

Full Length Research Paper

Antimicrobial activity of *Pityrocarpa moniliformis* leaves and its capacity to enhance the activity of four antibiotics against *Staphylococcus aureus* strains

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This work evaluated the antimicrobial activity of *Pityrocarpa moniliformis* leaves and the effects of extract and two active fractions in combination with four antibiotics against *Staphylococcus aureus* strains. The extract only inhibited Gram positive bacteria, whereas the fractions also showed activity against Gram negative organism. Only n-butanol fraction showed synergistic/additive effects with all tested drug. Certainly as a consequence of saponin presence (it has a great hemolytic capacity). This study showed at first time the antimicrobial activity extract/fractions of leaves from *P. moniliformis* and their capacity to enhance the anti-*S. aureus* activity of some protein inhibitors drugs.

Key words: Caatinga, saponins, synergism, *Pityrocarpa moniliformis*, antimicrobial.

INTRODUCTION

Pityrocarpa moniliformis is a valuable source of bioactive compounds with great diversity in the structure and physicochemical properties. The history of the discovery and use of drugs derived from plants date from several centuries. The oldest written evidence of the use of plants for drugs preparation has been found approximately 5000 years old, and it also has been described by Shen-Nung (2838-2698 BC) (Kelly, 2009; Petrovska, 2012; Lien, Lien and Adms, 2013). The high potential of plants is due to their great diversity in the structure and physicochemical properties (Newman and Cragg, 2012).

In recent years, resistance of pathogenic micro-

organisms to multiple drugs has increased due to the indiscriminate use of antimicrobials, commonly marketed and used in the treatment of infectious diseases (Paphitou, 2013; Toutain and Bousquet-Melou, 2013). Due to the increasing microbial resistance to drugs, the search for new antimicrobial agents from plants is intense (Radulovic et al., 2013), mainly because of the lower search costs, probable less risk of side effects and a great potential of synergistic effect (Saleem et al., 2010; Vuuren and Viljoen, 2011).

The Caatinga is a unique biome of Brazil and this region is marked by an accentuated dryness (rainfall is

usually less than 600 mm/year). The Caatinga is also named as Semi-arid region and occupies a large portion of Brazil's Northeast, comprising several plant species (Albuquerque et al., 2012). As a result of the environmental conditions to which they are exposed, the Caatinga plants have developed interesting chemical features and they have been described as excellent weapons against microorganisms (Almeida et al., 2012; Silva et al., 2012; Da Silva et al., 2012; Tretin et al., 2013). *Pityrocarpa moniliformis* (= *Piptadenia moniliformis*) Benth (Leguminosae - Mimosoideae) is a woody plant in Northeastern Brazil popularly known as angico-de-bezerro, which also occurs in dry forests in the region of Sucre (Venezuela). Our group has been the first to show that this plant is a source of active compounds with antimicrobial, anti-bacterial biofilms and antioxidant activities (Da Silva et al., 2011, 2012; Trentin et al., 2011). This study aimed to evaluate: (1) the antimicrobial activity of the hydroalcoholic crude extract of leaves and its fractions, (2) the synergetic potential with antibiotics that act on protein synthesis, and (3) test the hemolytic activity of extract and fractions that showed better results.

MATERIALS AND METHODS

Plant collection and plant storage

Leaves of *P. moniliformis* were collected in Parque Nacional do Catimbau, Pernambuco, Brazil, Northeastern Brazil, in September 2010. Botanical identification was made by staff of the Herbarium of Instituto de Pesquisa Agrônômica de Pernambuco (IPA), Brazil, and voucher specimens were deposited in the herbarium (IPA 84.048). Leaves were dried at room temperature. The dried plants were milled to a fine powder in a Macsalab mill (Model 200 LAB), Eriez®, Bramley, and stored at room temperature in closed containers in the dark until used.

Preparation of the crude hydroalcoholic extract

P. moniliformis leaves were dried at room temperature for 7 days, ground into a fine powder and used for extraction. The powder (20 g) was mixed with 50 ml ethanol:water (7:3) and submitted to agitation for 15 hours. Then the extracts were filtered and the powder residue was mixed again with 50 ml ethanol-water and the entire extraction process was repeated. The supernatants collected were mixed in a round bottom flask and concentrated at 45°C. The residue was dissolved in DMSO (dimethyl sulfoxide) and kept at -20°C until use.

Phytochemical analysis

The phytochemical tests to detect the presence of tannins, flavonoids, anthocyanins, saponins, coumarins, quinones, anthraquinones, reducers compounds and alkaloids were performed according to the method described by Kokate (1994) and Harborne (1998).

