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Medicinal Plants from the Brazilian Savanna with Antibacterial Properties

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Authors' contributions

This work was carried out as collaboration between all authors. Authors GSS, LNB, FCBA, BFMTA and MA performed the study (collection of plants, extract preparation, phytochemical analysis and susceptibility tests). Authors AFJ and GSS were the project leaders and were also responsible for designing the project. Author LCS was responsible for phytochemical analysis of the extracts. All authors have read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: Research on natural antimicrobials has increased due to the emergence of microbial strains resistant to conventional antibiotics. We investigated and report here the *in vitro* antibacterial properties of crude extracts from Brazilian savanna plants (*Achyrocline satureioides* (Lam.) DC ("macela"), *Stryphnodendron adstringens* (Mart.) Coville ("barbatimão"), *Miconia rubiginosa* (Bonpl.) DC ("quaresma branca"), *Davilla elliptica* A. St.-Hil. ("lixinha"), *Siparuna guianensis* ("negramina") and *Solanum lycocarpum* A. St.-Hil. ("lobeira").
Place and Duration of Study: Department of Microbiology and Immunology, Biosciences Institute, São Paulo State University from January 2010 to December 2011.
Methodology: Antibacterial activities were investigated using two methods: the disk diffusion method against American Type Culture Collection bacterial strains (*Staphylococcus aureus* – ATCC 25923, *Escherichia coli* – ATCC 22652, and *Pseudomonas aeruginosa* – ATCC 27853) and the susceptibility assays by agar dilution

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method (Mueller Hinton Agar (MHA) aiming the minimal inhibitory concentrations (MIC) and $MIC_{90\%}$ ($mg.mL^{-1}$), against *S. aureus*, *E. coli* and *P. aeruginosa* strains isolated from human clinical specimens. Qualitative phytochemical analysis of crude extracts was also performed.
Results: By agar dilution test, the *D. elliptica* leaf extract was efficient against all strains ($MIC_{90\%}$ values of 0.7, 2.6, and 2.1 $mg.mL^{-1}$ against *S. aureus, E. coli*, and *P. aeruginosa,* respectively), while the *S. guianensis* leaf extract showed the lowest activity (12.2, >32.0, and 26.0 $mg.mL^{-1}$ against *S. aureus, E. coli*, and *P. aeruginosa*, respectively). We detected flavonoids and phenolic compounds in all studied extracts.
Conclusion: All studied extracts showed antibacterial activity by the agar dilution method, although *M. rubiginosa* and *S. guianensis* leaves extracts did not show inhibitory action against *E. coli* and *P. aeruginosa* strains. There was a greater sensitivity of *S. aureus* and the *D.elliptica* leaf extract showed the highest efficiency over most of the strains tested. Additionally, the results show that it is possible to obtain conflicting results using the disk diffusion method and the dilution method.

Keywords: Bacteria; bacterial susceptibility; minimal inhibitory concentration; plant extracts; phytochemical analysis.

1. INTRODUCTION

The discovery of antimicrobial agents was the greatest medical success of the 20th century, and revolutionised infectious disease treatments. However, the gradual emergence of bacterial resistance to antibiotics, resulting from either inappropriate or indiscriminate use of these antimicrobials, is now a worldwide health problem [1]. Aimed at resolving this problem, effort to improve or develop macromolecules that can inhibit pathogens without incurring pathogen resistance is required and actively ongoing [2].

Natural products and related structures are essential sources of new pharmaceuticals, because of the immense variety of functionally relevant secondary metabolites of microbial and plant species [3]. Approximately half of all drugs that were recorded worldwide in the period before 2007 were from natural products or their synthetic derivatives [4].

Brazil has five areas of abundance of native plants, among which is the Brazilian savanna biome and the diversity of species from this biome with therapeutic potential is significant [5]. It is also important to study the possible uses of these plants in combating various diseases. *Achyrocline satureioides* (Lam.) DC (Asteraceae) is known as “macela” or national chamomile [6]. It is used in folk medicine as infusions for digestive, sedative, anti-inflammatory, antispasmodic, analgesic, and diuretic uses and also as a bronchodilator [7]. More recently, the cytoprotective effect of this plant was reported, and a special mixture of unglycosylated flavonoids could be a clue to the activity of this plant [8]. It is able to inhibit *Bacillus cereus*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* [9]. The bark decoction of *Stryphnodendron adstringens* (Mart.) Coville (Leguminosae) (“barbatimão”) is popularly employed to treat leucorrhoea, bleeding, diarrhoea, and hemorrhoids, and to clean wounds and conjunctivitis [10], and showed antibactericidal activity against *Enterococcus faecalis*, *Streptococcus salivarius*, *Streptococcus sanguinis*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus sobrinus*, and *Lactobacillus casei* [11], antifungal activity against *Candida albicans* [12], and antiviral activity against poliovirus and bovine herpesvirus [13]. Tannins and flavonoids are the main active compounds in this plant [14].

