Antimicrobial activity of *Marcetia* DC species (Melastomataceae) and analysis of its flavonoids by reverse phase-high performance liquid chromatography coupled-diode array detector

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ABSTRACT

Background: *Marcetia* genera currently comprises 29 species, with approximately 90% inhabiting Bahia (Brazil), and most are endemic to the highlands of the Chapada Diamantina (Bahia). Among the species, only *M. taxifolia* (A.St.-Hil.) DC. populates Brazil (state of Roraima to Paraná) and also Venezuela, Colombia, and Guyana. **Objective:** This work evaluated the antimicrobial activity of hexane, ethyl acetate, and methanol extracts of three species of *Marcetia* (*Marcetia canescens* Naud., *M. macrophylla* Wurdack, and *M. taxifolia* A.StHil) against several microorganism. In addition, the flavonoids were analyzed in extracts by HPLC-DAD. **Materials and methods:** The tests were made using Gram-positive (three strains of *Staphylococcus aureus*) and Gram-negative (two strains of *Escherichia coli*, a strain of *Pseudomonas aeruginosa* and another of *Salmonella choleraesius*) bacteria resistant and nonresistant to antibiotics and yeasts (two strains of *Candida albicans* and one of *C. parapsilosis*) by the disk diffusion method. Solid-phase extraction (SPE) was performed on the above extracts to isolate flavonoids, which were subsequently analyzed by high performance liquid chromatography coupled diode array detector (HPLC-DAD). **Results:** Results showed that extracts inhibited the Gram-positive bacteria and yeast. The hexane extracts possessed the lowest activity, while the ethyl acetate and methanolic extracts were more active. **Conclusion:** *Marcetia taxifolia* was more effective (active against 10 microorganisms studied), and only its methanol extract inhibited Gram-negative bacteria (*P. aeruginosa* and *S. choleraesius*). SPE and HPLC-DAD analysis showed that *M. canescens* and *M. macrophylla* contain glycosylated flavonoids, while the majority of extracts from *M. taxifolia* were aglycone flavonoids.

Key words: Antimicrobial activity, flavonoids, high performance liquid chromatography coupled-diode array detector, *Marcetia*

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INTRODUCTION

Brazil has a high biodiversity, comprising approximately 20% of the total species of terrestrial flora, including endemic plants, which have hardly been explored.[1] The Melastomataceae family comprises 166 genera and 4,500 species.[2] In Brazil, this family is the sixth major family between the Angiospermas, with 68 genera and more than 1,300 species.[3,4] The genus *Marcetia* is characterized by the combination of scored leaves without glandular tetramer flowers, by isomorphic or subisomorfos stamens, and by connective thickened dorsally, shortly or not prolonged below the teak.[5] Additionally, this genus currently comprises 29 species, with approximately 90% inhabiting

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Bahia (Brazil), and most are endemic to the highlands of the Chapada Diamantina (Bahia). Among the species, only *M. taxifolia* (A.St.-Hil.) DC populates Brazil (state of Roraima to Paraná) and also Venezuela, Colombia, and Guyana.[5,6]

The use of antibiotics has increased considerably in recent years due to developing microorganism resistance.[7] Microorganism resistance has intensified in severe medical cases, for example, in immunocompromised patients, opportunistic infections, post-transplant therapy and chemotherapy treatments, necessitating the search for new compounds. Thus, the secondary metabolism of plants or their synthetic derivatives have become a source of new active compounds against these microorganisms.[8]

Phenolic compounds are widely distributed in the plant kingdom,[9] and they possess biological action, especially antimicrobial[10] and antioxidant activities.[11] Sample preparation is a key procedure in biological and chemical analysis; however, various nonpolar compounds within plant materials affect their preparation. Thus, the method of sample preparation must be simple and effective to obtain the best response. Solid-phase extraction (SPE) is one of the simplest methods for concentrating natural products.[12] Components of interest within a plant extract can be separated from other metabolites by applying the extract to an appropriately chosen solid sorbent, which is prepacked and disposable in cartridges, and selectively eluting the desired components.[13] In the specific case of phenols, the coupling of SPE with reverse phase-high performance liquid chromatography (RP-HPLC) allows for crucial analytical characterization of flavonoids in plant extracts.[14,15] Furthermore, diode array detection (DAD) facilitates characterization of the main flavonoid structures present in a crude extract.[16,17] Thus, the HPLC-DAD analysis provides information about the flavonoid structure from retention time in a specific solvent phase and from absorption bands.[18] Band A is more significant in the following absorption ranges: 310-350 nm for flavones, 350-385 nm for flavonols, and 300-330 nm for flavanones and dihydroflavonols.[19] In addition, several authors have demonstrated that these techniques are rapid, inexpensive, and efficient. Unknown flavonoids can be identified based on their UV spectra and the correlation with standard compounds isolated from others fonts using other techniques.[20] Additionally, chemotaxonomic data of the family may aid in its identification.[21]