Fractionation of the hydroalcoholic extract

The hydroalcoholic extract was dissolved in water, producing a

solution that was submitted to liquid-liquid partitions successively with cyclohexane, ethyl acetate and n-butanol. The solutions produced were dried in anhydrous Na₂SO₄ and submitted to filtration under reduced pressure. Thereafter, the solvents were evaporated under reduced pressure in a rotary evaporator oven at 60°C, producing hexane, ethyl acetate, n-butanol soluble and n-butanol non-soluble phases. The residues obtained were kept at -20°C for future use.

Microbial strains

The antimicrobial activity of *P. moniliformis* leaves extract and its fractions were tested against the following microorganisms: *Staphylococcus aureus* (UFPEA02), *Micrococcus luteus* (UFPEA100), *Escherichia coli* (UFPEA 224), *Klebsiella pneumoniae* (UFPEA 396), *Salmonella enteritidis* (UFPEA 414), *Pseudomonas aeruginosa* (UFPEA416) and some *S. aureus* recently isolated strains (UFPEA 660, UFPEA 663, UFPEA 676, UFPEA 687, UFPEA 712, UFPEA 733) (Table 1). All strains were provided by Departamento de Antibióticos, Universidade Federal de Pernambuco (UFPEA) and maintained in Nutrient Agar (NA) and stored at 4°C.

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

MIC was determined by the microdilution method (CLSI, 2011). A twofold serial dilution of the extract/fractions was prepared in Mueller Hinton Broth (MHB) and 100 µl (approximately 1.5 × 10⁸ CFU/ml) of bacteria suspension was added. The samples were incubated for 24 h at 37°C. Resazurin solution (0.01%) was used as an indicator by color change visualization: any color changes from purple to pink were recorded as bacterial growth. The lowest concentration at which no color change occurred was taken as the MIC. Afterwards, cultures were seeded in MHA medium and incubated for 24 h at 37°C to determine the MBC which corresponds to the minimum concentration of extract/fractions that eliminated the bacteria.

Evaluation of combinatory effects of extract and drugs

The combinatory effects of extract/fraction and drug were tested by Checkerboard method (Vuuren and Viljoen, 2011). The drugs tested were: chloramphenicol (chlor), erythromycin (eryth), streptomycin (strep), and tetracycline (tetra). To this test, sample solutions (100 mg/ml) and drug (1 mg/ml) were mixed at 1:1 ratio, in a final volume 20 µl, and serially diluted. Interaction was assessed algebraically by determining the Fractional inhibitory concentration index (ΣFIC), according to the following equation:

$$\Sigma FIC = (MICE+D/ MICE) + (MICD+E/ MICD)$$

where MIC: minimal inhibitory concentration; MICE+D: minimal inhibitory concentration of extract/fraction in combination with drug; MICD+E: minimal inhibitory concentration of drug in combination with extract/fraction.

Data interpretation: synergism (ΣFIC ≤ 0.5), additive (0.5 < ΣFIC ≤ 1); noninteractive (1 < ΣFIC < 4); antagonist (ΣFIC ≥ 4).

In vitro hemolytic assay

Blood (5 to 10 ml) was obtained from healthy non-smoking volunteers by venipuncture, after written informed consent was obtained. Human erythrocytes from citrated blood were immediately

Table 1. Antimicrobial activity of *P. moniliformis* leaves hydroalcoholic extract and its fractions.

Microorganism UFPEDA	Hydroalcoholic crude extract		Ethyl acetate fraction ¹		Cyclohexane fraction		n-Butanol fraction		Aqueous fraction	
	MIC ²	MBC ²	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
02 <i>S. aureus</i>	1.56	6.25	3.12	6.25	3.12	12.5	25	12.5	25	25
100 <i>M. luteus</i>	0.39	1.56	6.25	>25	6.25	>25	6.25	>25	12.5	>25
224 <i>E. coli</i>	N/A ³	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
396 <i>K. pneumoniae</i>	N/A	N/A	12.5	>25	12.5	>25	12.5	>25	25	25
414 <i>S. Enteritidis</i>	N/A	N/A	6.25	>25	6.25	>25	6.25	>25	6.25	12.5
416 <i>P. aeruginosa</i>	N/A	N/A	6.25	>25	6.25	>25	6.25	>25	6.25	25
660 <i>S. aureus</i> ⁴	1.56	12.5	1.56	3.13	6.25	6.25	3.16	25	3.16	12.5
663 <i>S. aureus</i> ⁴	1.56	1.56	1.56	12.5	3.16	>25	1.56	>25	> 25	> 25
676 <i>S. aureus</i> ⁴	3.16	12.5	3.16	12.5	3.16	12.5	3.16	25	25	25
687 <i>S. aureus</i> ⁴	3.16	12.5	> 25	> 25	12.5	12.5	3.25	3.25	> 25	> 25
712 <i>S. aureus</i> ⁴	3.16	>25	3.16	>25	3.16	> 25	6.25	>25	6.25	>25
733 <i>S. aureus</i> ⁴	0.78	1.56	0.78	3.16	12.5	12.5	3.16	>25	0.78	6.25

