CHEMICAL STUDY AND ANTIMICROBIAL ACTIVITIES OF *Piper arboreum* (PIPERACEAE)SANDRA A. NASCIMENTO^a · EMERSON A. ARAÚJO^b · JANETE M. DA SILVA^b · CLÉCIO S. RAMOS^{a*}^aDepartment of Molecular Sciences, Rural Federal University of Pernambuco, Recife-Pe, Brazil^bAntibiotics Department, Federal University of Pernambuco, Recife-Pe, Brazil,

ABSTRACT

This study evaluated the antifungal and antibacterial activities of the extracts and their fractions, essential oils, and major constituents of *Piper arboreum* tissues. The chemical compositions of *P. arboreum* tissues and major constituent were identified using GC/MS, ¹H and ¹³C NMR. All the botanic material tested proved to be specific bactericidal agents against *Staphylococcus aureus*, with MBC/MIC = 2, including the amide piperetine, the major compound isolated of *P. arboreum* for the first time. The essential oil of leaves showed antifungal activity against *Microsporium gypseum* and *Epidermophyton floccosum*, with MFC values of 312.5 and 125 µg/mL, respectively.

Keywords: Piperaceae; *Piper arboreum*; Antimicrobial; Piperetine.

INTRODUCTION

The genus *Piper* (Piperaceae) includes about 1,400 species, distributed pantropically and taking the form of shrubs, herbs, and lianas, common in the understory of lowland wet forests. It is one of the most diverse lineages among basal angiosperms.^{1,2} *Piper* species have received much attention due to the potent activities and chemical diversity of their secondary metabolites, and several species have high economic value, such as *P. nigrum* (black pepper), *P. methysticum* (kava), and *P. betle* (betel nuts) *P. divaricatum*.³⁻⁵ In particular, *Piper arboreum*, a shrub that typically grows to 3 m height, popularly known as long pepper, rosemary-of-Angola or wood-of-Angola, has been used in traditional Brazilian medicine in the form of tea for treatment of rheumatism, bronchitis, colds and flu.⁶ Previous chemical investigations carried out on leaves of *P. arboreum* led to the identification of the amides *N*-[10-(13,14-methylenedioxyphenyl)-7(*E*),9(*Z*)pentadienyl]-pyrrolidine, arboreumine, *N*-[10-(13,14-methylenedioxyphenyl)-7(*E*)-pentaenyl]-pyrrolidine, *N*-[10-(13,14-methyl-methylenedioxyphenyl)-7(*E*),9(*E*)pentadienyl]-pyrrolidine and methyl 3-geranyl-4-hydroxybenzoate.^{7,8} Previous phytochemical studies of the leaf essential oil of *P. arboreum* revealed as major components the sesquiterpenes δ -cadinene, β -caryophyllene, germacrene D and bicylogermacrene.⁹⁻¹² Biological studies of *P. arboreum* have shown that their non-volatile and volatile constituents were active against the fungi *Cladosporium sphaerospermum*, *C. cladosporioides*, *Candida krusei*, *C. parapsilosis* and *Cryptococcus neoformans* and protozoan *Trypanosoma cruzi*.^{13,14} Despite the widespread use of *P. arboreum* in traditional medicine, very few studies of its biological potential have been performed. Therefore, we investigated the potential antifungal and antibacterial activities of the non-volatile and volatile compounds from *P. arboreum* tissues.

MATERIAL AND METHODS

Plant material

P. arboreum leaves and roots were collected in Recife, Pernambuco state, northeastern Brazil, in January 2012. Botanical identification was made by Dr. Ângela M. M. Freitas (Department of Forest Sciences – Pernambuco Federal Rural University) and a voucher specimen was deposited in the Sérgio Tavares Herbarium of that university (HST 18179). Leaves and roots were dried to 40 °C and the dried materials were milled to a fine powder in a Macsalab mill (Model 200 LAB).

