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Antimicrobial activities and preliminary phytochemical tests of crude extracts of important ethnopharmacological plants from Brazilian Cerrado

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Screening of native plants with therapeutical effects constitutes a valuable way to enhance biological attributes of medicinal herbs and discover new drugs. Therefore, ethanol and methanol extracts from ten plants collected from Brazilian Cerrado were tested to inhibitory effects against *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853) through disc diffusion. The crude extracts that showed antibacterial activities from *Anacardium humile*, *Psidium guineense* and *Myracrodruon urundeuva*, were tested on the standard strain *S. aureus* ATCC 25923. The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined and the interactions between the plant extracts and the main groups of secondary compounds present were investigated. The MICs of *P. guineense, M. urundeuva* and *A. humile* were all 4.1 μg/L for *S. aureus*. *P. guineense* and *A. humile* extracts tested against *P. aeruginosa* were 8.2 μg/L, whereas the extract from *M. urundeuva* had a MIC of 4.1 μg/L for the same strain. In addition, the observed MBCs were equivalent to the corresponding MICs. There were synergistic interactions in combinations of these plant extracts and tannins and flavonoids were identified in phytochemical analyses. These metabolites may be related to the biological activities that were found, indicating possible candidates for the development of strategies for treatment of infections caused by bacteria tested.

Key words: Crude plant extracts, biological activity, disc diffusion, traditionally use, popular medicine, Cerrado biome.

INTRODUCTION

The use of plants with therapeutic properties is an ancient practice and plants has been used as an important source of bioactive compounds (Pinto et al., 2002; Ncube et al., 2012; Khan et al., 2013; Li et al., 2016). Eating

herbs and leaves to relieve and to cure diseases was the earliest methodologies involving natural products (Viegas et al., 2006). It was through observation and experimentation that primitive peoples discovered the therapeutic properties of plants and disseminated this information from generation to generation (Turolla and Nascimento, 2006). The World Health Organization estimates that 80% of the world population uses medicinal plants as main resource in primary health care and they have been encouraging this practice (WHO, 2002). Medicinal plants and herbal medicines have an important role in therapy, as 25% of prescribed drugs worldwide are of natural origin, plants represent an important source of new biologically active compounds (Canton and Onofre, 2010). Selecting plant species for research and development based on allegations of a given therapeutic effect in humans may be a valuable shortcut to the discovery of pharmaceutical drugs (Schenkel et al., 2004; Viegas et al., 2006). The use of natural products as raw materials for the synthesis of bioactive substances has been widely reported (Schenkel et al., 2004; Viegas et al., 2006; Dias et al., 2012; Cragg and Newman, 2013).

During the last decades, the development of effective pharmaceutical drugs against bacterial infections has revolutionized medical treatment, resulting in a drastic reduction in mortality caused by microbial diseases (Dias et al., 2012; Cragg and Newman, 2013). However, the widespread use of antibiotics have caused bacteria to develop defenses, culminating in the emergence of resistance and imposing serious limitations on the options to treat infections, which represents a threat to public health (Silveira et al., 2006; Valgas et al., 2007). Because the use of plant extracts may constitute a viable alternative therapy to antibiotics (Nascimento et al., 2000), searching for new pharmaceutical drugs or prototypes from plant species has been suggested as a technological measure to solve the problem of multi-resistant bacteria (Silva et al., 2010).

In this context, this study aimed to test crude extracts of ten plants collected from the Brazilian Cerrado (Table 1) against the bacterial strains *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and 15 clinical strains of *S. aureus* isolated from patients at the Hospital Universitário Clemente de Faria (University Hospital) and from clinical laboratories at Montes Claros, State of Minas Gerais, Brazil. The minimum inhibitory concentrations (MICs) and minimum bactericidal concentration (MBCs) of the extracts were determined by microdilution method. The interactions between the extracts of these plants using the disc diffusion methodology and the existence of the main secondary metabolite groups present in the crude extract with antibacterial activity were investigated.

