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Full Length Research Paper

Myracrodruon urundeuva bark: an antimicrobial, antioxidant and non-cytotoxic agent

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This work evaluated the antioxidant, antimicrobial and cytotoxic activities of *Myracrodruon urundeuva* methanolic extract (MuBME) and identified its major phenolic compounds. The extract showed activity against all tested microorganisms (MIC: 0.39 to 3.13 mg/mL), and was able to enhance the erythromycin activity against some clinically isolated *Staphylococcus aureus* strains: fractional inhibitory concentration indices (ΣFIC) < 1. MuBME also showed a high antioxidant activity with 81.34 ± 0.2% of ascorbic acid activity (in phosphomolybdenum assay) and inhibited H_2O_2 and DPPH radical (IC_{50} of 0.038 and 0.0033 mg/mL, respectively). The phenolic and flavanoid content were 37.0 ± 0.47 mg/g gallic acid equivalents (GAE) and 43.88 ± 6.55 quercertin equivalents (QE)/mg, respectively. Additionally, we tested the cytotoxicity and observed IC_{50} of 1.94 (human erythrocytes) and 1.57 (Vero cells) mg/mL, much higher than the concentrations required for the biological responses tested. Gallic acid, protocatechuic acid, chlorogenic acid, catechuic acid and fumaric acid were detected by Ultra-fast liquid chromatography (UFLC) analysis and they are possible active compounds. Our results showed that *M. urundeuva* bark exhibits antimicrobial and antioxidant properties with application in pharmaceutical, food and cosmetics industries.

Key words: Caatinga, *Myracrodruon urundeuva*, antimicrobial, antioxidants, non-hemolytic compounds.

INTRODUCTION

Caatinga plants have been shown to contain rich sources of bioactive compounds for the treatment of diseases caused by bacteria, fungi and/or induced by oxidative stress (Da Silva et al., 2011; Trentin et al., 2011). *M. urundeuva* is a typical Caatinga plant characterized as bee- and wind-pollinated, dioecious species, which has a small flower size (Gaino et al., 2010). Monteiro et al. (2006) listed this plant as a valuable agent in combating various ailments including skin and subcutaneous lesions, coughing, neoplasia, inflammations in diverse systems (urogenital, respiratory, digestive, circulatory), etc., and they verified that the bark is the most commonly used part of the plant by studied communities, which correlated with the greatest amount of phenolic compounds found in the plant's tissues.

In this paper, we present the antimicrobial and antioxidant activities of the mehtanolic extract of *M.*

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urundeuva bark collected from Vale do Catimbau, Pernambuco, Brazil, a preservation area of the Caatinga biome. In addition, we also checked the effectiveness of this extract alone and combined with erythromycin against recently isolated *S. aureus* strains, its cytotoxicity and major phenolic compounds.

MATERIALS AND METHODS

Plant material and extraction

M. urundeuva bark was collect in Parque Nacional do Catimbau (Pernambuco, Brazil) and submitted in the Herbarium of Instituto de Pesquisa Agronômica de Pernambuco (voucher specimen: 84.059). The dried bark (20 g) was subjected to methanolic extraction (200 mL) by agitation at 180 rpm (rotations per minute). After 24 h, the extract was filtered (Whatman's No.1) and concentrated at 45°C under vacuum in a rotary evaporator. The obtained residue (extract) was kept at -20°C for future use.

Microorganisms

The tested microorganisms (*Aspergillus niger, Bacillus subtilis, Candida albicans, Enterococcus feacalis, Escherichia coli, Klebsiella pneumoniae, Micrococcus luteus, S. aureus*) were provided by Departamento de Antibióticos, Universidade Federal de Pernambuco (UFPEDA). The registration numbers are listed in Table 1.

Determination of antimicrobial activities

Disc diffusion method

Briefly, hundred microliters of microbial suspension (1.5×10^8 CFU/mL) were spread in Petri plates containing Mueller Hinton agar (MHA) medium, and sterile paper discs (containing 2000 µg of extracts) were added. After 18 h of incubation, the diameter of the zone of growth inhibition was examined. Dimethyl sulfoxide (DMSO) was used as the negative control (Bauer et al., 1966).

Minimum inhibitory concentration (MIC)

Microdilution method: In this assay, an aliquot of 10 µL of microbial suspension (1.5×10^8 CFU/mL) was added to a two-fold serial dilution of extract (50 to 0.20 mg/mL) prepared in Mueller Hinton broth (MHB) and incubated for 24 h at 37°C for bacteria, 48 and 96 h at 30°C for *C. albicans* and *A. niger*, respectively (CLSI, 2011). The growth was detected using a resazurin solution (0.01%): any color changes from purple to pink were recorded as microorganism growth. The lowest concentration at which no color change occurred was taken as the MIC. DMSO and antibiotics (chloramphenicol, tetracycline and nystatin) were used as the negative and positive controls.