Instrumentation and chromatography materials

IR spectra were measured in KBr pellets with a Varian infrared spectrometer. EI-MS were obtained with a Varian 220-MS mass spectrometer. ¹H and ¹³C NMR spectra were recorded at 300 and 75 MHz, respectively (Bruker DPX-300). CDCl₃ (Aldrich) was used as the solvent and TMS as internal standard. Chemical shifts were reported in δ units (ppm) and coupling constants (*J*) in Hz. Silica gel (Merck, 230-400 mesh) was used for column

chromatographic separations, while silica gel 60 PF254 (Merck) was used for analytic (0.25 mm) and preparative (1.0 mm) TLC chromatography. HPLC analyses of extracts and pure compounds were performed with a Shimadzu instrument using a C₁₈ column (250 x 4.6 mm, 5 mm) from Supelco eluted in a gradient mode starting with CH₃OH:H₂O (1:1) to 100% of CH₃OH in 30 min, with detection at 254 nm.

Extraction and isolation of constituents

The dried and powdered roots of *P. arboreum* (960 g) were extracted successively with CH₂Cl₂:CH₃OH, 2:1 (3 x 1000 mL), for two days at room temperature. The resulting solution was filtered and concentrated in vacuum to yield 28.3 g of the crude extract. A part of the crude extract (27.0 g) was partitioned by column chromatography over silica gel, using the following solvents: chloroform (9 fractions, 250 mL each); EtOAc (5 fractions, 250 mL each); and methanol (5 fractions, 250 mL each). Fractions 3 and 4 were grouped, yielding an amorphous white solid (189 mg), a mixture of α -sitosterol and stigmaterol. Fractions 5 to 8 were grouped (3.2 g) and submitted to column chromatography over silica gel using hexane with increasing amounts of EtOAc as eluent, yielding 100 fractions (20 mL each). Fractions 75 to 90 were grouped (540 mg) and submitted to column chromatography over silica gel using Cl₂CH₂:CH₃OH with increasing amounts of CH₃OH as eluent. Sub-fractions 9 to 15 from this column were grouped and submitted to preparative silica-gel TLC purification, using CH₂Cl₂:CH₃OH 94:6 as eluent. This purification procedure yielded 73 mg of amide piperetine, a yellow solid. The dried and powdered leaves of *P. arboreum* (60 g) were also extracted successively with ethanol (6 x 200 mL), yielding 1.9 g of crude extract. The crude ethanol extract was partitioned with hexane (1.16 g) and chloroform (0.38 g).

Piperetine amide

The chemical structure (Figure 1) was determined in comparison with the NMR spectra values in the literature.¹⁵ ¹H-NMR (CDCl₃, 300 MHz): δ 3.60 (4H, m, H-2); 1.91 (6H, m, H-3,4,5); 3.58 (4H, m, H-6); 6.20 (1H, d, *J* = 14.7 Hz, H-8); 7.41 (1H, dd, *J* = 14.7 and 11.0 Hz, H-9); 6.45 (1H, d, *J* = 14.7 Hz, H-10); 6.69 (1H, dt, *J* = 14.7 and 11.0 Hz, H-11); 6.72 (1H, dd, *J* = 14.7 and 11.0 Hz, H-12); 6.57 (1H, d, *J* = 14.7 Hz, H-13); 6.98 (1H, d, *J* = 1.7 Hz, H-2'); 6.78 (1H, d, *J* = 8.0 Hz, H-5'); 6.88 (1H, dd, *J* = 8.0 and 1.7 Hz, H-6'); 5.98 (2H, s, OCH₂O). ¹³C-NMR (CDCl₃, 75 MHz): δ 46.74 (C-2); 26.37 (C-3); 24.60 (C-4); 29.94 (C-5); 46.74 (C-6); 165.4 (C-7); 121.59 (C-8); 141.9 (C-9); 130.5 (C-10); 139.86 (C-11); 126.91 (C-12); 135.67 (C-13); 131.6 (C-1'); 105.75 (C-2'); 148.4 (C-3'); 148.4 (C-4'); 108.74 (C-5'); 122.31 (C-6'); 101.46 (OCH₂O).

Chemical analysis and obtaining essential oil

The essential oil from fresh leaves (100 g) was obtained using a modified Clevenger-type apparatus and hydrodistillation for 2 h. The oil layers were separated and dried over anhydrous sodium sulfate, stored in hermetically sealed glass containers, and kept under refrigeration at + 5°C until analysis.