Davilla elliptica St. Hil. (Dilleniaceae) ("lixinha" or "cipó-caboclo") is used in folk medicine for hemorrhoids, hernia, diarrhoea treatments, as an antiseptic for cleaning wounds, and as an astringent, tonic, laxative, sedative, and diuretic [15]. Flavonoids, triterpenoids, steroids, gallic acid, catechins, tannins, saponins, and coumarins were found in its leaves [16]. The chloroform extract of *D. elliptica* leaves showed effective action against *Mycobacterium tuberculosis* [17], while methanolic extracts of the leaves and bark were active also against *B. subtilis*, *B. cereus*, *Shigella* spp., *C. albicans,* and *E. faecalis* strains [18]. *Solanum lycocarpum* A. St.-Hil. (Solanaceae), known popularly as "lobeira" or fruit-the-wolf, is widely distributed in disturbed areas of the Brazilian savanna and has been widely employed for diabetes management and obesity, and to reduce cholesterol levels [19]. A methanolic extract of the fruits of *Solanum lycocarpum* showed an inhibitory effect on the increase in serum glucose levels in oral sucrose-loaded rats, and three known steroidal alkaloid oligoglycosides, solamargine, solasonine, and 12-hydroxysolasonine, were isolated from the active fraction, together with two new steroidal alkaloid oligoglycosides, robeneosides A and B [20]. Phenols are also commonly found in the leaves of the *Solanum* genus [21].

Studies with *Miconia rubiginosa* (Bonpl.) DC extracts (Melastomataceae), popularly known as "quaresmeira branca", revealed some biological properties including antimicrobial activities [22], specifically against the *Bacillus* genus. The flavonoids in *M. rubiginosa* extracts are the main antimicrobial compounds [23].

Siparuna guianensis Aubl. (Monimiaceae), popularly known as "limão bravo" or "negramina", is an aromatic and medicinal species from Brazilian flora [24] and is used as antimalarial agent because it has been shown to be effective against the trypomastigotes, killing approximately 100% of the parasites at the maximal concentration of 100 µg/mL [25]. Valentini et al. [26] reported this plant as a priority species for Brazilian savanna conservation and studies should be encouraged for this genetic resource to be available for future generations.

Despite the large number of species of plants with antimicrobial properties, there have been few studies on species from the Brazilian savanna. Thus, we aimed to evaluate the *in vitro* antimicrobial activities of extracts of six plants from the Brazilian savanna against *S. aureus, E. coli*, and *P. aeruginosa* strains isolated from human clinical specimens, including phytochemical analysis of each extract.

2. MATERIALS AND METHODS

2.1 Plants and Extract Preparation

The plant samples, including leaves of *S. lycocarpum* A. St.-Hil., leaves and bark of *S. adstringens* (Mart.) Coville, flowers of *A. satureioides* (Lam.) DC, leaves and fruits of *D. elliptica* St. Hil., leaves of *M. rubiginosa* (Bonpl.) DC, and leaves of *S. guianensis* Aubl. (negramina) were collected from Brazilian savanna areas (Botucatu/SP/Brazil). The plant organs samples, including a portion of a fresh sample of 10 g to check the moisture of the studied plant, were dried in a forced air circulation greenhouse (45ºC/48 hours) and ground. Extracts were prepared using 70% methanol and filtrates were obtained after 48 hours at refrigerator temperature (± 4ºC), when a rotary evaporator (Phoenix-Piracicaba/SP) [27] was used for solvent elimination. Five volumes of 1 mL of each extract was taken, kept in a forced air circulation greenhouse (45ºC), and weighed to obtain the dry weight in mg/mL.

