarins and flavonoids are present in all fractions. Alkaloids, steroids/ triterpenoids and tannins were found in dichloromethane and hydroethanol fractions. Alkaloids also present ethyl acetate fraction and tannins in hexane fraction. Saponins were absent in the samples.

Resistance profile of clinical isolates

Table 2 shows the profile resistance of the clinical isolates to different antibiotics classes. The profile revealed by antibiogram showed that the *E. coli* 3004 was the strain that has greater resistance to antibiotics, followed by *K. pneumoniae* 7845, *P. aeruginosa* 530, *A. baumannii* 7810, *E. faecalis* 3110 and *S. aureus* 8066.

Antimicrobial activity

The crude extract of *H. courbaril* displayed bacteriostatic activity against all bacteria, except *P. aeruginosa* 530 (Table 3). The MIC values found for *E. faecalis* 3110, *E. coli* 3004, *S. aureus* 8066, *A. baumannii* 7810 and *K.*

Table 2. Resistance profile of clinical isolates in front of different classes of antibiotics used in medical clinic.

Antibiotics	Clinical isolates					
	***A. baumannii* 7810**	***K. pneumoniae* 7845**	***P. aeruginosa* 530**	***E. coli* 3004**	***S. aureus* 8066**	***E. faecalis* 3110**
β-lactam						
Amoxicillin/Clavulanic acid	-	R	R	S	-	-
Ampicilin	R	R	R	S	-	R
Ampicilin/Clavulanic acid	-	-	-	S	-	
Ampicilin/Sulbactan	R	-	-	-	-	R
Aztreonam	R	R	R	S	-	R
Cefepime	R	R	R	R	-	-
Cephalothin	R	R	R	R	-	-
Ceftriaxona	-	-	-	R	-	-
Cefotaxime	R	R	R	R	S	-
Ceftazidime	R	R	I	R	-	-
Imipenem	R	-	-	R	-	-
Ertapenem	-	I	-	R	-	-
Meropenem	R	S	R	S	-	-
Piperaciclin/Tazobactam	R	R	R	R	-	-
Aminoglycosides						
Amikacin	I	S	S	-	-	-
Gentamicin	R	R	R	R	-	I
Fluorquinolones						
Nalidixic acid	-	R	R	R	-	-
Levofloxacin	-	R	R	R	-	S
Norfloxacin	-	-	R	R	R	R
Ciprofloxacin	R	R	R	R	R	R
Polymyxin						
Colistina	S	-	-	-	-	-
Nitrofurane						
Nitrofurantoin	-	R	-	R	-	-
Fosfomicin	-	-	-	R	-	-
Glycylcycline						
Tigecycline	S	-	-	S	S	-
Sulfonamides	-	R	R	R	-	S
Trimethoprim/Sulfametoxazole	-	-	-	-	S	-

S = sensivite; R = resistant; I = intermediate

pneumoniae 7845 were of 125, 250, 500, 750 and 1000 µg/ml, respectively. The bactericidal effect of *H. courbaril* was observed only for *E. faecalis* 3110, being the value of MLC found of 1250 µg/ml. The *S. adstringens* crude extract showed bacteriostatic activity in all bacterial tested (Table 3) with MIC values ranging from 250 to 1000 µg/ml. The best MIC, 250 µg/ml was obtained from *S. aureus* 8066 and the worst, 1000 µg/ml, from *E. faecalis* 3110. The MIC obtained to *A. baumannii* 7810 and *E. coli* 3004 was 500 µg/ml and to *K. pneumoniae* 7845 and *P. aeruginosa* 530 was 750 µg/ml. The bactericidal activity was obtained for all evaluated bacterial, except to *E. faecalis* 3110. The MLC value obtained to *A. baumannii* 7810 was 1000 and 1250 µg/ml to the others strains. The bacteriostatic activity was observed in all the fractions of *H. courbaril* against at

Table 3. Minimum inhibitory concentration (MIC) (µg/ml) and minimum lethal concentration (MLC) (µg/ml) of crude extracts of *S. adstringens* and *H. courbaril* against clinical isolates.

Bacteria	*S. adstringens*		*H. courbaril*		MIC Penicillin-Streptomycin solution
	MIC	MLC	MIC	MLC	
A. baumannii	500	1000	750	-	31.25
K. pneumoniae	750	1250	1000	-	31.25
P. aeruginosa	750	1250	-	-	31.25
E. coli	500	1250	250	-	7.81
S. aureus	250	1250	500	-	31.25
E. *faecalis*	1000	-	125	1250	31.25

(-) = absence of activity

least two bacterial species (Table 4). The MIC values ranged from 125 to 1000 µg/ml, being the smallest against *S. aureus* 8066 in the ethyl acetate (F3) fraction and against *E. faecalis* 3110 in fractions hexane (F1), ethyl acetate (F3) and hydroethanol (F4). The bactericidal effect was observed against *E. faecalis* 3110 in all the fractions tested and against *S. aureus* 8066 in ethyl acetate (F3).