Total oil yields were expressed as percentages (g/100 g of fresh plant material). All experiments were carried out in triplicate. The essential oils were analyzed by GC-MS (60–240 °C at 3 °C min. rate) in a Varian 431-GC coupled to a Varian 220-MS instrument using fused-silica capillary column (30 m × 0.25 mm i.d. × 0.25 µm.) coated with DB-5. MS spectra were obtained using electron impact at 70 eV with a scan interval of 0.5 s and fragments from 40 to 550 Da (all the samples were analyzed in triplicate). Quantitative analysis of the chemical constituents was performed by flaming ionization gas chromatography (FID), using a Perkin-Elmer instrument, under the same conditions and with the same column as reported for the GC-MS. The injection volume was 1.0 µL of diluted solution (1/100) of oil in *n*-hexane. The amount of each compound was calculated from GC peak areas in the order of DB-5 column elution and expressed as relative percentages of the total area of the chromatograms. Identification of the components was based on GC retention indices with reference to a homologous series of C₇–C₃₀ *n*-alkanes calculated using the Van den Dool & Kratz¹⁶ equation and by computer matching against the mass spectral library of the GC/MS data system (NIST 98 and Wiley) as well as other published mass spectra.¹⁷

Figure 1. Chemical structure of amide piperetine isolated of *P. arboreum*.

In vitro assay for antimicrobial activity

The antimicrobial activity of *P. arboreum* tissue extracts as well as their fractions, essential oils and pure compounds were tested against the following microorganisms: *Staphylococcus aureus* (UFPEDA02), *Bacillus subtilis* (UFPEDA82), *Enterococcus faecalis* (UFPEDA138), *Escherichia coli* (UFPEDA 224), *Candida albicans* (UFPEDA1007), *Fusarium oxysporum* (UFPEDA 2456), *Microsporum gypseum* (URM 6199) and *Epidermophyton floccosum* (URM 3182). All strains were provided by the Antibiotics Department of Pernambuco Federal University (UFPEDA) and maintained in nutrient agar (NA), stored at 4 °C. The antibacterial and antifungal activities are reported preliminarily utilizing the disc diffusion method. In this method, disks containing known amounts of an antimicrobial agent were placed on the surface of an agar plate that had been inoculated with a standardized suspension of each microorganism tested. Paper discs with only ethanol were used as negative controls. All experiments were carried out three times and repeated if the results differed. All sample having IDZs (inhibition diameter zones) greater than or equal to 12 mm were selected for minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) assay. A stock solution (10 mg/mL) of each test sample was prepared in ethanol as the solvent. Furthermore, the serial dilution of the test compounds was carried out and the concentrations used ranged from 2500 to 19.53 µg/mL. Test samples at various concentrations were added to culture media in test tubes and different strains were inoculated at 108 bacteria/mL concentration. Tryptic soy agar and nutrient agar (for antibacterial), and Sabouraud liquid medium (for antifungal) were utilized as culture media. The tubes were incubated at 37 °C (antibacterial) or 30 °C (antifungal) for 24–48 h and then examined for the presence or absence of growth of the organisms. Chloramphenicol and nystatin were used as antibacterial and antifungal substances, respectively. The MIC values were obtained from the lowest concentration of the test samples where the tubes remained clear, indicating that the bacterial or fungal growth was completely inhibited at this concentration. The MBC or MFC values were measured by inoculating the broths used for MIC determinations onto drug-free media. The MBC or MFC were the first dilution where no growth is observed. The MIC, MBC, and MFC values were expressed in µg/mL.

RESULTS AND DISCUSSION

Phytochemical analysis

The analysis by HPLC showed that the chemical profiles of leaf and root extracts of *P. arboreum* were identical, and a compound with retention time (*T_R*) at 22 min was detected on both extracts as the major compound.

The chloroform sub-fraction from the crude extract (CH₂Cl₂:CH₃OH, 2:1) of the *P. arboreum* roots was submitted to purification steps, resulting in the isolation of the amide piperetine (*T_R* = 22 min) and a mixture of α -sitosterol and stigmasterol. This is the first report of the three compounds isolated from *P. arboreum*. Structural elucidation of all compounds was based on interpretation of spectral data, mainly IR, MS, UV ¹H and ¹³C NMR, including comparison with values described in the literature.¹⁵ The results of the leaf essential oil analysis of *P. arboreum* obtained by hydrodistillation are described in Table 1. A total of eight compounds were identified, representing 99.98% of the total essential oil. The main constituents in the leaf essential oil were quantified to be curcumene (45.06%), *cis*-cadin-4-en-7-ol (12.07%) and germacrene B (9.73%), according to the results obtained from GC-MS.