The plant material vouchers were identified and deposited at Herbarium Irina Delanova Gemtchujnicov of Botany Department/IBB/UNESP/Botucatu/Brazil.

2.2 Phytochemical Analysis of the Crude Extracts

The methodology was carried out according Matos (1988) [28], aimed at detecting general classes of secondary metabolites. The extracts were prepared from 50 g of each material (dried and pulverised), subjected to extraction by cold maceration (4ºC) with pure methanol. After four extractions the filtered were concentrated on a rotary evaporator to about 100 mL. Two 15 mL aliquots of these extracts were dried in beakers in an oven at 50ºC. To the remaining 70 mL, distilled water was added to make up to 100 mL. From this hydroalcoholic solution, 40 mL were subjected to acid hydrolysis (HCl added to pH 1 to 3) under reflux and two 30 mL aliquots were separated for the remaining tests as shown in the general flowchart in Fig. 1.

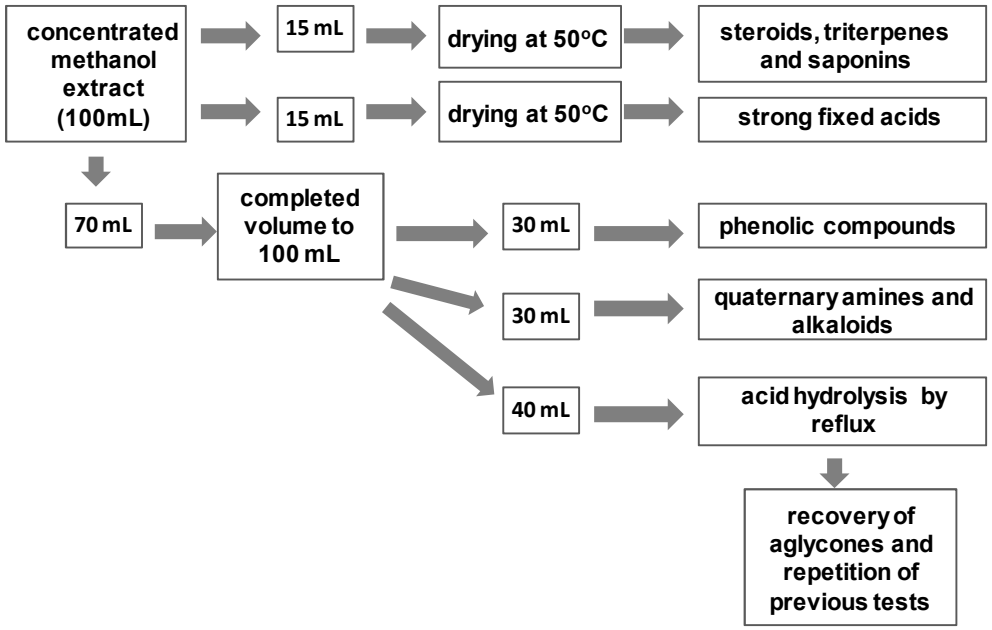

Fig. 1. Schematic procedures of phytochemical analysis (Matos, 1988)

2.3 Bacterial Susceptibility Tests

Two experiments were conducted to establish the antibacterial activity of the extracts. Initially, American Type Culture Collection standard strains (*S. aureus* – ATCC 25923, *E. coli* – ATCC 22652, and *P. aeruginosa* – ATCC 27853) were tested by the disk diffusion method and extract dilution assays on Mueller Hinton Agar (MHA), and the minimal inhibitory concentration (MIC) was recorded for each extract [29].

2.3.1 Disk diffusion method assays

Sterile paper disks with 6 mm diameter impregnated with 20 µl of the extracts were placed in Petri plates containing MHA and inoculated with the standard ATCC strains (standardised

bacterial suspensions by 0.5 McFarland standard). Positive controls were carried out with tetracycline (TET) disks. After 35°C/24 hours, the inhibition zones were recorded. The susceptibility assays were performed in triplicate and the results were the arithmetic average of the diameter of zones of inhibition.

