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Antibacterial and antioxidant activities and acute toxicity of *Bumelia sartorum*, a Brazilian medicinal plant

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Abstract: In order to validate the *Bumelia sartorum* Mart., Sapotaceae, traditional use for infection diseases, this study evaluates the antibacterial activity of the stem bark fractions against methicillin-sensitive (MSSA) and methicillin-resistant (MRSA) *Staphylococcus aureus* strains by using the agar dilution method and reported as MIC (minimal inhibitory concentration). In addition, the DPPH scavenging activity of these fractions was measured and the chemical composition and acute toxicity of the active fraction were also determined. The ethyl acetate (EtOAc) extract was chemically analyzed by LC/MS, direct ionization APCI/MS, ¹H NMR and ¹³C-NMR. All fractions, except butanol extract, presented high antioxidant activity, especially the methanol and the EtOAc extracts, which showed EC50 values (5.67 and 5.30 µg/mL, respectively) considerably lower than the Ginkgo-standard EGb 761® (38.58 µg/mL). The antibacterial activity against *S. aureus* strains was observed in EtOAc (MIC 256-512 µg/mL), which showed a very low toxicity. The chemical study of this fraction revealed the abundant presence of polyphenolic compounds. The antibacterial and antioxidant activities reported in this paper for EtOAc extract from *B. sartorum* and the low toxicity of this fraction opens the possibility that it could be helpful for the developing of new antibacterial agents for treating *S. aureus* infections.

Introduction

In Northeastern Brazil, *Bumelia sartorum* Mart., Sapotaceae, is commonly known as “quixaba” or “quixabeira” and has long been used in Brazilian folklore for treatment of Diabetes mellitus and inflammatory disorders (Almeida et al., 1985), ulceration (Costa-Neto & Oliveira, 2000), and bacterial infections (Ataide et al., 2007). Phytochemical studies are scarce and show the presence of triterpenoids and steroids such as the (2β,3β,4α)-2,3,23-trihydroxyoleana-5,12-dien-28-oic acid, bassic acid, identified as hydrolysis product from ethanol extract (Almeida et al., 1985; Naik et

al., 1991).

Staphylococcus aureus is one of the main causes of infection diseases and has developed resistance to many antibiotics, mainly due to the increased use of antimicrobial drugs (Harbarth & Samore, 2005). Its resistance has been related to the predominance of epidemic PFGE (pulsed field gel electrophoresis) clones able to expand largely around the world (Feng et al., 2008). Specifically in Brazil, a single PFGE clone A MRSA has been markedly detected as prevalent in several hospitals (Vivoni et al., 2006). The control of MRSA nosocomial infections is, many times, very difficult because of the presence of this prevalent clone. Additionally,

many antibiotics are associated with adverse effects (Starrels et al., 2008).

Based on that, the development of alternative antimicrobial drugs turns necessary. A large portion of the world population uses medicinal plants for the treatment of bacterial infections. Especially in developing countries, too much people are still dependent on traditional systems of medicine for the treatment of a variety of diseases (Machado et al., 2003). It is mostly vegetal species that are used and that can act as a rich source for the discovery of new agents for the treatment of difficult infectious diseases.

In order to validate its traditional use for infection diseases, this study evaluated the antibacterial activity of the methanol extract of the stem bark of *B. sartorum* as well as of the crude fractions obtained from the liquid-liquid partition of the mentioned extract. The antioxidative activity of the crude fractions and the acute toxicity of the active fraction were also determined. The chemical composition of the antibacterial and antioxidative EtOAc fraction previously established was further investigated.

Material and Methods

Plant material

The plant was collected in december 2006 in Cabrobó city (08°30' south; 39°18' west), Pernambuco State, Brazil, and an exsiccate (RFA-34154) was deposited in the herbarium of Biological Institute of Rio de Janeiro Federal University, Brazil. The ground barks of *Bumelia sartorum* Mart., Sapotaceae (2 kg) were extracted by maceration in methanol (6 L) for seven days at room temperature. The total methanol extract (2.5 L) was concentrated in rotative evaporator under reduced pressure and the residue (240 g) was successively partitioned between water (1.8 L) and *n*-hexane (1.5 L), dichloromethane (1 L), ethyl acetate (4 L) and *n*-butanol (2 L). The solutions were evaporated to give the respective dried fractions (1.5, 1.4, 23 and 3 g).