MATERIALS AND METHODS

Ethnobotanical information

The plant material was collected between August and September 2006 at cities of Jaíba (15°20'16"S and 43°40'26"W), Glaucilândia (16°51'00"S and 43°41'49"W) and Claro dos Poções (17°04'48"S and 44°12'32"W) (States of Minas Gerais, Brazil). The botanical material was treated using identification and herborization techniques and was placed in the Montes Claros Herbarium of the Universidade Estadual de Montes Claros, city of Montes Claros, state of Minas Gerais, Brazil.

Screening test (Plant collection)

Leaves from the 10 different species (Table 1) were separated, oven-dried at 50°C, pulverized with a Willey type grinder and crushed on 1:5 ratio (200 g pulverized material per 1000 ml solvent) and two different solvents were used: ethanol and methanol for seven days at room temperature. After filtration, the filtrate was evaporated in a forced-air oven at 50°C for 24 h, and the residue was reconstituted in dimethyl sulfoxide (DMSO) at a concentration of 300 mg/ml (Celloto et al., 2003). Bacterial inhibition tests were performed to *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923) and *P. aeruginosa* (ATCC 27853) by disc diffusion protocol (NCCLS, 2003) using 6 mm diameter blank discs sterile containing 10 µl of the plant extracts. DMSO and 30 µg cefoxitin discs were used as negative and positive controls, respectively. The extracts that showed an inhibition zone were considered active and selected to next step.

Extraction of crude metabolite

Leaves from species with positive antibacterial activities from screening test were dried in oven at 50°C, pulverized in a Willey type mill and ground in ethyl alcohol PA at a 1:5 ratio (200 g pulverized material per 1000 ml solvent) at room temperature for 48 h with occasional stirring. To determine this milling time, a pilot study was conducted for milling times between 48 and 72 h and seven days. There was no difference in the diameter of the inhibition zone formed, so we chose the shorter time (48 h) to perform the tests. After filtration with cotton, the filtrate was evaporated in a rotary evaporator at 70°C, 135 rpm and a negative pressure of -50 Kpa, and the residue was reconstituted in DMSO at a concentration of 300 µg/ml (Celloto et al., 2003). The extract obtained was subjected to filtration with a 0.22 µm Millex filter. The extracts were maintained at room temperature on dark condition.

Antibacterial activity: Disc diffusion

Bacterial inhibition tests were performed using *S. aureus* ATCC 25923 Müller-Hinton agar was used for bacterial growth incubated for 24 h in a growth chamber set at 35°C. Bacterial suspensions were prepared from fresh cultures with 0.98% NaCl saline solution with a turbidity of 0.5 McFarland Scale ($1.5x10^8$ cells/ml) (NCCLS, 2003). The suspensions were used to inoculate on Müller-Hinton agar plates using a sterile swab. Antibacterial activity was verified

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Table 1. Plant species studied, name and common uses.

Botanical species	Family	Common name	Common uses	Voucher: Unimontes number	References
Anacardium humile	Anacardiaceae	Cajuzinho-do-campo, cajueiro-anão, cajuí, caju-do-cerrado	Antidiarrheal, antidiabetic, Anti-syphilitic, in skin disorders	200	Rodrigues and Carvalho (2001); Lorenzi and Matos (2002)
Astronium fraxinifolium	Anacardiaceae	Gonçalo-alves	Anti-rheumatic and in contusions	449	Lorenzi and Matos (2002)
Boehmeria arborescens	Asteraceae	Assa-peixe	Fever-reducing, in bronchitis, pneumonia, the flu and cough	454	Rodrigues and Carvalho (2001)
Brosimum gaudichaudii	Moraceae	Mama-cadela	Blood cleanser, in bronchitis, the flu, colds, skin spots and vitiligo, Anti-rheumatic, in chronic intoxications, poor blood circulation	455	Rodrigues and Carvalho (2001)
Eugenia dysenterica	Myrtaceae	Cagaita	Anti-diarrheal	453	Tassara (1996).
Luehea divaricata	Malvaceae	Açoita-cavalo	Antidysenteric, anti-hemorrhagic, anti-rheumatic, in ulcers, burns and gangrenous wounds	450	Rodrigues and Carvalho (2001)
Myracrodruon urundeuva	Anacardiaceae	Aroeira, aroeira-preta, aroeira-do-campo, aroeira-verdadeira, urundeúva	Anti-inflammatory, healing agent, antiseptic for ulcers, in skin disorders, urinary tract problems and respiratory tract problems	448	Nunes et al. (2006); Fenner et al. (2006); Lorenzi and Matos (2002)
Psidium guineense	Myrtaceae	Brazilian guava	Anti-diarrheal, diuretic	456	Rodrigues and Carvalho (2001); Morton (1987)
Solanum lycocarpum	Solanaceae	Wolf apple	Emollient, anti-rheumatic, tonic for the flu, colds and asthma, anti-spamodic, anti-epileptic, in abdominal and renal cramps and urinary tract disorders	452	Rodrigues and Carvalho (2001); Lorenzi and Matos (2002)
Solanum paniculatum	Solanaceae	Jurubeba	Antidiabetic, fever-reducing, in jaundice, hepatitis, wound healing, in gastritis, anemia and uterine tumors	451	Rodrigues and Carvalho (2001); Lorenzi and Matos (2002)