The present study shows the evaluation of the *in vitro* antimicrobial activity of *Marcetia* extracts, including Gram-positive and Gram-negative resistant and nonresistant bacteria and yeast, using the disk-diffusion method. In addition, after clean-up by SPE, flavonoid analysis was performed on these extracts by HPLC-DAD.

MATERIAL AND METHODS

Plant material

Marcetia canescens Naud., *M. macrophylla* Wurdack, and *M. taxifolia* were collected in Morro-do-Chapéu and Rio-de-Contas (Chapada Diamantina, state of Bahia, Brazil). The plants were identified by Dr. Andrea K. A. Santos and Dr. Tânia R. S. Silva. Voucher specimens were deposited in the Herbarium of the Department of Biology of the State University of Feira de Santana (HUEFS) with the following numbers: T.R.S.Silva 246, A.K.A.Santos 269 and A.K.A.Santos 438, respectively.

Crude extract preparation

The dried aerial part of the plant (125 g of *Marcetia canescens*, 151 g of *M. macrophylla* and 44 g of *M. taxifolia*) was powdered and extracted at room temperature with hexane, ethyl acetate and methanol, successively for 48 hours for each solvent. The solvents were evaporated under reduced pressure to obtain the hexane extract [HE: 2.6 g (yielding 2.1%), 3.8 g (2.5%) and 0.9 g (2.0%), respectively)], ethyl acetate extract [EE: 1.4 g (1.1%), 4.1 g (2.7%) and 0.5 SS] g (1.1%), respectively] and methanol extract [ME: 3.0 g (2.4%), 5.2 g (3.4%) and 1.3 g (3.0%), respectively].

Microorganisms

The strains of bacteria and yeast used were obtained from the Culture Collection of Microorganisms of Bahia (CCMB) at the State University of Feira de Santana, Brazil. We tested four Gram-negative bacteria (*E. coli* CCMB 261 sensitive to trimethoprim and sulfonamide-resistant, *Escherichia coli* CCMB 258, *Pseudomonas aeruginosa* CCMB 268 and *Salmonella choleraesius* CCMB 281), three Gram-positive bacteria (*Staphylococcus aureus* CCMB 262 resistant to streptomycin and dihydrostreptomycin, *S. aureus* CCMB 264 resistant to novobiocin and *S. aureus* CCMB 263) and three yeast cultures (*Candida albicans* CCMB 266, *C. albicans* CCMB 286 resistant to fluconazole and amphotericin B and *C. parapsilosis* CCMB 288 resistant to fluconazole and amphotericin). Cultures were grown on Müeller-Hinton agar (MHA) at 37 ºC for 24 h for bacteria and at 28 ºC for 48 h for yeast.

Antimicrobial activity

The antimicrobial activity of extracts was carried out using the agar diffusion method.[22] This test was performed on sterile filter paper disks (6 mm), as recommended by the National Committee for Clinical Laboratory Standard.[23] An aliquot of each extract (100 mg/ml) was sterilized by 0.22 μm membrane filtration (TPP), and the filter paper disks were then impregnated with an aliquot of 5 μl. A suspension with 100 μl of the test microorganism (0.1 ml of 1.5×10^8 CFU ml-1 for bacteria and 0.1 ml of 1.5×10^5 CFU ml^{-1} for yeast) was spread on the surface of the Müeller Hinton Agar solid media (MHA) in Petri

dishes (15 ×90 mm). Afterward, the disk impregnated with extracts was placed on the plates inoculated with the test microorganisms. The plates were incubated at 37 ºC for 24 h for bacteria and 28 ºC for 48 h for yeast. After this period, visual readings were performed by observing the presence of a bacterial growth inhibition zone measured in millimeters, with the aid of a millimeter ruler. As a positive control, the disks were impregnated with 5 µl of antimicrobial substance at concentrations of 10 mg/ml erythromycin for bacteria and 20 mg/ml nystatin for yeast. As a negative control, the disks were impregnated with hexane (5 µl). All tests were performed in triplicate.