¹Crude hydroalcoholic extract was dissolved in water and were submitted to liquid-liquid partitions successively with cyclohexane, ethyl acetate and n-butanol. The solvents were evaporated and the remaining aqueous fraction was lyophilized. ²MIC and MMC of the samples were expressed in mg/ml (CLSI, 2011). ³N/A: No activity. ⁴Source of *S. aureus* strains: vaginal discharge, catheter tip, prosthesis secretion, ocular discharge, wound secretion transplant, bone fragment (respectively).

isolated by centrifugation at 1500 rpm for 10 min at 4°C. After removal of plasma and buffy coat, the erythrocytes were washed three times with phosphate-buffered saline (PBS; pH 7.4) and then resuspended using the same buffer and a 1% erythrocyte suspension was prepared. The hemolytic activity of the crude extract was tested under *in vitro* conditions. Each tube received 1.1 ml of erythrocyte suspension and 0.4 ml of extract of various concentrations (50 to 500 µg/ml) were added. The negative control was only solvent and the positive control received 0.4 ml of *Quillaja* species saponin (0.0025%). After 60-min incubation at room temperature, cells were centrifuged and the supernatant was used to measure the absorbance of the liberated hemoglobin at 540 nm. The average value was calculated from triplicate assays. The hemolytic activity was expressed in relation to ascorbic acid and calculated by the following formula (Oliveira et al., 2012):

$$\text{Hemolytic activity (\%)} = (A_s - A_c) \times 100 / (A_c - A_b)$$

where A_c was the absorbance of the control (blank, without extract), A_s was the absorbance in the presence of the extract and A_b was the absorbance of saponin solution.

Statistical analysis

Each experiment was performed in triplicate and results are expressed as the mean \pm standard deviation (SD). Statistical analysis was performed by Student's t-test. Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Several studies have been demonstrated that Caatinga plants are a rich source of biotechnology compounds, in special with remarkable antimicrobial activity (Da Silva et al., 2012; Silva et al., 2012; Jandú et al., 2013). The

preliminary antimicrobial activity of extract and fractions of *P. moniliformis* are showed in Table 1. The crude hydroalcoholic extract only inhibited Gram positive bacteria. The MIC and MBC were 1.56 and 6.25 mg/ml to *S. aureus* and 0.39 and 1.56 mg/ml to *M. luteus*. The fractions of this extract showed activity against all tested microorganism, except to *E. coli*. The MIC values against *S. aureus* were 3.12 mg/ml to ethyl acetate and cyclohexane, and n-butanol and 25 mg/ml to aqueous fractions. To *M. luteus*, the MIC values were 6.25 mg/ml (ethyl acetate, cyclohexane and n-butanol fractions) and 25 mg/ml to aqueous fraction. All fractions had the same MIC value against *P. aeruginosa* of 6.25 mg/ml. To *K. pneumoniae* and *S. enteritidis* inhibitory concentrations were 12.5 mg/ml (ethyl acetate, cyclohexane and n-butanol fractions) and 25 mg/ml (aqueous fraction). According to MBC/MIC ratio all extract and fractions may be classified as bactericidal agents (MBC/MIC \leq 4) (Gatsing et al., 2006).

The samples were also checked against six *S. aureus* strains recently isolated from human infections, because of the anti-*S. aureus* effectiveness (Table 1). *S. aureus* is an opportunistic bacteria well adapted to humans, which causes several hospital and community acquired infections. It can live as a commensal, but provided a suitable opportunity can initiate severe infection at various body sites due to the production of a large number of virulence factors and its ability to develop survival strategies, despite the availability of effective antimicrobial agents (Shimada et al., 2010; Price et al., 2012). In this context, the search for new effective anti-*S. aureus* compounds is extremely important. The MIC values ranged from 0.78 to 3.16 mg/ml to hydroalcoholic

Table 2. Combinatory effects of different drugs and extract/fractions against *S. aureus* strains.

