ISSN: 0976-3031

Available Online at http://www.recentscientific.com

CODEN: IJRSFP (USA)

International Journal of Recent Scientific Research
Vol. 9, Issue, 12(A), pp. 29791-29795, December, 2018

International Journal of Recent Scientific Research

DOI: 10.24327/IJRSR

Research Article

COMPARISON OF ANTIBACTERIAL AND ANTIBIOFILM POTENTIAL OF ORGANIC EXTRACTS OF *Pityrocarpa moniliformis* LEAFS OBTAINED BY DIFFERENT METHODS OF EXTRACTION

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DOI: http://dx.doi.org/10.24327/ijrsr.2018.0912.2931

ARTICLE INFO

Article History:

Received 6th September, 2018
Received in revised form 15th October, 2018
Accepted 12th October, 2018
Published online 28th December, 2018

Key Words:

Microorganisms, Minimum Bactericidal Concentration, Minimum Inhibitory Concentration, *Pityrocarpa moniliformis.*

ABSTRACT

The objective of this study was to determine the antimicrobial and antibiofilm properties of the organic extracts of *Pityrocarpa moniliformis* obtained by different techniques of extraction against several microorganisms. Antimicrobial and antibiofilm activities were determined using the serial microdilution method in 96-well culture plates and the crystal violet method, respectively. Most extracts showed antimicrobial activity in all evaluated microorganisms and Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were achieved for some extracts at the concentration of 2 mg/mL. In addition, the methanolic extracts showed antibiofilm activity against most of the tested microorganisms. Therefore, the extracts of *P. moniliformis* proved to be efficient for the activities tested, demonstrating their potential commercial application.

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INTRODUCTION

The caatinga is one of the biomes richest in biodiversity in Brazil, being the only large region fully inserted in the Brazilian national territory with a wealth of cultural knowledge accumulated by local communities. It is a semi-arid region, marked by a severe drought, with precipitation generally lower than 600 mm^{-1} per year (SAMPAIO *et al*., 2002), native plants developed strategies to compensate for this difficulty by producing unique chemical molecules that have made them an excellent source of bioactive compounds with antibacterial activity. In addition, studies have shown that caatinga plants have potential to prevent bacterial growth and adhesion (TRENTIN *et al*., 2011).

In recent years, biofilms have attracted significant interest, especially because of their high impact in medicine and public health. Bacteria in the form of biofilms contribute to the chronicity of persistent infections, such as those associated with implanted medical devices.

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The ability to form biofilm allows the pathogens to escape the immune defenses of the host and resist antibacterial treatments (HOIBY *et al.*, 2010).

The encouragement to identify new antibacterial agents with clear mechanisms of action, including anti-adherent compounds, raises the need for identification of innovative antimicrobials and highlights the potential of plant-derived molecules as a source of biofilm control products.

Pityrocarpa moniliformis is a tree plant in the Northeast of Brazil, with disjoint occurrence in dry forests of the region of Sucre (Venezuela). It is an endemic plant of the caatinga, a biome that has several plant species with therapeutic and pharmacological potential (MELO ET AL., 2010, COSTA *et al.*, 2017, MALAFAIA *et al.*, 2017, VIEIRA *et al.*, 2017) was not explored, evidencing the need to develop studies on its possible biological activities. Recent studies have shown promising antimicrobial and antibiofilm activities (SILVA *et al.*, 2011; SILVA *et al.*, 2013; TRENTIN *et al.*, 2015).

The objective of this study was to evaluate the antibacterial activity of the organic extracts of the leaves of *P. moniliformis*, obtained by different techniques of extraction against several pathogenic microorganisms, and to verify the antibiofilm activity of these extracts under viability of *Staphylococcus aureus*.

MATERIALS AND METHODS

Collect the material and extraction solvent

The leaves of *P. moniliformis* were collected in the *Parque Nacional do Catimbau*, in Buíque, Pernambuco. The material was taken to the forced air circulation oven (40-45 °C) for a period of three to four days. The samples were also identified according to the usual taxonomic techniques and deposited in the Herbarium *Instituto Agronômico de Pernambuco (IPA)*, Recife, Brazil. The plant material was processed in a bench mill and subjected to different extraction methods following the eluotropic order of the solvents: cyclohexane, ethyl acetate and methanol. The samples were rotated and left at room temperature for complete drying of the solvent. All extracts obtained were stored at -20 °C for further analysis.

