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Full Length Research Paper

Seasonal variation in the production of secondary metabolites and antimicrobial activity of two plant species used in Brazilian traditional medicine

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Guapira graciliflora and Pseudobombax marginatum **are two species used in the treatment of various diseases in traditional medicine of the Brazilian semiarid region, but no studies assessing their phytochemical and pharmacological properties have been reported. This study aimed to evaluate seasonal variation in the production of secondary metabolites and antimicrobial activity of these plants. The broth microdilution test was used against pathogenic microorganisms to evaluate the antimicrobial activity. The content of total polyphenols and flavonoids was determined by ultra violet (UV) spectrophotometry using gallic acid and quercetin as standards respectively. The concentration of polyphenols was higher in winter for *P. marginatum* and in summer for *G. graciliflora*, while for flavonoids the opposite occurred. Regarding the antimicrobial activity, only *P. marginatum* showed inhibition against seven tested strains and antibiosis against four, with variation in the minimum inhibitory concentration (MIC) and minimum microbicide concentration (MMC) between the two seasons. *G. graciliflora* showed no activity. The results show that the chemical composition of the extracts from *P. marginatum* and *G. gracilliflora* exhibits seasonal variation, with the first plant showing moderate antimicrobial activity.**

Key words: Seasonal variation, phenolic compounds, medicinal plants, *Gapira graciliflora, Pseudobombax marginatum*.

INTRODUCTION

The demand for natural products with antibacterial activity in fighting diseases has been highlighted, especially with the advent of multidrug-resistant strains. The use of plant resources in the Brazilian semiarid region for medicinal purposes has been described in several papers (Albuquerque et al., 2007a; b; Agra et al., 2007a; b; 2008; Araújo et al., 2008; Cartaxo et al., 2010; Siqueira et al., 2012). *Guapira gracilifora* (Mart.) Lundell (Nyctaginaceae) and *Pseudobombax marginatum* (A. St.-Hil., Juss. and Cambess.) A. Robyns. (Bombacaceae) present in the Caatinga, main biome of the Brazilian semiarid, are cited as medicinal by the human population living there (Agra et al., 2008; Siqueira et al., 2012) and appear as plant resources to be analyzed from the bioprospecting standpoint.

Caatinga in the Brazilian Northeast offers a wide variety of vital resources to the local population survival. Although the flora of this biome is closely linked to the cultural identity of the local population, the use of these species, in most cases, is based on unsustainable processes (Albuquerque and Andrade, 2002; Nunes et

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al., 2006) which is leading to rapid loss of endemic species, elimination of key ecological processes and formation of large clusters of desertification in various sectors of the region (Leal et al., 2003; Santana, 2007).

The need for evaluating the pharmacological and chemical potential of plants present in the residual Caatinga fragments includes not only the importance of finding new substances from non-studied plants, but also the ecological appreciation of this phytogeographical region since the knowledge of these species will allow the proposition of management plans for conservation of the existing phytodiversity by encouraging the sustainable use of plants which are found to have such potential.

Coupled with its richness and diversity (Leal et al., 2003), the Caatinga vegetation has also physiological peculiarities as a result of environmental conditions to which they belong (Trovão et al., 2007). These ecophysiological characteristics of species influence directly the secondary metabolism responsible for patterns and production processes of the constituents with medicinal properties. In fact, the secondary metabolites represent a chemical interface between plants and the environment; therefore, their synthesis is often affected by environmental conditions (Kutchan, 2001; Gobbo-Neto and Lopes, 2007).

Thus, this study aimed not only at enhancing ethnobotanical knowledge, but also assessing, within an ecophysiological perspective, the influence of environmental characteristics of the semiarid climate on the production of secondary metabolites, precursors of active compounds present in medicinal plants, as well as evaluating if the variation of these metabolites modifies the antimicrobial activity of the studied plants.

MATERIALS AND METHODS

Seasons in Cariri of Paraiba

The Cariri region has climate ranging from semiarid to dry sub-tropical exception sub-arid. Average annual temperatures are relatively high, 25 to 27°C, and average insolation is 2800 h/year. The relative humidity is about 50% and the average rates of evaporation are generally between 1500 and 2000 mm (Nascimento et al., 2008; Gariglio et al., 2010). As for the seasons, the Cariri is characterized by two distinct seasons, a rainy one concentrated in one period which can vary from 3 to 4 months, with the annual average rainfall commonly less than 300 mm, and a dry season that may exceed nine months.