by disc diffusion (NCCLS, 2003b) protocol using 10 µl of these 10 extracts dissolved in DMSO with concentration of 300 mg/ml added on 6 mm diameter blank sterile discs. Negative and positive controls were conducted with DMSO and 30 µg of chloramphenicol added on blank disk, respectively. The plates were incubated in growth chamber at 35°C for 24 h. After incubation, the inhibition zone formed around the colonies was measured (Table 2). The effect of the response dose of the prior effective extracts and its inhibitory activities against the same strains were determined according to disc diffusion, using increasing concentrations (5, 10, 15, 20, 25, 30, 35, 40 and 50%) of

Table 2. Inhibition zones[1] by disc diffusion of extracts from plants extracted with different solvents against ATCC bacterial strains*.

Plant species	Ethanol extract			Methanol extract		
	S. aureus	***E. coli***	***P. aeruginosa***	***S. aureus***	***E. coli***	***P. aeruginosa***
Myracrodruon urundeuva	15	0	14	15	0	15
Eugenia dysenterica	0	0	0	0	0	0
Solanum lycocarpum	0	0	0	0	0	0
Solanum paniculatum	0	0	0	0	0	0
Astronium fraxinifolium	0	0	0	0	0	0
Brosimum gaudichaudii	0	0	0	0	0	0
Luehea divaricata	0	0	0	0	0	0
Boehmeria arborescens	0	0	0	0	0	0
Anacardium humile	6	0	8	6	0	7
Psidium guineense	15	0	14	15	0	15
Cefoxitin (30 µg) [2]	24	22	22	25	22	22

*Tests performed in triplicates. [1]Diameters in mm. [2]Positive control antibiotic.

Table 3. Inhibition zones[1] of extracts plants and the positive control by NCCLS (2003b) disc diffusion protocol* against S. aureus ATCC 25923.

Extract concentration (µg/L)	Zone of inhibition measurement (mm)		
	P. guineense	***M. urundeuva***	***A. humile***
30	0	0.375	0
60	0.333	6.563	1.166
90	1.375	9.604	5.146
120	5.104	10.833	7.396
150	6.813	11.604	9.333
180	9	12.417	9.854
210	9.604	12.792	10.396
240	10.521	13.146	10.896
270	11.083	13.5	11.313
300	12.875	14.896	12.875
Chloramphenicol (30 µg)[2]	20.854	20.625	20.458

*Tests performed in triplicates.

the extracts diluted in DMSO that were done in three replicates for each test (Table 3).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

To determine the MICs) of the crude extracts, microdilution test was used following the standard protocol M7-A6 (NCCLS 2003a). Concentrations of extracts ranging from 1024 to 0.5 mg/ml were tested. Dilutions were performed with 96-well plates, which were added 0.1 ml of bacterial suspensions from fresh cultures in 0.98% NaCl saline solution with a turbidity of 0.5 McFarland Scale (1.5×10^8 cells/ml), and incubated at 35°C for 24 h at 0.2 ml final volume. The resazurin was used as indicator of microbial growth and MIC was defined as the lowest concentration which there was no microbial growth, indicated by blue resazurin. The dilutions that did not show growth were then streaked to evaluate the MBC. MBCs were determined in triplicate using the same CLSI recommended for MIC instructions. BHI was used for MBC bacterial quantification of all extract samples during 24 h at 35°C.