Evaluation of combinatory effects of extract and erythromycin

The checkerboard method was used to test the combinatory effect of MuBME and erythromycin against *S. aureus* strains. The extract and drug were added together to the medium to give a final concentration of 10 and 0.1 mg/mL, respectively, and a two-fold serial dilution was prepared (10 to 0.02 mg/mL to MuBME and 0.1 to 0.0002 mg/mL to drug). The interaction was assessed algebraically by determining Fractional Inhibitory Concentration indices (ΣFIC) according to the following equation:

MIC_E and MIC_D: MIC extract/erythromycin; $MIC_{E/D}$: MIC_E in combination with erythromycin; $MIC_{D/E}$: is the MIC of erythromycin in combination with extract;

Data interpretation: ΣFIC ≤ 0.5: synergism (syn); 0.5 < ΣFIC ≤ 1: addition (add); 1 < ΣFIC < 4: noninteraction (non); ΣFIC ≥ 4: antagonism (ant) (Vuuren and Viljoen, 2011).

Antioxidant assays

The total antioxidant capacity

Phosphomolybdenum assay: An aliquot of 100 µL of sample solution (100 mg/mL) was mixed with 1 mL of reagent (600 mM sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). After, the tubes were capped and incubated in a water bath at 95°C for 90 min.

After cooling to room temperature, the absorbance of the samples was measured at 695 nm against a blank (1 mL of reagent and 100 µL of the solvent) (Pietro et al., 1999). Total antioxidant activity was expressed in relation to ascorbic acid and calculated by the following formula:

$$TAC\ (\%) = (As - Ac) \times 100 / (Aaa - Ac)$$

where: A_c = control absorbance; A_s = sample absorbance; A_{aa} = ascorbic acid absorbance.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

In this assay the free radical-scavenging activity of the extract was measured in terms of hydrogen donating using the stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl; Sigma-Aldrich) (Blios, 1958). An aliquot of 250 µL of DPPH solution (1 mM) was mixed with 40 µL of different concentrations of extracts (0.2 to 0.025 mg/mL). Thirty minutes later, the absorbance was measured at 517 nm. Gallic acid was used as the reference compound. The DPPH radical scavenging was calculated using the following formula:

$$Scavenged\ [DPPH]\ (\%) = (A_c - A_s) / A_c \times 100$$

where: A_c = control absorbance; A_s = sample absorbance.

Hydrogen peroxide radical scavenging assay

Different concentrations of extract (0.2 to 0.025 mg/mL) were dissolved in 3.4 mL of phosphate buffer (pH 7.4; 0.1 M) and mixed with 600 mL of hydrogen peroxide (43 mM). The absorbance value (at 230 nm) of the reaction mixture was recorded after 10 min. For each concentration, a separate blank sample was used for background subtraction (Ruch et al., 1989). Gallic acid was used as the reference compound. The scavenging activity was measured by the following formula:

$$Scavenged\ [H_2O_2]\ (\%) = (A_c - A_s) / A_c \times 100$$

where: A_c = control absorbance; A_s = sample absorbance.

Table 1. Antimicrobial activity of MuBME against selected pathogens.

UFPEDA	Microorganism	Inhibition (mm)	MIC (mg/mL)			
			MuBME	Chloramphenicol	Tetracycline	Nystatin
02	*Staphylococcus aureus*	21±0.58	0.39	0.0031	0.0016	-
86	*Bacillus subtilis*	15±0.58	0.78	0.0016	0.0016	-
100	*Micrococcus luteus*	26±0.58	0.39	0.0004	0.0004	-
138	*Enterococcus feacalis*	14±1.32	0.78	0.0016	0.0008	-
224	*Escherichia coli*	13±1.15	1.56	0.0008	0.0008	-
396	*Klebsiella pneumoniae*	12±1.15	3.13	0.0016	0.0008	-
1007	*Candida albicans*	13±1.32	1.56	-	-	0.0008
2003	*Aspergillus niger*	12±0.50	0.78	-	-	0.0016