Plant material

The plant material collection was conducted in Vereda Grande, located in the rural municipality of Barra de Santana, Cariri of Paraiba, Brazilian semiarid region (Figure 1) during the dry (DP), February 2011, and rainy (RP), August 2011, periods, under the coordinates 7° 31,613′ S, 36° 2,991′ W (*G. graciliflora*) and 7° 32,013′ S, 36° 3,018′ W (*P. marginatum*). Exsiccates are deposited in the herbarium Arruda Camara of State University of Paraíba under the numbers 906 *(P. marginatum)* and 907 *(G. graciliflora).* The stem barks collected were subjected to the processes of drying in an oven with air circulation at 40°C and subsequently pulverized in a knife mill.

Extracts preparation

The plant samples were extracted with ethanol by percolation process for five days. The extracts were concentrated under vacuum using a rotary evaporator at 40°C and stored under refrigeration at -4°C for later use.

Phytochemical tests

Determination of total polyphenols

The total polyphenol content of plant extracts was measured using spectrophotometry in the visible region by the method of Folin-Ciocalteu described by Chandra and Mejia (2004) with minor modifications. The ethanolic extracts (25 mg) were dissolved in distilled water and filtered. These solutions were diluted to obtain a final concentration of 300 and 200 µg.mL^{-1} for *G. graciliflora* and *P. marginatum,* respectively. From each solution, a 1 mL aliquot was added to 1 mL of 1 mol.L^{-1} Folin-Ciocalteu reagent. This mixture remained undisturbed for 2 min before the addition of 2 mL of 20 % (w/v) Na_2CO_3 solution and left undisturbed for 10 min. Thereafter the reading was performed Spectrophotometer Shimadzu, model UV-mini 1240, at 757 nm. The calibration curve was obtained with a stock solution of gallic acid (1 mg.mL^{-1}), from which dilutions were made at concentrations of 1, 3, 6, 9, 12, 15, 20, 25, 30, 35 and 40 µg.mL^{-1}. The total content of polyphenols was expressed in microgram equivalents of the standard used.

Determination of total flavonoids

The total flavonoids were determined by the method described by Meda et al. (2005). The extracts were diluted with methanol, *G. graciiflora* for DP and RP and *P. marginatum* for DP at 1000 µg.mL^{-1} and *P. marginatum* for RP at 5000 µg.mL^{-1}. To the 5 ml of each test solution was added the same volume of 2 % (w/v) $AlCl_3$ solution in methanol. This mixture remained undisturbed for 10 min before the UV spectrophotometric reading at 415 nm wavelength. The total flavonoids was determined by the calibration curve using quercetin (Sigma-Aldrich) as standard at concentrations of 2, 4, 6, 8, 10, 13, 16, 19, 22, 26, 28 and 30 µg.mL^{-1} and expressed in µg equivalent of quercetin.

Antimicrobial activity

The microorganisms used in this study were: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Klebisiella pneumoniae* (ATCC 4352), *Streptococcus oralis* (ATCC 10557), *Streptococcus salivarius* (ATCC 7073), *Enterococcus faecalis* (ATCC 29212), *Candida albicans* (ATCC10231), *Candida guilliermondii* (ATCC 6260) and *Candida krusei* (ATCC 34135).

The broth microdilution method described by CLSI (2003) was performed with adaptations to determine the minimum inhibitory concentration (MIC). The microbial inocula were prepared in test tubes with 5 mL of 0.9 % saline solution and standardized a final concentration close to 10^6 CFU.mL^{-1}. The extracts of *P. marginatum* were dissolved in 10% Dimethylsulfoxide (DMSO) and the extracts of *G. graciliflora* in chloroform to yield 200 mg.mL^{-1} stock solution for *P marginatum* and 50 mg.mL^{-1} for *G graciliflora*. Serial dilutions were performed for each sample in BHI broth. Later were added 10 µL of the respective microbial inoculum. The plaques containing