Interactions between plant extracts

Synergistic interactions (agonism or antagonism between plant extracts) against standard strain *S. aureus* ATCC 25923 were evaluated following procedures from several reports (Ahmad and Aqil, 2007; Aqil et al., 2005; Zhao et al., 2001). The extracts were combined two by two varying concentrations and each combination was tested adding on 6 mm diameter blank discs following a disc diffusion assay protocol. Control tests were conducted with discs soaked in DMSO or 30 µg chloramphenicol and all plates were incubated at 35°C for 24 h. After incubation, the inhibition zones formed around colonies were measured. Three replicates were performed for each test.

Phytochemical analyses

The characterization of the main groups of plant substances with antibacterial activity was performed with dry and pulverized leaves using qualitative chemical reactions that resulted in the development of colors and/or precipitates characteristic for each compound

Table 4. MICs and MBCs from crude extracts1 against ATCC strains (Staphylococcus and Pseudomonas)*.

Plant sample	Plant portion	*S. aureus* MIC MBC	*P. aeruginosa* MIC MBC
A. humile	Leaves	4.1	8.2
P. guineense	Leaves	4.1	8.2
M. urundeuva	Leaves	4.1	4.1
Control	Chloramphenicol[2]	-	-

*Tests performed in triplicates. [1]Concentration (µg/L). [2]Concentration (30 µg/ml).

groups. The material was subjected to chemical processes of identification for the following classes of chemical components: saponins, tannins, flavonoids, alkaloids, polysaccharides, anthraquinones, organic acids and reducing sugars, all according to the protocols established by Mouco et al. (2003).

Statistical analyses

The data were analyzed by the statistical system R 2.5 (R Development Core Team, 2008) via generalized linear models (Crawley, 2007). For the analysis of extract inhibitory activities, the presence or absence of activity was used as response variable. In this case, a model with binomial error distribution and logit link was used. To examine the responses of tested strains to the pure extracts and to the positive control, a normal error distribution model was used. Interactions between the plant extracts were analyzed taking into consideration inhibition zone diameters, in which the presence of agonism, antagonism or lack of interaction follows a quadratic trend.

RESULTS

Antibacterial activities of the metabolites crude from plants

According to the results of the antibacterial test, extracts from *Piper guineense* and *Myracrodruon urundeuva* plants resulted in growth inhibition of *S. aureus* and *P. aeruginosa* with the two solvents used (ethanol and methanol) with no apparent difference between them. The *Anacardium humile* extract, although less effective, showed inhibition against the same bacterial strains. None of the plants evaluated showed antibacterial activity against *E. coli*. The negative control did not show antibacterial activity against any bacteria tested (Table 2). The results of the agar diffusion antibacterial test were expressed by measuring the diameters of the inhibition zones of the tested plant extracts and the positive control. There was an increase in the inhibitory responses of the extracts as their concentrations increased (Table 3). The negative control did not show antimicrobial activity against the microorganisms tested, and the positive control showed inhibition as expected. Figure 1 shows the relationship between the presence/absence of antibacterial activity and the different concentrations of plant extracts. The *M. urundeuva* extract began its antibacterial action at a lower concentration (30 µg/L), followed by the *A. humile* and *P. guineense* extracts in disc diffusion assays.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

All results for MIC and MBC are shown in Table 4. The MICs for the *P. guineense, M. urundeuva* and *A. humile* extracts were all 4.1 µg/L for *S. aureus*. For the *P. guineense* and *A. humile* extracts tested against *P. aeruginosa*, the MICs were both 8.2 µg/L, and for the *M. urundeuva* extract, it was 4.1 µg/L. As shown from the MICs and MBCs, the extracts had effective antibacterial activities compared to the control; the samples obtained had MBCs up to 16 times lower than the initial values of the crude extracts tested. According to the classification proposed by Aligiannis et al. (2001), MIC values ≤ 100 mg/ml are considered strongly inhibitory when testing fractionated plant material. In this specific case, MICs of ≤ 10 mg/ml for crude, unpurified extracts were considered promising.