Obtaining organic extracts by different methods

Cold Extraction (CE): 30 g of the dry and ground vegetable material was added to 300 mL of cyclohexane and subjected to successive extractions, remaining under mechanical agitation for 72 h at room temperature. The hexane extract (Hex) obtained was filtered and maintained at a temperature of ± 4 °C. To the residue was added cyclohexane again and the same extraction procedure repeated 3 more times. The same procedure was performed for solvents ethyl acetate (AcOEt) and methanol (MeOH) following the eluotropic series. The extracts were dried in a rotary evaporator coupled to a water bath at 40 °C and kept in desiccator until constant weight for subsequent calculation of their yield.

Extraction in Soxhlet Apparatus (ESA): The vegetable material (30 g) was added in 30 mL of cyclohexane in soxhlet apparatus at 40 ºC for 72 h in a water bath. Subsequently, the extract was filtered and concentrated to dryness in a rotary evaporator coupled to a water bath at 40 °C. The residue was kept in desiccator until constant weight for further calculation of its yield. The same procedure was repeated to obtain extracts with ethyl acetate and methanol.

Ultrasonic Assisted Extraction (UAE): Ultrasonic bath (model USC-1400/Family USC-1400A/USC-1450A) was used as the source of ultrasound to obtain extracts. 30 g of the plant material was added to 300 mL of solvent, following the eluotropic series (Cyclohexane, ethyl acetate and methanol) and sonicated (3x30 min) at a temperature of 40 °C and power of 150W, then the samples were rotated and left at room temperature for complete drying of the solvent and subsequent calculation of the yield.

Accelerated Solvent Extraction (ASE): 20 g of the vegetable material together with 10 g of diatomaceous earth was subjected to extraction in ASE 350 Dionex automatic extractor, following the eluotropic order of the solvents: cyclohexane, ethyl acetate and methanol, under a temperature of 40 ºC for 15 minutes under a pressure of ± 1500 psi and solvent luxury of 5 mL/min. The extracts filtered on the ASE were dried on Rocket Evaporator ™ and left at room temperature for complete drying of the solvent.

Supercritical Fluid Extraction (SFE): Supercritical extraction was performed on a Spe-ed SFE unit from Applied Separations model 7071 (Allentown, PA, USA) with a 115 mL extraction cell. 30 g of dry plant material were loaded into the SFE cell at a temperature of 50 °C under a pressure of 5000 psi with a static period of 30 min. With dynamic mode (continuous flow) $scCO_2$ was used as solvent and cyclohexane, ethyl acetate and methanol as co-solvent following the eluotropic series. Flow rates were 4 mL/min for $scCO_2$ and 1 mL/min for solvents. The extracts were then evaporated at room temperature until complete drying.

Antibacterial activity

Minimum Inhibitory Concentration (MIC)

The antimicrobial activity of *P. miniliformis* organic extracts obtained by different techniques was determined by the serial microdilution method (CLSI, 2011) in 96 well flat bottom culture plates. The pathogenic bacteria tested against the extracts were obtained from the culture collection of the Department of Antibiotics of the Federal University of Pernambuco (UFPE).Gram-negative bacteria, *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and Gram-positive, *Staphylococcus aureus* (ATCC 29213) were tested 100 µL of the Heller-Hinton broth (MH) HIMEDIA® medium was distributed to all wells of the 96-well plate, then 100 µL of each extract (20 mg/mL) was added to the 1st row of the plate (horizontal direction). Subsequently, serial dilution of the metabolites to the wells of row G was carried out, obtaining concentrations of 2000; 1000; 500; 250; 125; 62.5; 32.25 and 15.625 µL/mL. A bacterial suspension corresponding to 0.5 tube of the Mac Farland scale was used with 0.85% saline, corresponding to a concentration of approximately 1.5 x 10^8 CFU/mL. This solution was diluted 1:90 in order to obtain the standard suspension. Then, 20 µL of the bacterial suspension was dispensed into each well on the plate, except the control wells. The plate was sealed and incubated in the oven at 37 °C for 18-24 h. The assays were performed in triplicates at all concentrations for each strain.