2.3.2 Agar dilution method assays

The susceptibility tests were carried out by the agar dilution method [30], and the MIC of 10 *S. aureus*, 11 *E. coli*, and 11 *P. aeruginosa* strains isolated from human clinical specimens from patients of the Clinical Hospital Campus-UNESP Botucatu/SP were recorded. Bacterial identifications were according Koneman (2001) protocol [31]. These samples were stored at -70°C in the Department of Microbiology and Immunology, IBB/UNESP/Botucatu-SP. Because these were microorganisms isolated from human materials, permission was obtained from the Ethics Committee in Research (CEP) of the Medicine School of Botucatu/UNESP/Botucatu Campus, protocol number 3098/2009-CEP.

MHA plates were prepared with concentrations ranging from 0.25 to 32 $mg.mL^{-1}$ and the concentration range tested was chosen according to preliminary tests. Strains were subsequently standardised by 0.5 McFarland standard and a new dilution (1:20) was performed in Brain Heart Infusion (BHI) to 10^5 and 10^6 colony forming units (CFU) per mL on the agar plates. Inoculation procedures were performed using a Sterr multi-inoculator with a capacity for 32 concurrent strains, followed by incubation at 37ºC for 18–24 hours. Individual MIC values were recorded according to bacterial colony formation and the $MIC_{90\%}$ values of bacterial species were calculated. The assays were performed in duplicate.

2.4 Statistical Analysis

Statistical analyses were performed using the Kruskal-Wallis test or nonparametric variance analysis.

3. RESULTS AND DISCUSSION

The results are those obtained from antibacterial activity assays with the crude plant extracts from the Brazilian savanna. These plants were chosen because they were popularly used for the treatment of infectious diseases.

The extracts' dry weights are important because they allows to calculate the MIC values in $mg.mL^{-1}$ in microbiological assays. These were calculated after evaporation of the water present in the extracts, and even after the use of a rotary evaporator for elimination of methanol. The results are presented in Table 1. There was variation in the dry weights of the extracts studied; the lowest value was with *D. elliptica* leaf extract (35 $mg.mL^{-1}$), and the highest was bark from *S. adstringens* (179.5 $mg.mL^{-1}$). Also shown are the dry weight values of plants from the drying out of a total of 10 g of fresh plant material, to determine the dry weights of the plants collected and the percentages of moisture of these plants. The same profile of results was not observed in the dry weight of all the collected plants, with a slight variation: the lowest value was observed for leaves of *S. lycocarpum* (2.6 g and 74% moisture) and the highest was for *M. rubiginosa* (5.4 g and 46% moisture).

The different results from crude extract dry weights is possible due to various parameters, especially those resulting from the preparation process adopted (e.g., quantity of plant

material, evaporation of the solvent on a rotary evaporator, solubilisation ability of the solvent used, etc.) as well as the characteristics of the plant samples used in extract preparation (moisture content and dry matter of the plant studied, etc.).

Table 1. Dry weight values from plant organs after drying of 10 g of fresh vegetable sample (five replicates) and concentrations of crude extracts (five replicates)

Plants (organs)	Dry weight from plants (% moisture)	Dry weight from extracts ($mg.mL^{-1}$)
S. adstringens (bark)	3.7 (63)	179.5
S. adstringens (leaves)	5.1 (49)	99.7
D. elliptica (leaves)	3.2 (68)	35.0
D. elliptica (fruits)	3.5 (65)	43.0
S. lycocarpum (leaves)	2.6 (74)	47.3
A.satureioides (flowers)	3.2 (68)	40.0
M. rubiginosa (leaves)	5.4 (46)	104.0
S. guianensis (leaves)	4.3 (57)	81.0

The results of susceptibility assays by disk diffusion method are shown in Table 2. In *S. aureus* assays, the largest size of inhibition zone diameter (mm) was found with *S. adstringens* leaf extract (18.6 mm), and in decreasing order of diameter were: *S. adstringens* (bark) (16.6 mm), *D. elliptica* (leaf) (14.6 mm), *S. lycocarpum* (leaf) (14.0 mm), *D. elliptica* (fruit) (13.0 mm), *M. rubiginosa* (leaf) (11.0 mm), *S. guianensis* (leaf) ((11.0 mm), and *A. satureioides* (flower) (9.3 mm). For the *E. coli* strains, there was also a higher antimicrobial efficiency for *S. adstringens* leaf extract, with an inhibition zone of 8.6 mm, followed by *D. elliptica* (leaf) (7.3 mm), and finally *S. adstringens* (bark) with 7.0 mm. The other extracts did not form an inhibition zone. With *P. aeruginosa*, the extract with the largest inhibition zone was *S. guianensis* (13.0 mm) followed by, in descending order: *D. elliptica* (fruit) and *M. rubiginosa* (10.6 mm), *S. adstringens* (leaf) (9.6 mm), *S. adstringens* (bark) (8.6 mm), and *D. elliptica* (leaf) (8.0 mm). The *S. lycocarpum* and *A. satureioides* extracts did not form inhibition zones, allowing us to conclude in disk diffusion tests, they were not active against *P. aeruginosa*. Regarding these results, the disk diffusion method represents an attempt to highlight possible inhibitory effects from plants, especially when there are a large number of plants to be tested. Furthermore, we established that the size of the inhibition zones was dependent on the diffusion ability of the compounds in the extract, independent of their antimicrobial potential.