Interactions between plant extracts

The results of the assessment of the interactions between plant extracts are illustrated in Figure 2. All combinations resulted in synergistic interactions, which the results of the combined action were greater than the sums of the effects of each isolated compound. According the analysis of variance of the plant extracts as a function of the bacterial strain of *S. aureus* tested (Table 5), there was no significant difference between them, demonstrating a similar behavior to the extracts tested. In the analysis of deviance for the interaction between the plants extracts studied, all interactions were significant.

Phytochemical analyses

All extracts were positive for the presence of tannins and flavonoids and negative for alkaloids. The detection of tannins with ferric chloride indicated the presence of

Table 5. Analysis of Deviance from results by disc diffusion protocol between the extracts plants, S. aureus ATCC 25923 and the combination of extracts*.

Plant extract	GL	Residual GL	Deviance	Residual deviance	p
P. guineense	15	512	288	14422	0.8063
M. urundeuva	15	512	340.4	15522.4	0.735
A. humile	15	512	237	13409	0.8732
Chloramphenicol (30 µg)[1]	15	512	1568.8	5667.2	<0.0001
P. guineense + *M. urundeuva*	1	563	420.6	7406.5	<0.0001
P. guineense + *A. humile*	1	563	466.5	6112.0	<0.0001
M. urundeuva + *A. humile*	1	563	317.4	6010.7	<0.0001

*Tests performed in triplicates. [1]Positive control.

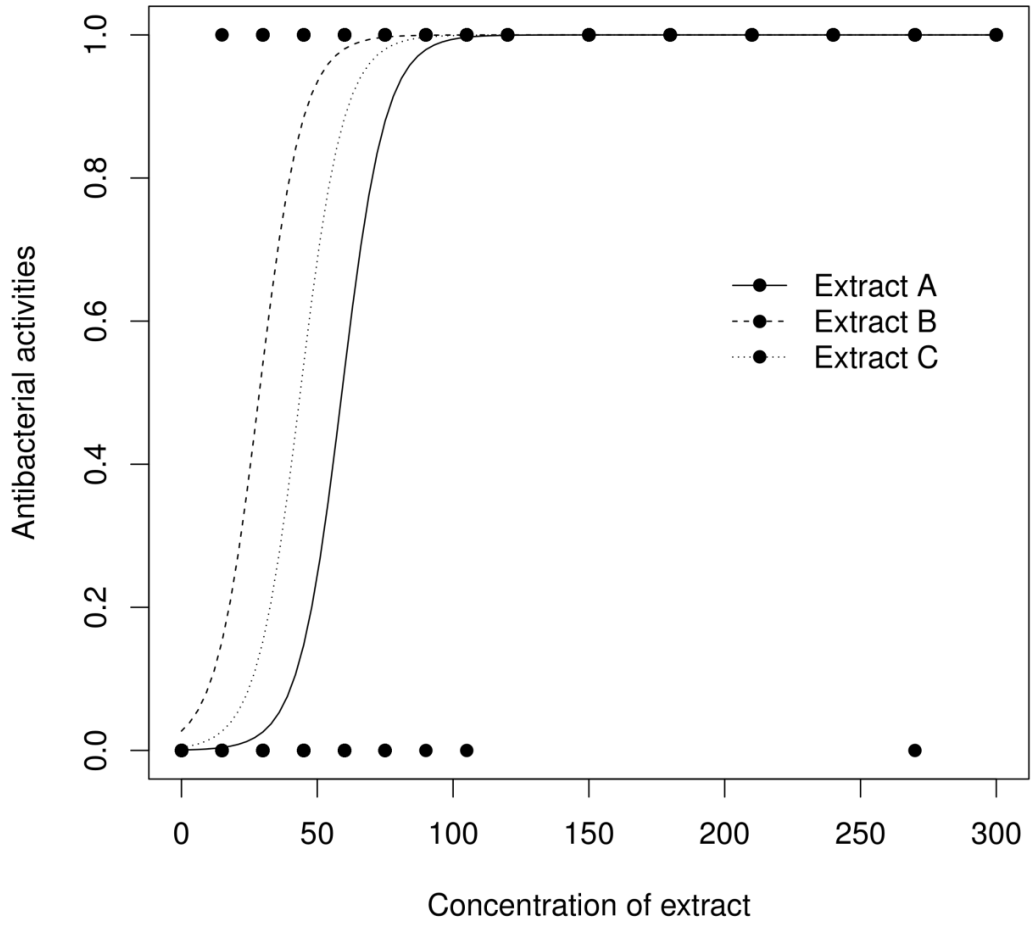

Figure 1. Relationship between bacterial activity and plants extract concentration, where A, B and C represent *Psidium guineense*, *Myracrodruon urundeuva* and *Anacardium humile*, respectively by disc diffusion protocol against *S. aureus* ATCC 25923.