Evaluation of cytotoxic effects

In vitro hemolytic assay

Blood (5 to 10 ml) was obtained from healthy volunteers by venipuncture and placed in heparinized tubes, after written informed consent was obtained. Human erythrocytes were isolated by centrifugation (1,500 rpm, 10 min at 4°C). The erythrocytes were washed three times with phosphate-buffered saline (PBS; pH 7.4). Each tube received 1.1 mL of erythrocyte suspension (1%) and 0.4 mL of various extract concentrations (0.5 to 0.05 mg/mL). The controls were only solvent (negative) and Quillaja saponin (0.0025%, positive). After 60 min incubation cells were centrifuged and the absorbance of supernatant was recorded at 540 nm. The hemolytic activity was expressed by the following formula:

Hemolytic activity (%) = (As - Ab) × 100 / (Ac - Ab)

A_b = solvent absorbance, A_s = sample absorbance; Ac = saponin absorbance.

Cell viability by MTT assay

In this assay was used cell of a monkey kidney fibroblast line (Vero). Cell viability was then estimated via an 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay. The cells (1.0×10^5 cells/mL) (cultivated in 96-wells microplate) were treated with the extract at difference concentrations (4.0 to 0.25 mg/mL) and incubated for 24 h at 37°C. MTT stock solution (10 µL; 5 mg/mL) was then applied and after 4 h, the supernatants were aspirated, the formazan crystals were dissolved in 100 µL of dimethylsulfoxide (DMSO), and the absorbance was measured at 540 nm. The optical density of the formazan generated in the control cells was considered to represent 100% viability.

Phytochemical analysis

Determination of total phenol content

The total phenolic content was determined by the Folin-Ciocalteu method described in Da Silva et al. (2011). The amount of total phenol was expressed as mg/g gallic acid equivalents (GAE) using the calibration curve of gallic acid ($y = 1.6221x + 0.0084$, $r^2 = 0.9934$).

Determination of total flavonoid content

The total content of flavonoids was measured using a method proposed by Kumaran and Karunkaran (2007). The amount of flavonoids in the extract was expressed as quercertin equivalents (QE)/mg plant extract, and calculated by the following formula:

Total flavonoid content = $(A_s \times m_c) / (A_c \times m_s)$

A_s = sample absorbance; A_c = quercetin absorbance; m_s = mass of plant extract; m_c = mass of quercetin.

Qualitative determination of Phenolic composition by UFLC

The detection of phenolic content was performed by a Prominence Ultra Fast Liquid Chromatographic system (UFLC Shimadzu inc. Japan). This system comprises of binary pump (model LC-20AD), diode array detector (model SPD 20A), auto-sampler (model SIL-20A HT), oven (model CTO-20A), controller (model CBM-20A), degasser (model DGU-29A3). The LC Solution Software version 1.2 was used to control the auto-sampler, detector, data acquisition and run settings. An octadecil silane (C_{18}) reverse-phase column (Shimadzu inc., Japan, XR, ODS 50 ×3.0, 2.2 µm particle size) was employed for all chromatographic analysis.

Extract powder (0.5 g) was diluted in a metanol/water solution (20%, v/v) in ultrasonic bath sonicator for 30 min. Afterwards, extracts were filtered and liquid fraction were directly loaded onto solid phase extraction (SPE) cartridges (Strata C18-E cartridge Phenomenex™ Torrance – California, USA) in order to separate and concentrate target groups as phenolic acids and flavonoids. Cartridges were eluted with 2 mL of 1% trichloracetic (HCl) followed by 2 mL of acetone and 10 mL of methanol. Eluted fractions were concentrated under vacuum at 40°C until dryness, and re-suspended in methanol.

Chromatographic runs were performed according to the settings: isocratic elution mode; flow rate of 0.6 mL min^{-1}; oven temperature of 40°C; dihydrogen-potassium phosphate (KH_2PO_4) 5 mmol, as solution A and acetonitrile/water (12% v/v) as solution B. Prior to injection, samples (200 µL) were filtered with Polytetrafluoro-ethylene (PTFE) syringe 0.22 µm filters (Phenomenex, UK). Phenolics compounds were identified by comparison of their retention times and their UV spectra obtained with the diode array detector-DAD (SPD-M20A), ranging from 210 to 310 nm. Gallic, vanillic, protocatechuic, chlorogenic, coumaric and ferulic acids,

Table 2. Effects of MuBME and erythromycin combination against *S. aureus* strains.