After incubation time, the plates were read in spectrophotometer at 600 nm (A600) for each well. Percent growth inhibitions at different concentrations of the extracts for each microorganism were calculated as: % Growth Inhibition = [1-Ac/A_0 x 100], where Ac represents the absorbance of the well with a concentration of the extract and A_0 is the absorbance of the well with the control (without extract). Minimum inhibitory concentration (MIC) was determined for each strain as the lowest extract concentration that completely inhibits measurable growth (A600 = 0).

Minimal Bactericidal Concentration (MBC)

The Minimum Bactericidal Concentration (MBC) was determined according to the technique described by Koo *et al*. (2000) and Duarte *et al*. (2003). With the aid of sterile loops, the blending of each well of the microplate was replicated on the plate Petri dishes containing Mueller-Hinton agar medium for all tested microorganisms. Plates were incubated at 37 °C for 24 h. The lowest concentration of the extract that did not allow growth was considered the CBM for each strain. The spaces that presented growth were considered from wells with bacteriostatic concentration of the extract.

Determination of anti-biofilm formation

The *S. aureus* isolate with a strong biofilm formation was tested with the methanolic extracts of *P. moniliformis*. The activity was performed through the adaptation of the methodology described by Trentin *et al*. (2011), where 20 µL of extracts concentrations were added to the microtiter plate together with 20 µL of the bacterium and 160 µL of Trytone Soy Broth (TSB) growth medium. The final concentration of extracts was then 4 and 0.4 mg/mL. The plate was incubated for 24 hours at 37 °C, after incubation the contents were removed and the plate washed three times with 0.85% saline. For biofilm fixation the plate was incubated at 55 °C for one hour. The biofilm visualization is revealed with 0.4% violet crystal for 10 minutes, and then removed, washed three times and ethanol (100%) added, in which the optical density is visualized at 570 nm.

RESULTS AND DISCUSSION

Antimicrobian activity

The antimicrobial activity of the organic extracts (CE, ESA, UAE, ASE and SFE) was determined by calculating the percentage inhibition of growth obtained for various microorganisms. From these results, the MIC for each microorganism was determined (Table 1). In addition, where possible, CBM has also been established. Organic extracts of *P. moniliformis* were effective for most of the microorganisms tested, although to varying degrees. It was observed that the methanol extract obtained by the UAE technique showed more than 90% inhibition of the growth of three microorganisms (*S. aureus*, *E. coli*, *K. pneumoniae*) at a concentration of 2 mg/mL. The same occurred for ESA-MeOH and ASE-MeOH, but these inhibited only *S. aureus* bacteria. The CE-Hex extract also showed satisfactory results for all strains tested, except for *K. pneumoniae*.

The *S. aureus* bacteria showed the highest sensitivity against most of the extracts tested. Most phytochemicals perform better against Gram-positive bacteria and there are still a limited number of plant-derived molecules that act as high-activity antimicrobial compounds against Gram-negative species (LEWIS, 2013).

The antimicrobial activity of extracts obtained with methanol has been reported in several studies. Pareek *et al*. (2000) reported the antibacterial activity of the methanolic extract of the leaves of *Syzygium cumini* against the bacterial strains of *Raoultella planticola*, *Pseudomonas aeruginosa*, *B. subtilis* and *Agrobacterium tumifaciens*. Ibrahim, Hong and Kuppan (2013) also referred to the antibacterial efficiency of the *Phyllanthus niruri* plant's methanolic extract against pathogenic bacteria responsible for common skin infections and gastrointestinal and urinary tract infections, demonstrating antibacterial action against all tested Gram-positive and Gram-negative bacteria. It is believed that the actions of many antimicrobial agents results in the formation of pores in the bacterial membrane and stimulate the leakage of cellular content (YENUGU *et al*., 2006).