Table 2. Inhibitory zones (mm) values from disk diffusion tests of plant extracts against standard ATCC bacterial strains

Plants (organs)	*S. aureus* ATCC 25923	*E. coli* ATCC 22652	*P. aeruginosa* ATCC 27853
S. adstringens (bark)	16.6	7.0	8.6
S. adstringens (leaves)	18.6	8.6	9.6
D. elliptica (leaves)	14.6	7.3	8.0
D. elliptica (fruits)	13.0	0.0	10.6
S. lycocarpum (leaves)	14.0	0.0	0.0
A.satureioides (flowers)	9.3	0.0	0.0
M. rubiginosa (leaves)	11.0	0.0	10.6
S. guianensis (leaves)	10.3	0.0	13.0
tetracycline (control drug)	26.6	21.3	11.3

Therefore, we present the results from the second phase (dilution agar method) on MHA, including the $MIC_{90\%}$ values (Table 3). The $MIC_{90\%}$ values against *S. aureus* strains differed between extracts, with the greatest efficiency with *D. elliptica* leaf extract (0.7 $mg.mL^{-1}$), and a lower efficiency with *S. guianensis* extract (12.2 $mg.mL^{-1}$). With *E. coli*, the $MIC_{90\%}$ values were higher than with *S. aureus*; again, *D. elliptica* leaf extract showed the highest efficiency (2.6 mg/mL^{-1}), followed by *S. adstringens* leaf extract (4.0 $mg.mL^{-1}$). With *P. aeruginosa*, the inhibitory effects were relatively satisfactory with most extracts, but with the *M. rubiginosa* extract, the result was above the highest concentration tested (>32 $mg.mL^{-1}$). The *S. adstringens* leaf extract showed the highest efficiency (2.0 $mg.mL^{-1}$).

Bacterial growth inhibition with extracts from *Baccharis dracunculifolia, Vernonia polyanthes, Matricaria chamomilla*, and *Eugenia uniflora*, was also higher against the Gram-positive bacteria *S. aureus* and can be directly related to the cell wall structure of Gram-positive bacteria, considering that this is the main feature that differentiates the two bacterial groups [32]. Another relevant aspect is the presence of the outer membrane in Gram-negative bacteria, which serves as a barrier to some antibiotics, digestive enzymes, detergents, and heavy metals.

A further obvious explanation is the presence of antimicrobial compounds in the extracts. Phytochemical characterisation, although qualitative analysis, showed variations in the extracts' compositions. However, flavonoids were found in all extracts (Table 4). Flavonoids are known to be synthesised by plants in response to infection of a wide variety of microorganisms, and their activity is due to the ability to form complexes with proteins and soluble extracellular and bacterial cell walls, as well as the ability to disrupt microbial membranes [33].

In addition to flavonoids with antioxidant, anti-inflammatory and anticancer properties [34], other compounds associated with numerous biological properties have also been found, such as saponins with haemolytic, antiviral, and anti-inflammatory activities. Many of them are able to cause disruption of cell membranes, which underlies their antibacterial and antifungal activities [35]. Tannins accelerate the healing process [36]; phenolic compounds have antioxidant, antibacterial, and wound healing properties [37]; coumarins act as antioxidants, as they chelate iron ions and prevent lipid peroxidation [38]; alkaloids have anti-inflammatory, antiviral, and antimalarial activities [39]; and quinones have microbicidal, trypanocidal, virucidal and antitumor properties [40].