Table 1: Chemical composition of the essential oil from the *P. arboreum* leaves.

Compounds ^a	Relative area (%)	IR ^b	IR ^c
Isolodene	7.55	1379	1374
Rotundene	5.92	1458	1459
Curcumene	45.06	1478	1479
Germacrene D	7.27	1494	1484
Premnaspiradiene	8.26	1504	1505
λ -Cadinene	4.12	1508	1513
Germacrene B	9.73	1555	1559
<i>Cis</i> -Cadin-4-en-7-ol	12.07	1644	1635

^aCompounds are listed in order of their elution from a DB-5 column; ^bRI = retention indices relative to C₇ – C₃₀ *n*-alkanes; ^cRI = retention indices from literature.

Antimicrobial activities

All the extracts and their fractions as well as essential oil from *P. arboreum* tissues exhibited narrow activity against the bacterium *S. aureus*, except the crude extract (CH₂Cl₂:CH₃OH, 2:1) of the roots, which showed no inhibition diameter zones (IDZs) (Table 2). The essential oil from the leaves showed IDZs for the bacterium *Bacillus subtilis* (8 mm) and the fungi *M. gypseum* (23 mm) and *E. floccosum* (15 mm), while the crude ethanol extract from the leaves and the hexane fraction exhibited IDZs of 10 mm for the bacterium *E. faecalis*. All compounds having IDZs greater than or equal to 12 mm were selected for MIC and MBC or MFC. The results of MIC and MBC for the bacterium *S. aureus* of all the extracts tested as well as the amide piperetine were identical, with values of 250 and 500 µg/mL, respectively (Table 3). The essential oil from the leaves showed greater activity against *S. aureus*, with MIC and MBC values of 62.5 and 125 µg/mL, respectively. Since antimicrobial substances are considered to be bacteriostatic agents when the MBC/MIC ratio > 4 and as bactericidal agents when the same ratio ≤ 4, the extracts from *P. arboreum* tissues proved to be strong bactericidal agents against *S. aureus* (MBC/MIC = 2). Additionally, the essential oil from the leaves showed antifungal activity against *M. gypseum* and *E. floccosum*, with MFC values of 312.5 and 125 µg/mL, respectively.

Table 2. Antimicrobial activity of *P. arboreum* tissues using disc diffusion assay

Diameter of inhibition zone (mm) / Concentration of 20 mg/mL							
Micro-organisms	1	2	3	4	5	6	7
<i>Staphylococcus aureus</i>	0	12	15	15	12	15	15
<i>Bacillus subtilis</i>	0	0	0	0	0	0	8
<i>Enterococcus faecalis</i>	0	0	10	10	0	0	0
<i>Escherichia coli</i>	0	0	0	0	0	0	0
<i>Candida albicans</i>	0	0	0	0	0	0	0
<i>Fusarium oxysporum</i>	0	0	0	0	0	0	0
<i>Microsporum gypseum</i>	0	0	0	0	0	0	23
<i>Epidermophyton floccosum</i>	0	0	0	0	0	0	15

1. Root-dichloromethanic extract; 2. Root-chloroformic fraction; 3. Leaf-ethanoic extract; 4. Leaf-hexanic fraction; 5. Leaf-chloroformic fractions; 6. Amide piperetine; 7. Leaf-essential oil.

Table 3. MIC, MBC and MFC of the extracts and essential oil obtained of *P. arboreum* tissues.

Micro-organisms	Samples tested	MIC (µg/mL)	MBC (µg/mL)	MFC (µg/mL)
<i>S. aureus</i>	Leaf ethanolic extract	250	500	-
	Leaf hexanic fraction	250	500	-
	Leaf chloroformic fraction	250	500	-
	Root chloroformic fraction	250	500	-
	Amide piperetone	250	500	-
	Leaf essential oil	62.5	125	-
	Chloramphenicol	20	25	-
<i>M. gypseum</i>	Leaf essential oil	156.25	-	312.5
	Nistatin	1.1	-	1.1
<i>E. floccosum</i>	Leaf essential oil	62.5	-	125
	Nystatin	1.4	-	1.4

CONCLUSION

In particular, *P. arboreum* tissues showed specific narrow against the bacterium *S. aureus*, and specifically the essential oil of the plant showed greater activity to *S. aureus* and the fungus *M. gypseum* and *E. floccosum*.

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