Fractionation and identification of the compounds

A part of the EtOAc fraction (0.8 g) was solubilized in H₂O:MeOH (9:1) and chromatographed on a Diaion HP-20 column with stepwise gradient with H₂O:MeOH (9:1→0:1). A total of 400 mL of each proportion of solvents were eluted through the column and evaporated until dryness to furnish ten fractions named successively as: F1 (209.3 mg), F2 (27.7 mg), F3 (46.3 mg), F4 (203.5 mg), F5 (216 mg), F6 (148 mg), F7 (86.8 mg), F8 (39 mg), F9 (80 mg) and F10 (23 mg). The fractions were evaporated until dryness,

solubilized in MeOH (1 mg/mL) and submitted to direct infusion ionspray ionization mass spectrometry in negative mode with a LC-MS-System API-150EX Single Quadrupole MS with Turbo-Ionspray and APCI-Quelle sowie HPLC (Agilent 1100) and Autosampler (CTC-PAL). ¹H-NMR and ¹³C-NMR spectra (acetone-d₆ or DMSO-d₆) of some of them were recorded in a 400-MHz-NMR-Spectrometer Varian Mercury-VX 400 when necessary to identify the substances.

Qualitative analysis of EtOAc extract was performed using an LC-ESI-MS with a 250 x 4 mm, 5 mm, Lichrospher RP-18 column (Merck, Darmstadt, Germany). The mobile phase consisted of (A) methanol and (B) 0.1% aqueous acetic acid. The flow rate was 1 mL/min with a gradient profile consisting of B with the following proportions (v/v) of A: 0-30 min, 5-35%; 30-35 min, 35-65%; 35-50 min, 65-100%; 50-55 min, 100%. Mass spectra were obtained with a LC-ESI-MS/MS-System TSQ Quantum Ultra AM, Finnigan, Triple Quadrupole MS operated in negative ion mode.

Bacterial strains

In this study 28 *S. aureus* isolates were obtained from the culture collection of Hospital Infection Laboratory located at the Institute of Microbiology of Rio de Janeiro Federal University, Brazil. From that, five methicillin-sensitive *S. aureus* (MSSA) and 21 methicillin-resistant *S. aureus* (MRSA) isolates from different clinical sources and two reference strains [ATCC 29213 (MSSA) and ATCC 33591 (MRSA)] were analyzed. The isolates had been identified previously by traditional biochemical tests (Bannerman & Peacock, 2007). In order to verify the antibiotic susceptibility patterns the tests were performed according to CLSI standards (CLSI, 2003). The description of the Brazilian prevalent clone of MRSA as well as the other clones (Machado et al., 2003) tested in this study is presented in the Table 1. The antibacterial activity evaluation of the *B. sartorum* extracts was determined in order to attribute the minimal inhibitory concentration (MIC) using the agar dilution method in Mueller-Hinton agar medium (Difco), according to the guidelines of the National Committee for Clinical laboratory Standards (NCCLS, 2008). Before gelling, Mueller-Hinton agar medium (19 mL) was added to each of the Petri dishes plus plant extract (1 mL) in the desired concentration. The Petri dishes were swirled until the agar began to set. Concentrations of 128, 256 and 512 µg/mL were set. The bacterial samples solutions (10⁴ CFU/mL) were inoculated using a Steers replicator that placed 2 µL of each bacterial strain on the agar surface (Machado et al., 2005; Leal et al., 2010). The plates were incubated during 24 h at 35 °C (Table 1).

DPPH photometric assay

The 2,2-diphenylpicrylhydrazyl (DPPH; Sigma) scavenging activity was measured according to Mensor et al. (2001). Sample stock solutions (1 mg/mL) of the plant fractions were diluted to the final concentrations of 250, 125, 50, 25, 10 and 5 µg/mL in ethanol. *Ginkgo biloba* extract (Egb 761®) was used as standard and samples were prepared using the same dilution procedures. One mL of 0.3 mM DPPH solution was added to 2.5 mL of sample solutions, and allowed to react at room temperature. After 30 min the absorbance values were measured at 518 nm in UV-VIS spectrometer Shimadzu UV-2200 and converted into percentage antioxidant activity (AA%) using the formula:

$$AA\% = 100 - \left\{ \left[\frac{ABS_{\text{sample}} - ABS_{\text{blank}}}{ABS_{\text{control}}} \right] \times 100 \right\}$$

Ethanol (1 mL) plus plant extract solution (2.5 mL) was used as blank. DPPH solution (1 mL) plus ethanol (2.5 mL) was used as negative control. The EC50 values were calculated by linear regression. The results are given as mean±standard deviation (SD). Student's *t*-test and ANOVA were used for comparison between means. A difference was considered statistically significant when $p < 0.05$.