Table 3. Minimal inhibitory concentration of 90% ($MIC_{90\%}$) ($mg.mL^{-1}$) by the agar dilution method against bacterial strains isolated from human clinical specimens

Microorganisms	***S. adstringens (bark)***	***S. adstringens (leaves)***	***D. elliptica (leaves)***	***D. elliptica (fruits)***	***S.lycocarpum (leaves)***	***A. satureioides (flowers)***	***M. rubiginosa (leaves)***	***S. guianensis (leaves)***
S. aureus (n=10)	$1,8\pm0,00^{f}$	$1,0\pm0,00^{b}$	$0,7\pm0,14^{a}$	$1,8\pm0,00^{e}$	$7,1\pm0,00^{g}$	$1,6\pm0,00^{d}$	$1,1\pm0,00^{c}$	$12,2\pm0,00^{h}$
E. coli (n=11)	$7,2\pm3,13^{c}$	$4,0\pm0,59^{b}$	$2,6\pm0,28^{a}$	$8,0\pm1,76^{c}$	$18,0\pm3,39^{d}$	$16,0\pm0,00^{d}$	$>32,0\pm0,00^{e}$	$>32,0\pm0,00^{e}$
P. aeruginosa (n=11)	$3,6\pm3,76^{b}$	$2,0\pm0,86^{a}$	$2,1\pm0,94^{a}$	$3,5\pm2,45^{b}$	$7,1\pm0,71^{c}$	$9,5\pm3,22^{d}$	$>32,0\pm0,00^{e}$	$26,0\pm1,24^{e}$

The symbol '>' shows that it was not possible to verify the real value of MIC because it exceeded the highest concentration tested.
The same letters on the same line show that the $MIC_{90\%}$ values did not differ ($P< .05$).

Table 4. Phytochemical characterisation of the plant extracts

	Phenolic compounds	**Flavonoids**	**Saponins**	**Triterpenes and free steroids**	**Tannins**	**Quinones**	**Coumarins**	**Alkaloids**
S. adstringens (bark)	+	+	+	-	+	-	-	-
S. adstringens (leaves)	-	+	+	Triterpenes	+	+	+	-
D. elliptica (fruits)	+	+	+	-	+	-	-	-
D. elliptica (leaves)	+	+	+	Steroides	+	+	-	-
A.satureioides (flowers	+	+	-	Triterpenes	-	-	-	-
M. rubiginosa (leaves)	-	+	+	Steroids	+	-	+	-
S. lycocarpum (leaves)	+	+	-	Triterpenes	-	-	+	-
S. guianensis (leaves)	+	+	-	Steroids	-	-	-	+

**The symbols represent the presence (+) or absence (-) of the chemical compound.*

It has been reported that the chemical composition of antimicrobials from plants varies according to climatic and edaphic factors [41]. Thus, the results are important, including an attempt to explain the antimicrobial activity of these extracts, although studies should also be conducted to quantitatively characterise these antimicrobial products, considering that their levels vary significantly.

The results of the disk diffusion method and the agar dilution method were discrepant. Therefore, it was necessary to obtain the MIC values with both methods. For example, we found that with *S. lycocarpum* extract against *P. aeruginosa*, in the disk diffusion method there was no inhibition zone formation, while in the agar dilution method the $MIC_{90\%}$ was 7.1 $mg.mL^{-1}$. Another example was with *A. satureioides* extract against *S. aureus*, which had the smallest inhibition zone in the disk diffusion method, but in the agar dilution method had the fourth best $MIC_{90\%}$ value (1.6 $mg.mL^{-1}$).

Thus, we found that microbial susceptibility testing of plant extracts required attention regarding their standardisation. It should also noted that the MIC is the most reliable method for testing antimicrobial products from plants and tests using disks impregnated with extracts or other plant derivatives should be used with some restraint and only in very preliminary testing when the researcher is initiating studies of antimicrobial action with some plant or plants, in particular.

4. CONCLUSIONS

The most important result was that *D. elliptica* leaves extract revealed significant antimicrobial action against bacteria strains, even with the use of crude methanol extracts, and the *S. aureus* strains are highly susceptible to all tested extracts. Phytochemical analysis of the extracts found wide variation between the studied plants, allowing us to infer that the biological properties of a plant is due its particular phytochemical composition, and is essential to conduct tests to clarify the chemical composition. Therefore, the range of antimicrobial activity of the different extracts must be due to the amount and/or concentrations of active compounds found in the plant and not only their presence or absence. Finally, the results are encouraging and further studies aiming to understand the antimicrobial mechanisms of the compounds found at studied plants are necessary and the use of proteomic analysis protocols on bacterial cell could explain the mechanisms of antibacterial action of the plant extracts studied.