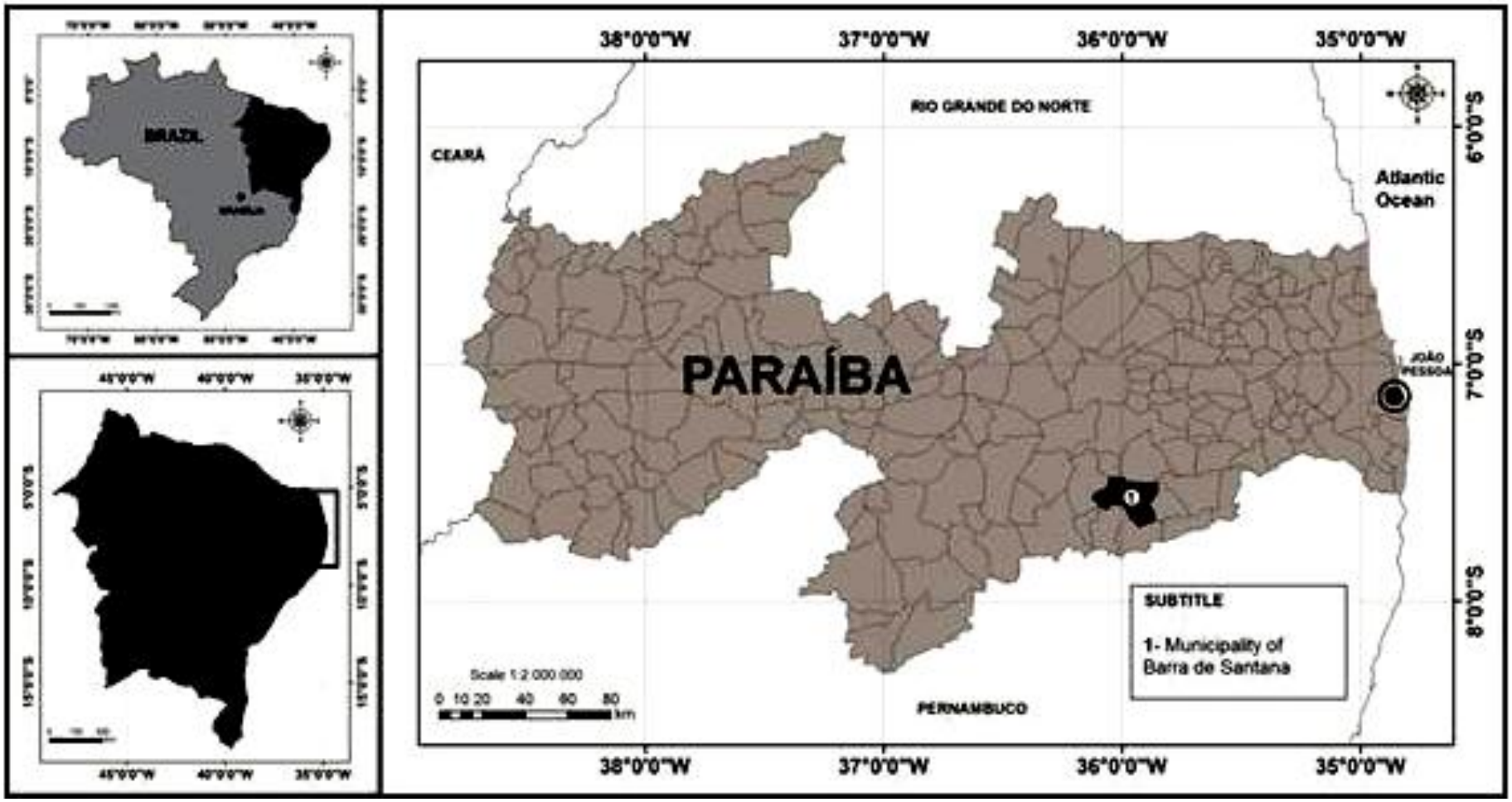

Figure 1. Map showing the site location of the plant material collected in the municipality of Barra de Santana, Cariri of Paraiba, Brazilian semiarid.

bacteria were incubated at 37°C and those with fungi at 35°C, both for a period of 24 h. Chloroform and 10% DMSO were used as negative control. As positive controls, 0.12% chlorhexidine gluconate was used for *S. oralis, S. salivarius and E. faecalis;* Cephalothin for *S. aureus;* Gentamicin for *E. coli*, *P. aeruginosa* and *K. pneumoniae* and Nystatin for fungi. Microbial growth was indicated by the addition of 20 µL of resazurin aqueous solution (Sigma-Aldrich) at 0.01% in each well. The change from blue to pink coloration, characterized by the dye reduction, indicates the presence of viable microbial cells. MIC was considered the lowest extract concentration able to inhibit microbial growth.