Flavonoid analysis

The flavonoid used as a reference was purchased from Sigma-Aldrich. The calycopterin was previously obtained in our laboratory from *Marcetia latifolia* Naud.[24] Prior to HPLC-DAD analysis from the *Marcetia* ethyl acetate and methanol extracts, a purification step was carried out using solid-phase extraction (SPE) cartridges to concentrate the flavonoid compounds. One milliliter of each extract was passed through a C_{18} Sep-pak cartridge (Strata-X, Phenomenex). Flavonoids were adsorbed onto the column and eluted with methanol. The methanol was removed under vacuum, and the flavonoid content was redissolved in Milli-Q water. The flavonoid analyses were made using a reverse-phase HPLC column on a Hitachi equipped with an autoinjector, a photodiode array detector (PAD) and Lachrom software. Spectra data were recorded from to 200 to 400 nm during the entire run. An Elite LiCospher 100 RP_{18} (5 µm, 150 × 4 mm) column (Merck) and a 4.6 mm × 2.0 mm guard column were used for flavonoid analysis at 30°C. The mobile phase was composed of solvent (A) H_2O/H_3PO_4 0.1% and solvent (B) MeOH. The solvent gradient was composed of A (75-0%) and B (25-100%) for 25 minutes. A flow rate of 1.0 ml/min was used, and 20 µl of each sample was injected. Samples and mobile phases were filtered through a 0.22 µm Millipore filter prior to HPLC injection. Flavonoids were characterized by comparing their retention time and UV-Vis spectral data to that of known standards.

RESULTS

The data for antibacterial and antifungal activities for the crude extracts of *Marcetia canescens, M. macrophylla* and *M. taxifolia* are shown in Tables 1 and 2, respectively. The antimicrobial activities assay was performed for the crude extract against three strains of Gram-positive bacteria, four strains of Gram-negative bacteria and three strains of yeast. The SPE procedures were applied to ethyl acetate and methanol extracts to obtain concentrated flavonoids in *Marcetia* species. The samples of each extract were later analyzed by HPLC-DAD. Figure 1 shows chromatograms of the samples, and Figure 2 shows the UV λ_{max} values of peaks eluted in *Marcetia* samples.

DISCUSSION

The extracts that were studied had lower inhibition against yeast, except for the methanolic extract of *M. taxifolia* that inhibits the microorganisms *P. aeruginosa* (8.20 mm) and *S. choleraesius* (12.92 mm). All extracts inhibited the growth of *S. aureus* that is resistant and nonresistant to antibiotics, but they were inactive against Gram-negative bacteria. Erythromycin, which was used as a positive experimental control against all assayed bacteria strains, produced a zone of inhibition of 14-31 mm [Table 1].

The inhibition zone analysis of the organisms tested suggest that the ethyl acetate extracts and methanol extracts of

Table 1: Antibacterial activity of the crude extracts from *Marcetia* species using the diffusion method[h]

Specie/extract	Microorganisms						
	E. coli	*E. coli*[1]	*S. aureus*[2]	*S. aureus*	*S. aureus*[3]	*P. aeruginosa*	*S. choleraesius*
M. canescens							
HE	-	-	11.25±1.88	-	10.25±1.75	-	-
EE	-	-	-	19.85±0.76	11.50±0.50	-	-
ME	-	-	8.50±0.00	9.75±0.43	12.5±1.52	-	-
M. macrophylla							
HE	-	-	-	-	7.95±0.14	-	-
EE	-	-	-	-	-	-	-
ME	-	-	7.70±0.38	7.33±0.29	-	-	-
M. taxifolia							
HE	-	-	-	8.50±0.43	-	-	-
EE	-	-	10.25±0.66	11.33±1.15	9.20±0.29	-	-
ME	-	-	9.25±0.25	8.67±0.58	10.33±0.58	8.20±1.26	12.92±1.23
Eritromicina	14.48±1.58	24.16±0.70	27.47±0.97	31.55±1.36	34.50±1.44	9.47±0.49	10.05±1.38

h: Inhibition zone (mm); -: not active, HE: Hexane extract; EE: Ethyl acetate extract; ME: Methanolic extract; 1: sensitive to trimethoprim and sulfonamide-resistent; 2: resistant to streptomycin and dihydrostreptomycin; 3: resistant to novobiocin.