Drug	Strain	FIC (Extract/Drug)	FIC (Cyclohexane fraction/Drug)	FIC (Butanol fraction/Drug)
Chloramphenicol	02	0.5	0.5	0.5
	687	1.0	4.0	1.0
	733	2.1	8.4	0.9
Erythromycin	02	0.5	0.5	0.5
	687	0.5	0.5	0.5
	733	1.0	1.0	1.0
Streptomycin	02	1.2	0.5	0.6
	687	2.4	1.2	0.6
	733	2.5	0.7	1.0
Tetracycline	02	0.5	1.0	0.5
	687	1.0	0.5	0.5
	733	0.5	0.5	0.5

Table 3. Phytochemical analysis of *Pityrocarpa moniliformis* leaves.

Positive	Negative
Saponins	Alkaloids
Flavonoids	Carbohydrates
Triterpenes	Cinnamic acid derivatives
Gallics tannins	Caffeine

extract and cyclohexane fraction; 1.56 to 6.25 mg/ml to n-butanol fraction; 3.13 to 12.5 mg/ml to ethyl acetate fraction; and 0.78 to 25 mg/ml to aqueous fraction. Both aqueous and cyclohexane fractions did not inhibit the 687 strain.

Given the high capacity of *S. aureus* to developing drug resistance, which contributes to clinical infections, is also extremely important to find news alternative substances that can enhance the activity of drug, and have been reported by various studies (Agboke et al., 2011; Chung et al., 2011). The *P. moniliformis* leaves hydroalcoholic extract and two of its fractions (cyclohexanic and butanolic fractions) were evaluated to their capacity to enhance the anti-*S. aureus* of standard antibiotics by determination of the Fractional Inhibitory Concentration indices (Σ FIC). The results showed that the combinatory effects were strain-dependent (Table 2). The crude extract showed synergetic or additive effect with tetracycline against all strains whiles the cyclohexane fraction showed with tetracycline and erythromycin. On other hand, the butanolic fraction showed synergetic or additive effect with all tested drug, which may be

explained by saponin presence, which can cause membrane permeabilization (Hassan et al., 2010).

In the hemolytic assay, the hydroalcoholic extract and butanolic fraction had the lowest HC₅₀ (the concentration needed for 50% of hemolysis) (1.91 and 3.92 mg/ml). This effect may be attributed by saponin presence in crude extract (Table 3), which is known as hemolytic agent (Hassan et al., 2010) and is frequently soluble in ethanol and butanol (Levy et al., 1989). The cyclohexanic fraction showed HC₅₀ of 6.11 mg/ml (Figure 1). The HC₅₀ of active anti-*S. aureus* fractions were higher than the MIC values.

Phytochemical analysis of *P. moniliformis* leaves revealed the presence of triterpenes, saponin, flavonoids (luteolin, proanthocyanidin), and gallics tannin. All these classes of compounds are reported as antimicrobial agents. Triterpenes are reported as antimicrobial and synergetic compounds (Chung et al., 2011). Flavonoids, soluble in ethyl acetate and n-butanol, are bacteriostatic and has multiple bacterial targets (nucleic acid synthesis, cytoplasmic membrane function or energy metabolism) (Tim and Lamb, 2005). Saponins antimicrobial activity is reported and usually is related to the aglycone part (Hassan et al., 2010). Finally, gallic tannin is able to inactivate microbial adhesins, enzymes, cell envelope transport proteins, etc (Cowan, 1999).

Conclusion

P. moniliformis leaves are an important source of substances possessing antimicrobial activity, including against *S. aureus* clinical isolates. The crude extract,

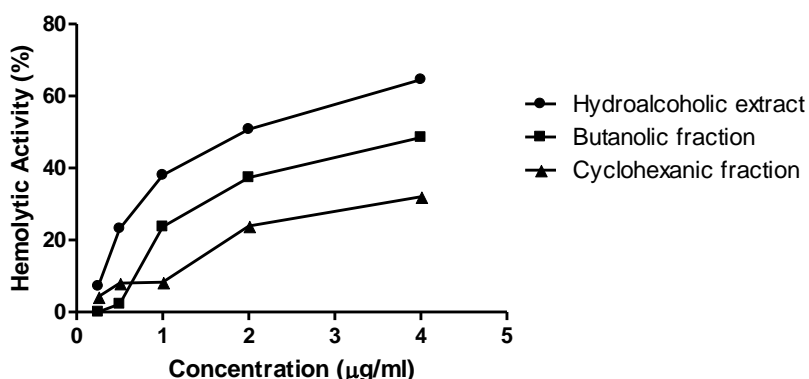


Figure 1. Hemolytic activity of *P. moniliformis* leaves hydroalcoholic extract and its fractions.

cyclohexanic and butanolic fractions are able to enhance the activity of erythromycin and tetracycline. Butanolic fraction showed synergetic effect with all tested antibiotic. The purification and structural characterization of compounds from active fractions are being performed by our group.

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