For detecting the minimum microbicide concentration (MMC), 20 µL of the wells suspensions that showed no coloration change were transferred into Petri dishes containing BHI agar, which were incubated under the same conditions described above. The MMC was determined by

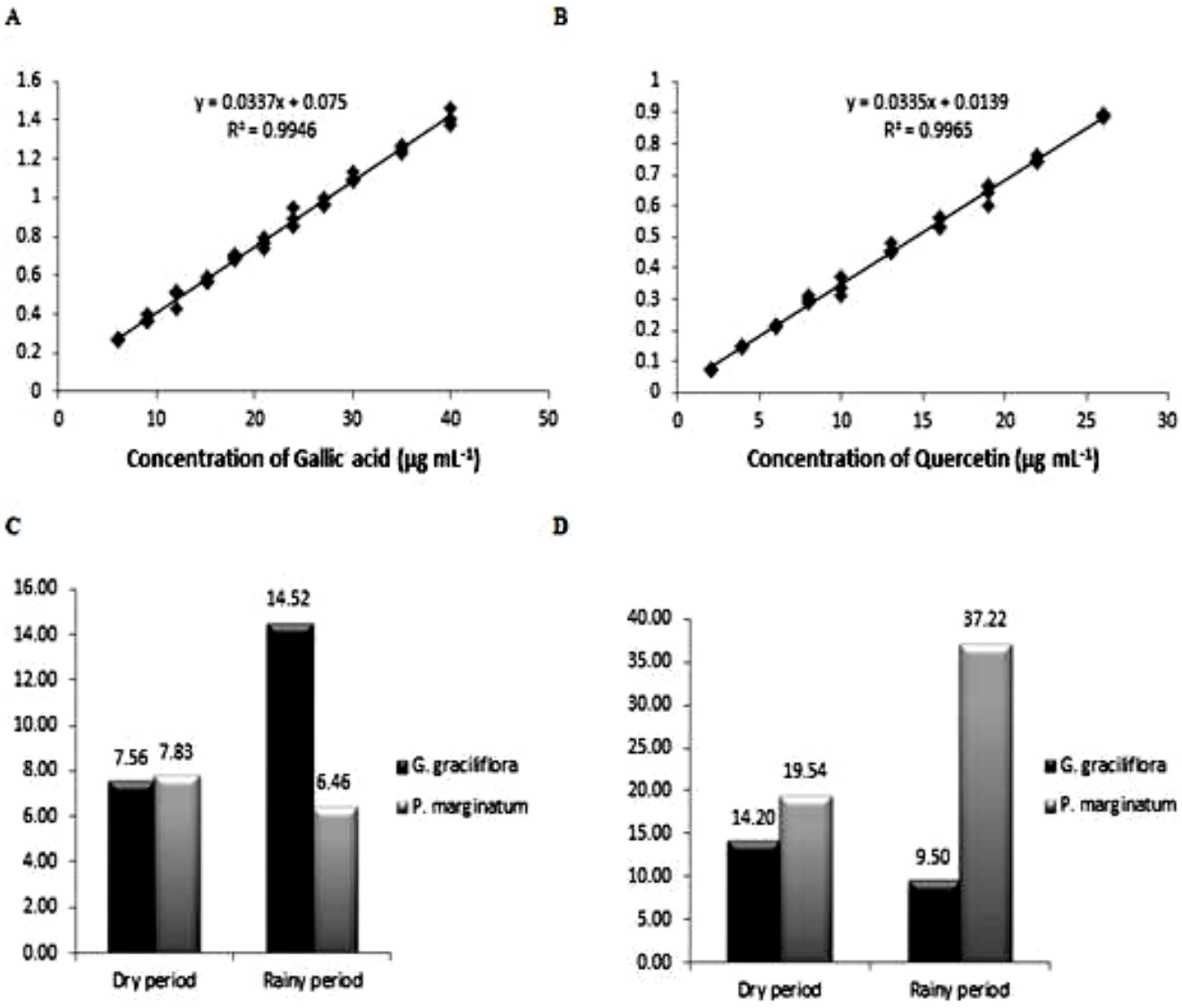

Figure 2. Calibration curves with gallic acid (A) and quercetin (B) and concentration of total flavonoids (C) and total polyphenols (D) and *Guapira graciliflora Pseudobombax marginatum* in DP and RP.

the lowest concentration of extracts that inhibited 100% microorganisms' growth.