CONSENT

Not applicable.

ETHICAL APPROVAL

The permission, because it is microorganisms isolated from human materials, was obtained from the Ethics Committee in Research (CEP) of Medicine School of Botucatu / UNESP / Botucatu Campus, protocol number 3098/2009-CEP.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX

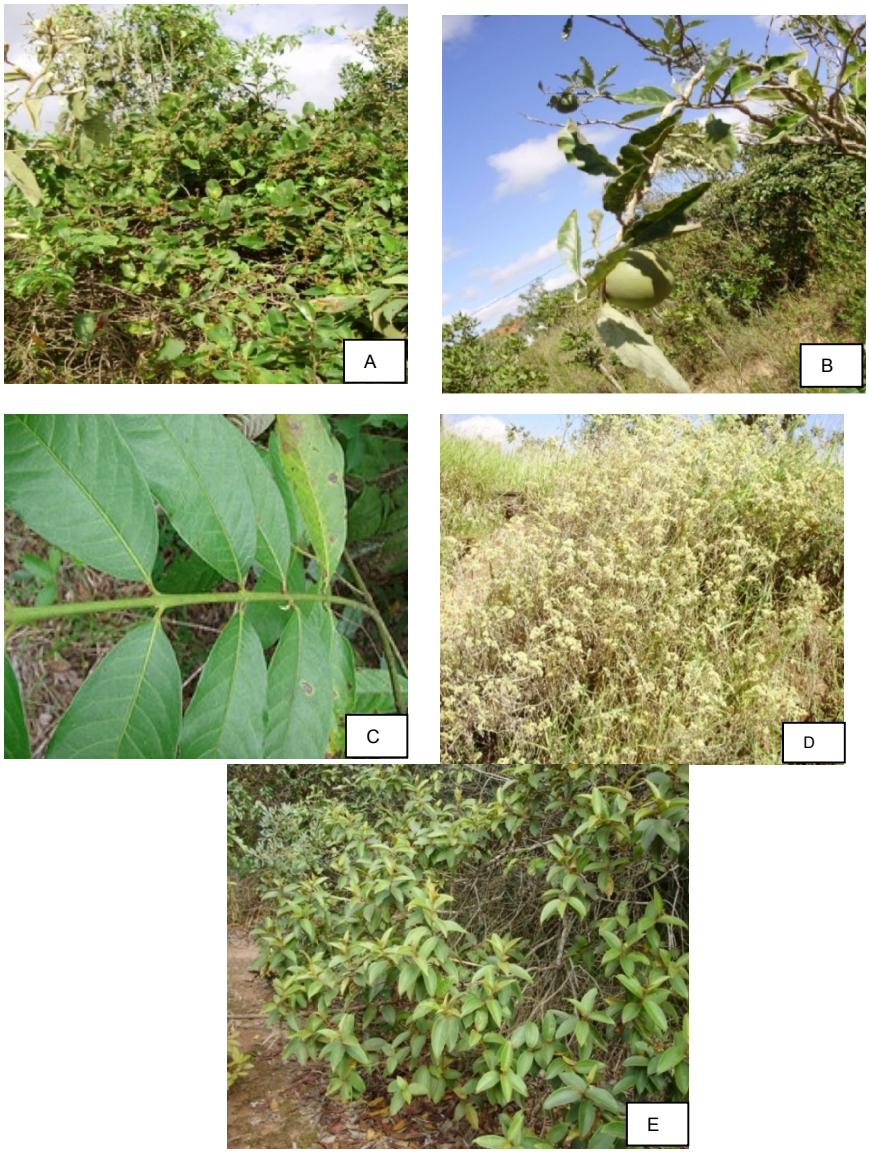

Fig. 2. Brazilian Savanna plants used in the study: A) *Davilla elliptica* (lixinha); B) *Solanum lycocarpum* (lobeira); C) *Siparuna guianensis* (negramina); D) *Achyrocline satureioides* (macela) and E) *Miconia rubiginosa* (quaresma-branca) (Source: Ary Fernandes Júnior, 2010)

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