UFPEDA	Source	MIC[1] Extract	MIC Drug	MIC E/D	MIC D/E	ΣFIC
672	Blood	1.56	0.0016	0.16	0.000156	0.20
676	Prosthesis Secretion	0.78	0.0016	0.078	0.00078	0.59
677	Wound secretion	1.56	0.0016	0.08	0.0008	0.55
679	Wound secretion	0.78	0.1	0.63	0.0063	0.87
687	Ocular discharge	3.125	0.5	1.25	0.0125	0.43
697	Chest drain secretion	0.39	0.0016	0.02	0.0002	0.18
709	Purelent exudate	1.56	0.0016	0.02	0.0002	0.14
712	Wound secretion transplant	1.56	0.0016	0.02	0.0002	0.14
730	Wound secretion	1.56	0.1	1.3	0.013	0.96
733	Bone fragment	1.56	0.1	0.31	0.0031	0.23

[1]MIC values are expressed in mg/mL, MIC = Minimum inhibitory concentration, FIC = Fractional Inhibitory Concentration indices

quecertin and rutin were used as standard compounds (all purchased from Sigma-Aldrich).

Statistical analysis

Each experiment was performed in triplicate and results were expressed as the mean ± SD (standard deviation). Statistical analysis was performed using the Student's t-test. Differences were considered significant at $p < 0.05$. The concentration needed for 50% of response (IC_{50}) was estimated graphically by linear or nonlinear regression analysis.

RESULTS AND DISCUSSION

Antimicrobial activity

The MuBME showed inhibitory effects on the growth of all microorganisms tested. The inhibition diameter zone (IDZ) ranged between 12 and 26 mm, while the MIC values ranged between 0.39 and 3.13 mg/mL (Table 1). The most susceptible organisms were *M. luteus* (IDZ: 26 ± 0.58 mm) and *S. aureus* (IDZ: 21 ± 0.58 mm) with MICs of 0.39 mg/mL, followed by *B. subtilis* (IDZ of 15 ± 0.58 mm), *E. faecalis* (14 ± 1.32 mm) and *A. niger* (IDZ: 12 ± 0.50 mm) with MIC of 0.78 mg/mL ($p < 0.05$). The Gram-negative organisms and *C. albicans* were less susceptible with MIC values of 1.56 mg/mL for *E. coli* (IDZ: 13 ± 1.15 mm), and *C. albicans* (IDZ: 13 ± 1.32 mm); and 3.13 mg/mL for *K. pneumonia* (IDZ: 12 ± 1.15). These results indicate that MuBME has a broad-spectrum activity. This broad-spectrum activity in the same plant extract may be explained by the presence of a widespectrum of bactericidal substances, or by the action of toxins produced by the plant (Kostova and Dinchev, 2005).

In order to study the combinatory effect of MuBME and erythromycin, we performed a Checkerboard assay against ten clinical isolates of *S. aureus*. We observed that the extract showed a synergistic effect against 60% of tested strains and an addictive effect against the others (Table 2).

S. aureus, an ubiquitous Gram-positive bacteria, is one of the main causes of diseases in humans in a wide spectrum way, acting in both hospital- and community-acquired infections (Novick and Geisinger, 2008). Since the worldwide emergence and spread of resistant *S. aureus* strains, research on new sources of anti-*S. aureus* compounds have become very important. MuBME demonstrated a high ability to enhance the erythromycin activity (synergistic and addictive effects). Interactions of plant extract with antibiotics are an important parameter in the investigation of plant extract action mechanism due the potential of enhanced therapy efficacy (Vuuren and Vijoen, 2011).

Antioxidant assays

Free radicals, when in excess can cause oxidative damage of macromolecules (DNA, proteins, lipids) which is implicated in various diseases, such as cancer, atherosclerosis, diabetes, gastric ulcers, ageing, cardiovascular diseases, and other conditions (Halliwell, 1994; Valko et al., 2007). For this reason, interest has increased considerably in finding natural antioxidants for use in foods or medicinal materials to replace synthetic antioxidants which are being restricted due to their side effects such as carcinogenicity (Botterweck et al., 2000). The MuBME was evaluated in phosphomolybdenum, hydrogen peroxide and DPPH radical scavenging assays. The MuBME showed a total antioxidant capacity of 81.34 ± 0.2% in relation to ascorbic acid activity. MuBME showed a strong hydrogen peroxide scavenging ability with an IC_{50} of 0.038 mg/mL (Gallic acid: 0.008 mg/mL).