M. taxifolia were the most active, inhibiting the grown of five and seven microorganisms, respectively. Ethyl acetate extracts of *M. canescens* and methanol extracts of *M. taxifolia* yielded the largest inhibition zone diameters, with a 19.85 mm diameter against *S. aureus* and a 15.5 mm diameter against C. *parapsilosis*.

Table 2: Antifungal activity of the crude extracts from *Marcetia* species using the diffusion method[h]

Specie/extract	Microorganisms		
	C. albicans	*C. albicans*[1]	*C. parapsilosis*[1]
M. canescens			
HE	-	-	-
EE	8.33±0.38	-	-
ME	-	-	-
M. macrophylla			
HE	-	-	-
EE	-	-	7.33±0.29
ME	-	-	-
M. taxifolia			
HE	-	-	-
EE	-	-	15.50±1.00
ME	8.25±0.25	9.17±0.29	-
Nistatin	20.09±1.40	23.50±1.34	8.83±0.49

h: Inhibition zone (mm); -: no active, HE: Hexane extract; EE: Ethyl acetate extract; ME: Methanolic extract; 1: resistant to fluconazole and amphotericin.

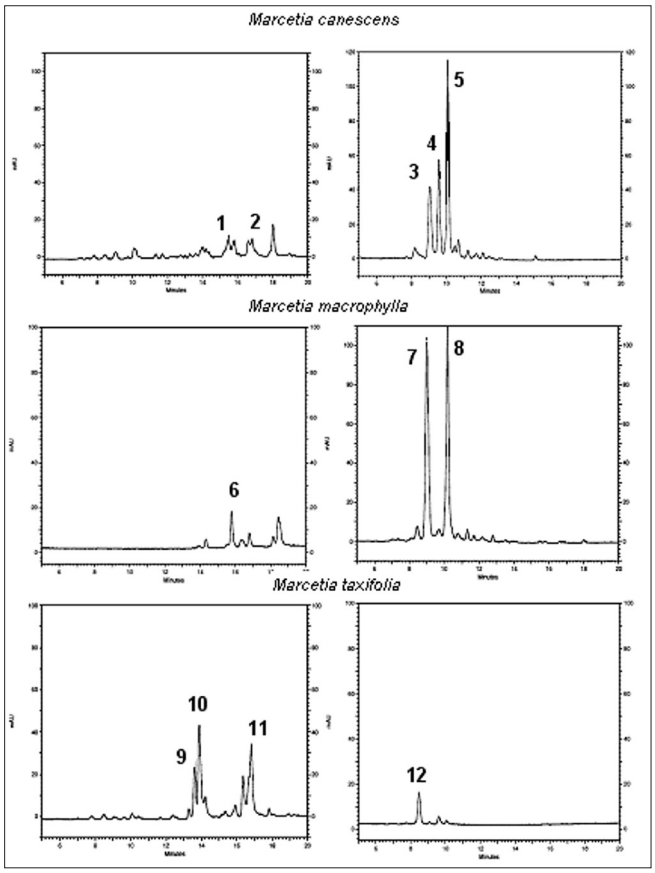

Figure 1: High performance liquid chromatography coupled Chromatogram of *Marcetia* species. Chromatograms to the left represent the ethyl acetate extraction, and chromatograms to the right represent the methanolic extraction

After the evaluation and comparison of antimicrobial activity between different extracts of *Marcetia* species collected in the Brazilian semiarid region, it was notable that a principal class of natural products was responsible for their actions. Furthermore, the large variety of natural products may each possess antimicrobial activity. In the present study, the hexane extracts (apolar) of three *Marcetia* species showed that the low ranges of antimicrobial properties were different for the more methanol extracts. Gas chromatography analysis of these extracts showed the presence of a majority of hydrocarbonets and a minority of terpenoids, which are common in the Melastomataceae family.[25] On the other hand, polar extracts from species within this family exhibited a wide range of antimicrobial activity with varying strengths against the tested Gram-positive and Gram-negative bacteria and also some fungi.[26-29] Chemical investigation of the antimicrobial active extract of Melastomataceae cited in literature showed the presence of flavonoid, e.g., *Miconia cabucu* Hoehne, *M. rubiginosa* (Bonpl.) DC, *M. stenostachya* DC,[30] and *Tibouchina grandifolia* Cogn.[31] These facts suggest that flavonoid is truly active against several microorganisms