hydrolyzable tannins in *M. urundeuva* and condensed tannins in *A. humile.* These results were confirmed by the reactions with lead acetate and glacial acetic acid. The detection of flavonoids with Shinoda reagent indicated the presence of flavonoids in *A. humile* and flavones in *M. urundeuva.* These results were confirmed by the reactions with ferric chloride. No foaming saponins, polysaccharides, organic acids, anthraquinones or reducing sugars were detected in the plant species evaluated.

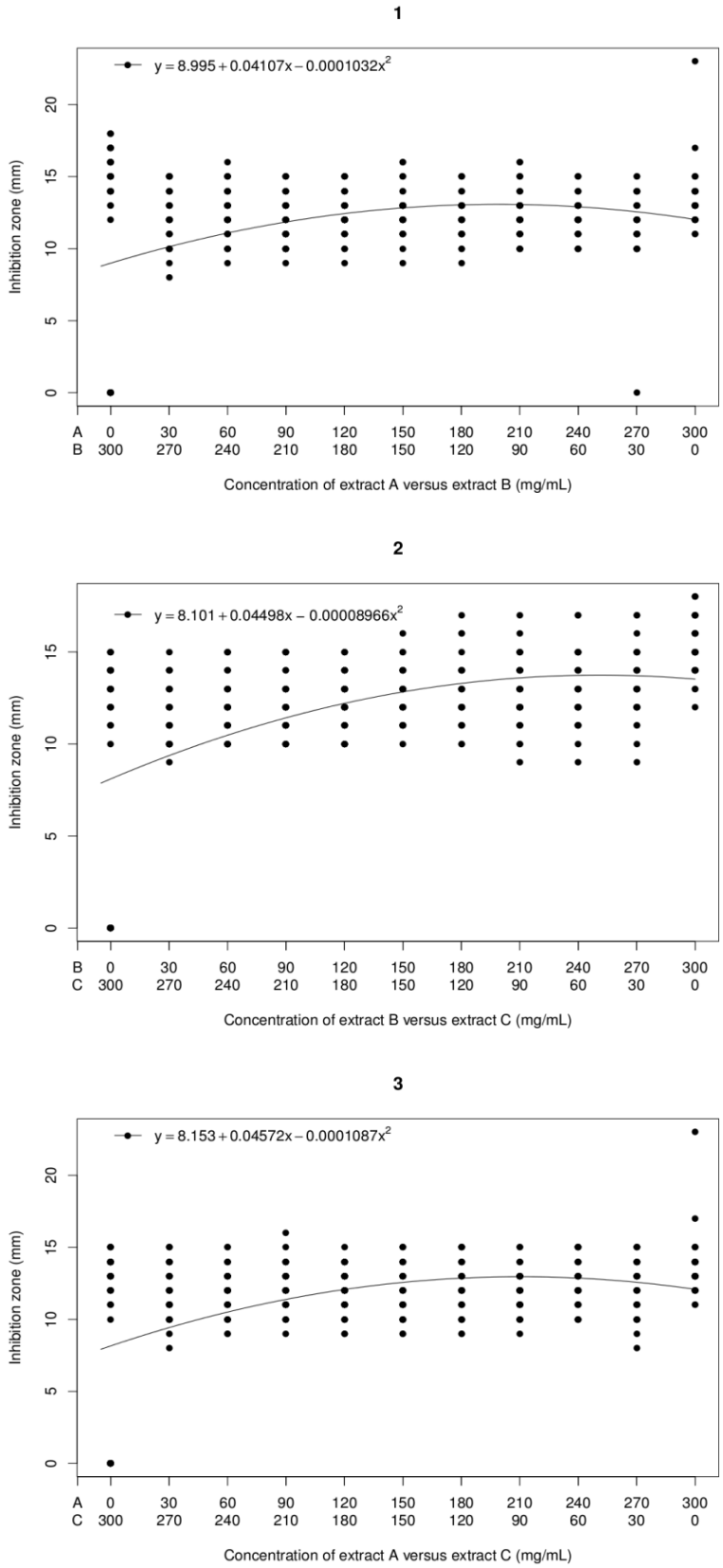

Figure 2. Interactions between plant extracts (A: *Psidium guineense*, B: *Myracrodruon urundeuva* and C: *Anacardium humile*) by disc diffusion protocol against standard strain *S. aureus* ATCC 25923. Tests performed in triplicates.

DISCUSSION

To elucidate the pharmacological activities of chemical components present in plant species is the objective of several studies. A preliminary phytochemical analysis can identify the relevant metabolite groups that may be related to the identified biological activities, thus guiding the research to obtain an effective and safe herbal medicine, in addition to identifying possible toxic active ingredients (Cragg and Newman, 2013). Previous phytochemical researches with the plant species in this study confirm the results found in the phytochemical analysis like antibacterial activity (Al-Mariri and Mazen, 2014; Rodrigues et al., 2014; Tankeo et al., 2016). Ferreira (2005) while studying the anti-ulcerogenic activity of *A. humile*, detected the presence of tannins and flavonoids. Correia et al. (2006) reported that substances with antibacterial and antifungal activities were present in species from the Anacardiaceae family. Lignins and tannins were isolated from *M. urundeuva* by Morais et al. (1999) and Queiroz et al. (2002). Vergas et al. (2007) and Rodrigues et al. (2014) reported the presence of tannins in leaves from plants of the genus *Psidium.*

The results obtained in this study corroborate with use of plants as antimicrobials in popular therapy. Pathogenic bacteria such as *S. aureus* and *P. aeruginosa* frequently shows resistance to the antibiotics used against them (Oliveira et al., 2007), however, they were inhibited by the three ethanol and methanol extracts tested. The *M. urundeuva* crude extract showed the largest inhibition zone against the microorganisms tested, and its inhibition started at