RESULTS AND DISCUSSION

Phytochemical tests

Figure 2 shows the concentration of polyphenols and total flavonoids determined in the plants studied. It was observed that the highest concentration of polyphenols in *P. marginatum* was detected in RP reaching 37.22 µg.mL^{-1}, while in the DP the value measured was 19.54 µg.mL^{-1}. With *G graciliflora* the opposite has occurred: concentration reached 14.20 µg.mL^{-1} in the DP and 9.5 µg.mL^{-1} in the RP. The content of flavonoids in *G. graciliflora* nearly doubled from 7.56 µg.mL^{-1} in the DP to 14.52 µg.mL^{-1} in the RP, while in *P. marginatum* that number was less variable and slightly higher during DP with 7.83 µg.mL^{-1} and decreasing to 6.45 µg.mL^{-1} in the RP.

It is observed that the afore mentioned plants respond differently to environmental changes, and according to Gobbo-Neto and Lopes (2007) and Ncube et al. (2010) the physiological characteristics associated with genetic conditions responsible for the variations mentioned above, which arise probably of different climate characteristics in both seasons in the region under study, since the metabolism is influenced in many ways by those conditions. Factors such as water level in the soil, evapotranspiration rate, light intensity, photosynthetic

Table 1. MIC and MMC determination of extracts from P. *marginatum* and *G. graciliflora* collected in the summer and winter on the microorganism tested.

Microorganisms	MIC (MMC) mg.mL^{-1}					
	DP		RP		PC	NC
	P. m.	*G. g.*	*P. m.*	*G.g.*		
S. s.	50.0 (100.0)	N.a. (N.a.)	50.0 (100.0)	N.a. (N.a.)	< 1 (< 1)	-
S. o.	100.0 (N.a)	N.a. (N.a.)	100.0 (N.a)	N.a. (N.a.)	< 1 (< 1)	-
S. a.	25.0 (50.0)	N.a. (N.a.)	12.5 (50.0)	N.a. (N.a.)	< 1 (< 1)	-
E. f.	50.0 (N.a)	N.a. (N.a.)	100.0 (N.a)	N.a. (N.a.)	< 1 (< 1)	-
E. c.	50.0 (N.a.)	N.a. (N.a.)	50.0 (100.0)	N.a. (N.a.)	< 1 (< 1)	-
P. a.	N.a. (N.a.)	N.a. (N.a.)	N.a. (N.a.)	N.a. (N.a.)	< 1 (< 1)	-
K. p.	12.5 (100.0)	N.a. (N.a.)	N.a. (N.a.)	N.a. (N.a.)	< 1 (< 1)	-
C. a.	N.a. (N.a.)	N.a. (N.a.)	N.a. (N.a.)	N.a. (N.a.)	< 1 (< 1)	-
C. k.	N.a. (N.a.)	N.a. (N.a.)	N.a. (N.a.)	N.a. (N.a.)	< 1 (< 1)	-
C. g.	100.0 (N.a)	N.a. (N.a.)	100.0 (N.a)	N.a. (N.a.)	< 1 (< 1)	-

DP – Dry period**; RP –** Rainy Period; **N.a.** Not active; **PC** – Positive Control; **NC** – Negative Control; ***P. m. –*** *Pseudobombax marginatum*; ***G. g. –*** *Guapira graciliflora;* ***S. s.*** *– Streptococcus salivarius,* ***S. o.*** *– Streptococcus oralis;* ***S. a.*** *- Staphylococcus aureus;* ***E. f.*** *– Enterococcus faecalis;* ***E. c.*** *- Escherichia coli;* ***P. a.*** *– Pseudomonas aeruginosa;* ***K. p.*** *– Klebisiella pneumoniae;* ***C. a.*** *– Candida albicans;* ***C. k.*** *– Candida krusei;* ***C. g.*** *– Candida guilliermondii.*

efficiency, plant water potential and plant stage, directly respond to these variations (Ferri, 1986; Larcher, 2004; Taiz and Zeiger, 2004). Ncube et al. (2010) have found variation in the production of polyphenols in *Tulbaghia violacea*, *Hypoxis hemerocallidea*, *Merwilla plumbea* and *Drimia robusta* in different seasons, the explanation lies precisely in the climate differences, biotic and environmental conditions in addition to those genetic. Other authors Ma et al. (2003), Brooks and Feeny (2004), Ercisli et al. (2008), Ruiz-Terán et al. (2008), Santos and Kaye (2009), Siatka and Kašparová (2010) and Chavarria et al. (2011) have also attributed the variation in the production of secondary metabolites to environmental factors.