It should be noted that the results obtained corroborate with the studies by Silva *et al*. (2012 and 2013) that evaluated the antibacterial activity of *P. moniliformis* against clinical strains of *S. aureus*, so that the samples were active for all strains of *S. aureus* (minimum concentration of inhibition: 0.38-3.13 mg/mL^{-1}), suggesting that the cell wall was the main target of extracts. Moreover, the harmful activity of methanolic extracts to the bacterial membrane can be attributed mainly to the presence of phenolic and flavonoid compounds, which have characteristics similar to detergent (YADAV *et al*., 2017).

Table 1. Growth inhibition percentages obtained with organic extracts in bacterial isolates at the concentration of 2 mg/mL.

Extracts		*S. aureus*	*E. coli*	*K. pneumoniae*	*P. aeroginosa*
CE	Hex	93.4 ± 5.0	72.3 ± 2.8	33.4 ± 2.3	59.4 ± 2.0
	AcOEt	40.3 ± 7.8	20.4 ± 1.7	6.0 ± 1.5	9.7 ± 3.8
	MeOH	60.0 ± 5.5	52.1 ± 4.1	17.9 ± 3.1	23.9 ± 1.7
ESA	Hex	73.4 ± 3.9	4.3 ± 1.8	49.3 ± 3.5	17.9 ± 1.4
	AcOEt	51.7 ± 4.7	8.4 ± 0.7	6.0 ± 2.7	4.7 ± 1.6
	MeOH	96.2 ± 2.9	55.2 ± 3.1	5.5 ± 3.7	20.8 ± 3.0
UAE	Hex	47.8 ± 2.2	17.9 ± 4.6	22.5 ± 3.4	22.7 ± 4.9
	AcOEt	31.4 ± 5.6	91.3 ± 1.6	92.2 ± 1.2	12.2 ± 3.6
	MeOH	98.9 ± 4.9	94.2 ± 2.8	94.5 ± 1.4	41.1 ± 2.7
ASE	Hex	40.5 ± 2.0	55.4 ± 2.8	58.8 ± 3.0	1.4 ± 2.4
	AcOEt	45.6 ± 1.4	34.4 ± 5.0	45.3 ± 2.2	23.1 ± 1.8
	MeOH	94.2 ± 2.5	3.3 ± 2.1	60.8 ± 2.5	20.2 ± 4.3
SFE	Hex	53.8 ± 3.1	64.7 ± 4.4	12.3 ± 4.7	20.4 ± 4.7
	AcOEt	33.7 ± 2.1	49.8 ± 1.5	8.8 ± 3.6	34.8 ± 4.3
	MeOH	75.3 ± 3.6	0.2 ± 3.9	47.7 ± 4.6	21.7 ± 3.0

The results are expressed as means ± standard deviations of the values obtained from the triplicate assays.

The Minimum Bactericidal Concentrations (MBC) was performed for the extracts that showed activity against at least two microorganisms. Figure 1 shows the plating performed to determine the CBM.

Extracts tested for CMB were CE-MeOH, ESA-Hex, ESA-AcOEt, ESA-MeOH, UAE-MeOH, ASE-MeOH, SFE-AcOEt and SFE-MeOH. It was observed that the SFE-MeOH extract showed bactericidal activity, while the others were considered bacteriostatic.

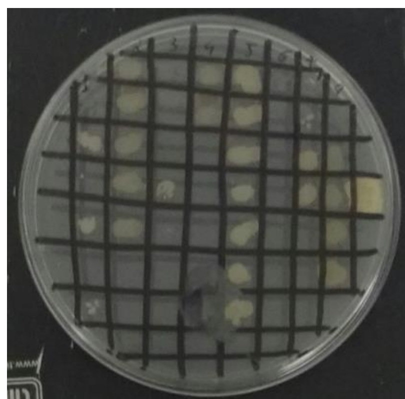

Figure 1. Representation of the plating performed to determine the Minimum Bactericidal Concentration (MBC) of the organic extracts of *P. moniliformis*.