S. adstringens exhibited greater bacteriostatic activity in hexane (F1) and ethyl acetate (F3) with MIC values ranging from 250 to 1250 µg/ml. The hexane (F1) fraction was active against all bacteria evaluated and ethyl acetate (F3) just not demonstrated activity against *E. faecalis* 3110. No effect was observed for dichlorometane (F2) and the hydroethanol (F4) was active only against *E. faecalis* 3110. The bactericidal effect was observed in the hexane (F1) fraction against *E. coli* 3004 and on ethyl acetate (F3) fraction against *K. pneumoniae* 7845, *E. coli* 3004 and *S. aureus* 8066. The results of the combined effect of crude extracts can be observed in Table 5. The combination of extracts exhibited antibacterial activity potential, with MIC value of 31.25 µg/ml against *A. baumannii* 7810, *E. coli* 3004 and *S. aureus* 8066, indicating interaction of the type synergistic between the extracts (FIC index < 0.9).

Cytotoxicity analysis by the MTT assay

The evaluation of cytotoxic effects of crude extracts and fractions were conducted by mitochondrial reduction technique through the MTT reagent (data not show). The CC_{50} of the hydroethanol crude extract of *H. courbaril* was 4.33 ± 3.4 mg/ml. The more cytotoxic fraction derived from this extract was the hexane with CC_{50} of 3.37 ± 2.25 mg/ml. The Vero cell line showed low sensitivity to hydroethanol fraction, where the CC_{50} observed was 1.67 ± 3.5 mg/ml. The CC_{50} resulting from the combination of two plant species under study was 0.0082 ± 3.19 mg/ml. The hydroethanol crude extract of *S. adstringens* presented CC_{50} of 0.094 ± 3.3 mg/ml. The most cytotoxic fraction of this species was the hexane with CC_{50} of 0.016 ± 5.2 mg/ml. On the other hand, the sample that showed least cytotoxicity against cell line tested was the ethyl acetate fraction with CC_{50} of 0.234 ± 1.3 mg/ml.

DISCUSSION

Extracts with MIC less than 100 µg/ml, the antimicrobial activity is good, from 100 to 500 µg/ml the antimicrobial activity is moderate, from 500 to 1000 µg/ml the antimicrobial activity is weak, and over 1000 µg/ml the extract is considered inactive (Holetz et al., 2002; Aleixo et al., 2014). In this work, it was necessary to use a mix of streptomycin and penicillin as controls because some bacteria isolated from clinical samples showed resistance to one of these antibiotics when used individually.

There are different approaches to cure and control the infection caused by the multidrug-resistant (MDR) strains bacteria, one of which is by isolation of active phytochemicals that can help to prevent the spread of infection. Bacteria presented in this study showed resistance to different antibiotics classes, which makes them important models to mimic the infections that have been occurred in hospitals. Another method is to formulate new synergistic combinations using active phytochemicals that have antimicrobial properties. In this work, the synergistic effect of the crude extracts of *H. courbaril* and *S. adstringens* showed a reduction of MIC value (<100 µg/ml) in three of the four tested microorganisms (Table 5). Such synergistic combinations may result in increased therapeutic effects and reduce the chances of toxicity dose-dependent (Boucher and Tam, 2006).

The results showed by fractions of *H. courbaril* were more heterogeneous as compared with those of *S. adstringens,* however with a lower number of bacteria. The hexane and ethyl acetate fractions of *S. adstringens* were most active. These were active against the bacterial strains that showed a profile of multi-resistance to several classes of antibiotics, indicating that the mechanisms of action of antibacterial substance are able to overcome

Table 4. Minimum inhibitory concentration (MIC) (µg/ml) and minimum lethal concentration (MLC) (µg/ml) from fractions hexane (F1), dichloromethane (F2), ethyl acetate (F3) and hydroethanol (F4) derived from crude extracts of *S. adstringens* and *H. courbaril* against clinical isolates.