Gobbo-Neto and Lopes (2007) report that these factors have correlations with each other and do not act in isolation; they may jointly influence the secondary metabolism. As the plant material analyzed in this study was collected from plants growing under natural conditions, it is not easy to separate the effects of individual factors from the multifactorial influence of the environment.

Antimicrobial activity

Table 1 shows the values of MIC and MBC of extracts from *G. graciliflora and P. marginatum.* At the concentrations tested, the extracts of *P. marginatum* led to growth inhibition of all bacterial strains tested, except for *P. aeruginosa*. The extract obtained in the DP had the lowest MIC against *K. pneumoniae*, with value of 12.5 mg.mL^{-1} a while for extract in the DP the lowest MIC was obtained against *S. aureus* in the same concentration. The lower MMC for both extracts was 50.0 mg.mL^{-1} against *S. aureus*. From the fungi tested, *C. guilliermondii* was only inhibited by both extracts in a concentration of 100.0 mg.mL^{-1}, although this concentration has not been fungicide. None of the isolates tested was sensitive to extracts of *G. graciliflora* in the concentrations tested.

Although *G. graciliflora* and *P. marginatum* having secondary metabolites with proved antimicrobial activity (Cowan, 1999; Monteiro et al., 2005) the activity presented here was not clinically significant according to the study of Fabry et al. (1998) and Ríos and Recio (2005) which suggest the CIM of extracts expressive less than 8 mg.mL^{-1} and 1mg.mL^{-1} respectively. This may be explained since the metabolites that have the biological activity are not present in sufficient quantities to the extract show antimicrobial activity, which may be related to the organic solvent used in their preparation. Another probable hypothesis is that the activity cannot be attributed only to a single compound, but compounds or different combinations with the same effects and/or synergistic effects on the microorganism (Ncube et al., 2010). According to Silva et al. (2003), the antimicrobial activity of plant extracts occurs by the combined action of chemical compounds present in plants, and not by the activity of isolated compounds.

The antimicrobial activity by *P. marginatum* was slightly better in the DP, where flavonoids showed higher concentration that RP, showing that the seasonality influenced the biological activity in question, which has not happened with *G. graciliflora.* Studies addressing the action of seasonality on the antimicrobial potential of plant extracts are still very scarce. Hess et al. (2007) evaluating the seasonality effect on the antibacterial potential of ethanol extracts obtained from aerial parts of

Elyonurus miticus observed that the extracts produced in the spring were more effective on Gram-positive bacteria tested. In contrast, Schmidt et al. (2008) observed that the antimicrobial activity of *Baccharis trimera* (Less.) DC was not significantly altered depending on the collection period.

Ordoñez et al. (2004) assessing the antimicrobial activity of *Boerhavia erecta* L. (Nyctaginaceae), reported low bacteriostatic activity against *K. pneumoniae* (MIC = 100 mg.mL^{-1}) and resistance of *C. albicans* to all extract concentrations tested. On the other hand, the extract of *Bougainvillea glabra* Choisy, showed antimicrobial activity against *E. coli, S. aureus, K. pneumoniae* (Edwin et al., 2007) as well as *Mirabilis jalapa* L. that in addition to these microorganisms, showed activity against *P. aeruginosa* and *C. albicans* (Walker et al., 2009).

Microbiological studies on species of the family Bombacaceae were similar to those of *P. marginatum,* the extracts of which showed better activity against *S. aureus.* In the microbiological screening, performed by Leal et al. (2011), with extract from stem bark of *Ceiba glaziovii* Kuntze K. Schum. observed that *S aureus* was sensitive while *E. coli and C. albicans* were resistant to that extract. The extract of *Adansonia digitata,* evaluated by Masola et al. (2009), showed significant antimicrobial activity against several microorganisms, including *S. aureus*.

G. graciliflora and *P. marginatum* have secondary metabolites that confer different pharmacological activities, which can justify its use in folk medicine. The groups of active substances assessed for seasonal variation showed concentration differences between the DP and RP in both species. As regards the antimicrobial activity, the MIC and MMC also showed some variation, but only *P. marginatum* showed inhibitory and bactericidal activity against Gram positive and Gram negative bacteria tested, but at high concentrations. No microorganism was inhibited by extracts of *G. graciliflora* at the concentrations tested.

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