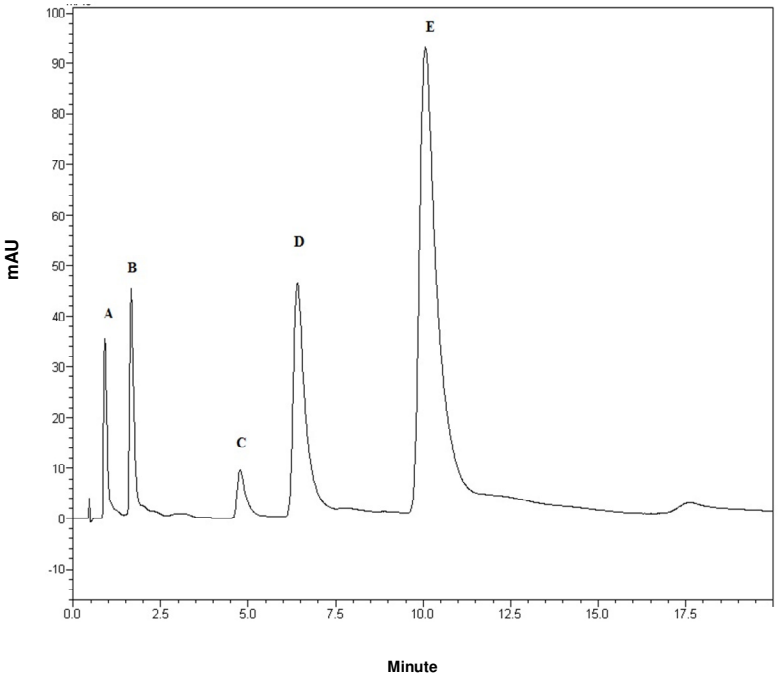

Figure 1. UFLC analysis of *Myracrodruon urundeuva bark*. **A**- Gallic acid; **B**- Catechuic acid; **C**- Protocatechuic acid; **D**- Fumaric acid; **E**- Chlorogenic acid,

Table 3. Phenolic and flavanoid contents, antioxidant and hemolytic activity of MuBM.

Activity	Result
Total phenolic content	37.0 ± 0.47 mg/g GAE
Flavonoid content	43.88 ± 6.55 QE/mg
%TAC	81.34 ± 0.2
H_2O_2 IC_{50}	0.038 mg/mL
DPPH IC_{50}	0.0033 mg/mL
IC_{50} (Vero Cells)	1.57 mg/mL
IC_{50} (erythrocytes)	1.94 mg/mL

Additionally, this extract showed a high capacity to convert DPPH radicals to their more stable molecular counterparts 2,2-diphenyl-1-picrylhydrazines with an IC_{50} of 0.0033 mg/mL (Gallic acid: 0.0012 mg/mL) (Table 3).

Evaluation of cytotoxic effects

In citotoxic evaluations, the extract showed an IC_{50} of 1.94 mg/mL against erythrocytes and 1.57 mg/mL against mammalian Vero Cells, much higher than the concentrations required for the biological responses (Table 3). These activities were assayed because compounds possessing potent antimicrobial and antioxidant activities may not be useful in pharmacological preparations if they possess a hemolytic or cytotoxic effect.

Phytochemical analysis

Among natural antioxidants, polyphenols have been shown to be of great importance because of their ideal structural chemistry for free radical-scavenging activity by donating a hydrogen atom or an electron. The health effects of medicinal plants have been attributed in part to the presence of phenolic compounds which may exert their effects as a result of their antioxidant properties (Kaur et al., 2008). MuBME showed a high phenolic content equivalent to 37 ± 0.47 mg/g GAE. MuBME showed a flavonoid content of 43.88 ± 6.55 QE/mg. Flavonoids are present in photosynthesizing cells and therefore occur widely in the plant kingdom, they have been documented as antioxidant agents (Ferreira et al., 2010). The UFLC analysis detected the presence of bioactive compounds: gallic acid, protocatechuic acid, chlorogenic acid, catechuic acid, fumaric acid (Figure 1). Earlier authors have demonstrated a lot of pharmacologic application for these phenolic acids as antioxidant and antimicrobial (Yilmaz and Toledo, 2004; Liu et al., 2005; Karthikesan et al., 2010; Lin et al., 2009; He et al., 2011; Lou et al., 2011).

Conclusion

Our work showed that *M. urundeuva* bark is a great source of antimicrobial and antioxidant agents. We also demonstrated a high capacity to enhance erythromycin-induced death against *S. aureus*. In addition, this extract did not show cytotoxicity. Gallic acid, protocatechuic acid, chlorogenic acid, catechuic acid and fumaric acid are possible active compounds. A bioassay-guided fractionation procedure for the identification and structural elucidation of biologically active molecules present in this extract is a target for additional research of our group.

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