Activity Antibiofilm

The antibiofilm activity of extracts of *P. moniliformis* that were considered bacteriostatic was evaluated against S. aureus bacteria. All extracts tested inhibited biofilm formation at the two concentrations tested (Table 2).

Biofilm inhibition was higher for the ESA-MeOH extract at concentrations of 4 mg/mL and 0.4 mg/mL (81.1% and 79.0%, respectively).The lowest activity was presented by SFE-MeOH with 65.8% for the concentration of 4mg/mL and 74.4% for the concentration of 0.4 mg/mL, this may be related to the bactericidal action of this extract, preventing the viability of the microorganism and consequently preventing it from forming the biofilm.

Table 2. Percentages of biofilm inhibition after exposure of organic extracts to *S. aureus*.

Extracts		*S. aureus*	
		4 mg/mL	**0,4 mg/mL**
CE	MeOH	76.5 ± 0.6	73.2 ± 1.6
ESA	MeOH	81.1 ± 1.2*	79.0 ± 0.8*
UAE	MeOH	67.2 ± 1.6	78.0 ± 1.6
ASE	MeOH	73.6 ± 1.0	67.8 ± 2.1
SFE	MeOH	65.8 ± 1.4	74.4 ± 0.4
GC	-	0.0 ± 0.9	

Growth Control = GC. Results are expressed as means ± standard deviation of triplicate results.

Studies have demonstrated the antibiofilm activity against different microorganisms of extracts of plants of the caatinga, indicating that this biome has several species with a high potential of use in the development of prototypes of drugs with property antibiofilms (TRENTIN *et al*., 2014; SILVA *et al*., 2015). Malafaia *et al*. (2017) verified the antibiofilm activity in 22 extracts of caatinga plants against *Ralstonia solanacearum*, one of the most destructive pathogens identified so far, due to its ability to induce fast and fatal symptoms in host plants. In the present study, researchers also confirmed their antibiotic activity against resistant strains of *Staphylococcus epidermidis*, *P. aeruginosa* and *K. pneumonia*, demonstrating this as a plant species rich in biomolecules with such activity (TRENTIN *et al*., 2011 and 2015). Inhibition of bacterial adhesion with consequent inhibition of biofilm formation by a pathway that does not cause bacterial destruction is an important feature in relation to a novel concept of antiviral therapies. This technique explores new mechanisms of action that may hinder the rapid development of bacterial resistance. In addition, this approach allows bacterial growth, maintains cells in planktonic state, and admits the extinction of virulence expression and attenuation of the pathogen, making the microorganisms more susceptible to other antimicrobials and the immune system (CLATWORTHY *et al*., 2007; MARTIN *et al*., 2008, MACEDO, ABRAHAM, 2009).

In this context, natural products are an important source of bioactive molecules and the medicinal plants commonly used in folk medicine can facilitate the search for new functional agents.

Some of the molecules that may be involved with the antibiofilm effect are: polyphenols, coumarins, steroids and terpenes. Sampaio *et al*. (2009) demonstrated that extracts rich in fruit polyphenols from *Caesalpiniea ferrea* showed antibiofilm activity in a multispecies biofilm model involving *Streptococcus sp.*, *Candida albicans* and *Lactobacillus casei*. Considering the coumarins, some studies were found with reaction to antibiofilm activity (GIRENNAVAR *et al*., 2008; PRAUD-TABARIES *et al*., 2009).

The study highlighted the potential application of *P. moniliformis* as a new and valuable source of prototype compounds. Further studies are needed in order to isolate the components of the plant species and elucidate the most efficient molecules in the fight against pathogenic microorganisms and antibiofilm activity.

CONCLUSIONS

The antimicrobial and antibiofilm properties of the organic extracts of the leaves of *P. moniliformis* obtained by different techniques against several pathogenic microorganisms were evidenced, suggesting the possible use of this plant as antimicrobial agent of commercial application. In addition, due to the antibiofilm action, it is considered an alternative for the coating of bioactive compounds and mainly hospital materials that are commonly contaminated by bacteria, causing serious health risks to patients.

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