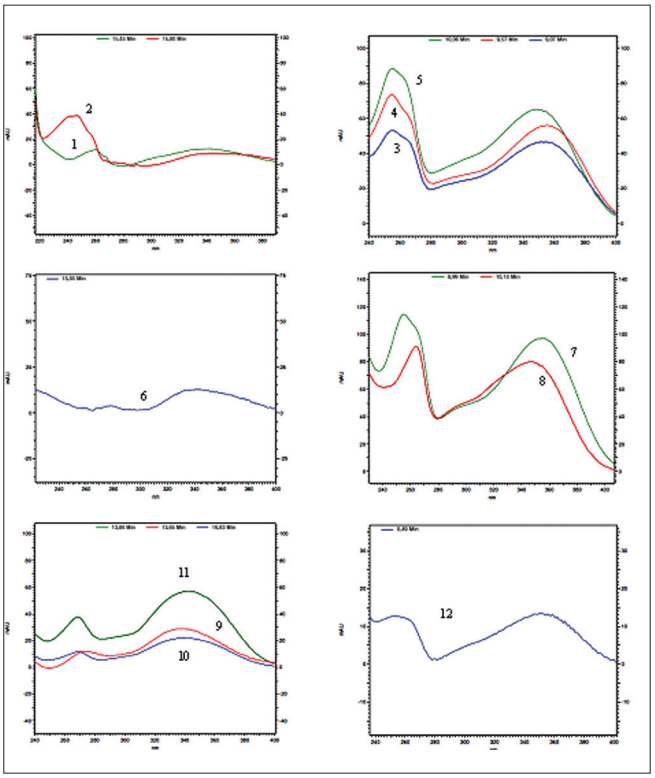

Figure 2: UV spectra of the peaks shown in the high performance liquid chromatography coupled chromatogram of *Marcetia* species

due to its ability to react with extracellular and soluble proteins and to form complexes with bacterial cell walls,[32] allowing us to investigate the presence of flavonoids in the extracts of *Marcetia*.

To perform HPLC-DAD analysis, the same patterns of flavonoids were eluted in the same experimental HPLC-DAD conditions: three glycosylated flavonoids (naringin t_R 8.5 minutes, hesperidin t_R 8.9 minutes and rutin t_R 9.2 minutes), one polyhydroxylated flavonoid (quercetin t_R 11.9 minutes), and one polymethoxylated flavonoid (t_R 15.5 minutes calycopterin). This polymethoxylated flavonoid was isolated from *M. latifolia*.[24] Thus, three regions of elution were determined in this system: glycosylated (between 8 and 10 minutes), hydroxylated (approximately 12 minutes) and polymethoxylated (14-17 minutes) flavonoids.

Thus, the ethyl acetate extract of *M. taxifolia* (peaks 9, 10, and 11) primarily contained polymethoxylated flavonoids, while the methanol extracts of *M. canescens* (peaks 3, 4, and 5) and *M. macrophylla* (peaks 7 and 8) contained glycosylated flavonoid. The other chromatograms lacked flavonoids. It can be deduced from the UV spectra that flavonoids present in chromatograms were of the flavonol class due the absorption between 342 and 359 nm (band I), characteristic of this flavonoid class.

There was a correlation between the presence of glycosylated flavonoids and good antimicrobial activity from the extracts against *S. aureus*, except the methanol extract of *M. taxifolia* did not show glycosylated flavonoid and showed good inhibition against this microorganism.

CONCLUSION

This is the first study focused on antimicrobial activity and flavonoid profile in the *Marcetia* species. The hexane, ethyl acetate, and methanolic extracts of the species of *Marcetia* tested in this work showed some antimicrobial activity. The flavonoids present in the extracts were extracted using SPE followed by HPLC-DAD analysis. This method illustrates a good and rapid technique to analyze flavonoids in *Marcetia* species. Thus, *M. canescens* and *M. macrophylla* were used to derive glycosylated flavonols, while the majority of extracts from *M. taxifolia* were aglycone flavonols.

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