Bacteria	Fractions derived from crude extracts															
	S. adstringens								*H. courbaril*							
	F1		F2		F3		F4		F1		F2		F3		F4	
	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC	MIC	MLC
A. baumannii	750	-	-	-	250	-	-	-	-	-	-	-	-	-	-	-
k. pneumoniae	1250	-	-	-	750	1000	-	-	-	-	-	-	-	-	-	-
P. aeruginosa	1000	-	-	-	750	-	-	-	-	-	-	-	-	-	-	-
E. coli	500	1250	-	-	750	1000	-	-	750	-	750	-	500	-	-	-
S. aureus	1250	-	-	-	250	1250	-	-	-	-	1000	-	125	1250	250	-
E. *faecalis*	1250	-	-	-	-	-	500	-	125	1250	500	1250	125	1250	125	1250

(-) = absence of activity.

Table 5. Effect resulting from combination of crude extracts of *S. adstringens* and *H. courbaril* against clinical isolates.

Bacteria	MIC in combination (µg/ml)	FIC Sa	FIC Hc	FIC index	Interaction
A. baumannii	31.25	0.06	0.12	0.18 < 0.9	sinergic
K. pneumoniae	-	-	-	-	-
P. aeruginosa	nt	-	-	-	-
E. coli	31.25	0.02	0.02	0.05 < 0.9	sinergic
S. aureus	31.25	0.12	0.06	0.18 < 0.9	sinergic
E. *faecalis*	125	0.12	1.0	1.12	additive

(-) = absence of activity. nt = not tested. FIC Sa = FIC *S. adstringens*. FIC Hc = FIC *H. courbaril.* FIC index = sinergic (≤ 0.5), additive, (0.5 to 1.0) and antagonistic (≥ 4.0)

the various barriers of resistance.

Several classes of secondary metabolites are present in the extracts and the fractions of *H. courbaril* and *S. adstringens,* such as alkaloids, coumarins, flavonoids, steroids/triterpenoids and tannins. Cecílio et al. (2012) also observed the presence of coumarins, flavonoids, triterpenoids and tannins in ethanol extract of *H. courbaril* and flavonoids, triterpenoids and tannins in ethanol extract of *S. adstringens*, corroborating with results showed in this study. Tannins are known for antimicrobial properties, acting by different mechanisms (Scalbert, 1991). Triterpenoids from *Callicarpa farinosa* showed antimicrobial activities against different strains of *S. aureus*, with MIC ranging from 2 to 512 µg/ml (Chung et al., 2014). Flavonoids have been reported to possess antimicrobial activity against a wide range of pathogens as flavonoids from *Dorstenia* species that showed activity against methicillin-resistant *S. aureus* (MRSA) strains with MICs values ranged between 0.5 to 128 µg/ml (Dzoyem et al., 2013). Coumarins from *Angelica lucida* showed antimicrobial activity (Widelski et al., 2009). Alkaloids isolated from *Litsea cubeba* presented antibacterial activity against *S. aureus* (Zhang et al., 2012). The results of the antibacterial activity of liquid-liquid fractions showed that *S. adstringens* presented the greater antibacterial effect in hexane fractions and ethyl acetate,

suggesting that the metabolites responsible for this activity are present in these fractions.

Regarding *H. courbaril*, all fractions were active, indicating that this species has a greater diversity of secondary metabolites with antimicrobial activity. Furthermore, a higher number of active fractions of *H. courbaril* against tested Gram positive bacteria was observed. This fact may be due to composition of bacterial wall cell, where the lipopolysaccharide outer membrane that Gram negative bacteria have, restricts the diffusion of hydrophobic compounds, which could lead to greater resistance to antimicrobial substances (Biswas et al., 2013; Tajkarimi et al., 2010). Usually, Gram negative bacteria are more resistant to plant-derived antimicrobials compared to Gram positive bacteria (Biswas et al., 2013; Vlietinck et al., 1995).

Considering that the compounds with intermediate polarity of *S. adstringens* (ethyl acetate) were effective, with lower MIC (Table 4), compared with the most nonpolar compounds (hexane), this may indicate that there are effective components that act on the membranes of microorganisms or affect any transport mechanism. Although less effective, the same fractions (hexane and ethyl acetate) for the species *H. courbaril* also had the same trend as for the antimicrobial effect. This indicates first that the active principle is preserved within the family Fabaceae, which corroborates previous results (Máximo et al., 2006).

This work provides the first reports of potent antimicrobial activity resulting from the combination of the two vegetal species, *H. courbaril* and *S. adstringens* against multi-resistant Gram negative and Gram positive bacterial strains. These results encourage additional studies of extract and fractions from the barks of *H. courbaril* and *S. adstringens* for isolation of the bioactive compounds with antibacterial potential.

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Conflict of interest

The authors do not have any conflicts of interest.

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