a lower concentration (30 µg/L), followed by the *A. humile* and *P. guineense* extracts (60 µg/L). The antibacterial activity detected in these crude extracts may be related to the identified compounds (tannins and flavonoids). Studies performed with extracts from *Psidium* species plants showed antibacterial activities against Gram-positive and Gram-negative bacteria as well as antifungal action (Oliveira et al., 2007; Nair and Chanda, 2007; Carvalho et al., 2008; Rodrigues et al., 2014). González et al. (2005) attributed the antibacterial activity observed in *P. guineense* extracts to secondary metabolites such as tannins and flavonoids. Soares et al. (2006) reported the antibacterial activity of *M. urundeuva* Allemao (Aroeira) against *S. aureus*. Antibacterial activities were also reported in studies with plants of the genus *Anacardium* (Melo et al., 2006). A better understanding of the antimicrobial activities of plants aids in the selection of new substances for this purpose (Gonçalves et al., 2005; Dias et al., 2012; Cragg and Newman, 2013). Given that bacteria are resistant to multiple antimicrobials compounds become a problem in the treatment of infections, being clear that there is a need to find new substances with these properties to be used in the treatment against these pathogenic micro-organisms.

The antibacterial potentials of substances produced naturally in several plant species must be explored, and the relevant components or active fractions must be identified (Ríos and Recio, 2005; Zago et al., 2009; Dias et al., 2012; Cragg and Newman, 2013). Research with medicinal plants involves investigating traditional and popular medicine (ethnobotany); isolating, purifying and characterizing active ingredients (organic chemistry and phytochemistry); investigating pharmacological extracts and isolating chemical compounds (pharmacology); chemically transforming active ingredients (synthetic organic chemistry); studying the relationships between structure, activity and the mechanisms of action of the active ingredients (medicinal and pharmacological chemistry); and finally, formulating herbal medicines. Integrating these areas into the study of medicinal plants leads to a promising and effective path for the discovery of new medications.

Conflict of Interests

The authors have not declared any conflict of interests.

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