Acute toxicity evaluation

The evaluation of acute toxicity of the EtOAc fraction was carried out in the Clinical and Toxicological Analysis Department of Rio de Janeiro Federal University.

Table 1. Description of *S. aureus* strains and their antimicrobial susceptibility pattern.

Strain type (N° of isolates)	Clinical Source (N° of isolates)	Genotype pattern PFGE Clones	Antimicrobial resistance pattern
MRSA (21)	Surgical site infection (4)	A ₄	VCa, RIFb
		A ₁	VC, MUPc
		A ₂	VC, RIF, MUP
	Tracheal secretion (1) Blood (3)	J ₁	VC, TSxd, MUP, AMe, CIPf
		A ₃	VC, RIF
		A ₁	VC
	Nasal colonization (6)	A ₂	VC, MUP
		A ₃	VC, ChOg, TTh, CIP, CLIf, MUP
		A ₂	VC, RIF, MUP
		A ₂	VC, AM, MUP
		A ₂	VC
		A ₁	VC
	Rectal colonization (3)	I	VC
		A2	VC
		A2	VC, MUP
Cutaneous colonization (1)	B	VC, ChO, MUP	
	H	VC, TT, Elj, AM, MUP, RIF	
	C	VC, RIF	
	B	VC, MUP, RIF	
	A ₂	VC, RIF	
MSSA (5)	Abscess secretion (2)	A ₃	VC, MUP, ChO
		ND ^k	ND
	Blood (1)	ND	ND
	Catheter tip (1)	ND	ND
Bronchoalveolar aspirate (1)	ND	ND	
MRSA	Reference – ATCC 33591	–	methicillin-resistant
MSSA	Reference – ATCC 29213	–	methicillin-sensitive

^aVC (vancomycin); ^bRIF (rifampicin); ^cMUP (mupirocin); ^dTSx (trimethoprim-sulfamethoxazole); ^eAM (amikacin); ^fCIP (ciprofloxacin); ^gChO (chloramphenicol); ^hTT (tetracycline); ⁱCLI (clindamycin); ^jEI (erythromycin); ^kno determined; ^{ATCC}American Type Culture Collection; ^{PFGE}Pulsed field gel electrophoresis; ^{MRSA} methicillin-resistant *S. aureus*; ^{MSSA} methicillin-sensitive *S. aureus*.

The solution was prepared dissolving the fraction in sterile water. The test was carried out *in vivo*, using female mice of Swiss type with nine weeks and approximately 20 g of physical weight. Animals were housed at 25 °C and allowed free access to food and water. The animals were divided in seven groups of five mice. The six test groups received the aqueous solution intraperitoneally in the final doses of 50, 100, 150, 500, 1000 and 1500 mg/kg, respectively, and the control group received only sterile water. They were observed three times a day during one week. The LD50 was calculated (95% confidence) using the GraphPad Prism® software. This study was performed in accordance with IMPPG 014 guidelines following approval by the University Ethics Committee of Rio de Janeiro Federal University.

Results

Identification of the compounds - a chemical profile

LC/MS analysis of the EtOAc extract showed a chromatogram with epicatechin (RT 14.90 min, [M-H]⁻-289), ellagic acid (RT 16.12 min, [M-H]⁻-301) and procyanidins B [M-H]⁻-577 (a broad peak of unseparated compounds at 17.7 min) as the main compounds. Direct diffusion APCI/MS of the samples eluted from Diaion HP-20 column afforded a phenolic profile. The Table 2 shows the most prominent peaks observed in each one of the vegetal samples. In F1 and F2, several low molecular weight compounds were found. ¹H-NMR and ¹³C-NMR (DMSO-d₆) of F-3 pointed to the presence of a prodelphinidin dimer. Flavonoids and phenylpropanoids could not be detected. The samples containing ellagic acid derivatives were analyzed by ¹H-NMR (DMSO-d₆) and singlets at 7.5 ppm and 3.9 ppm (OCH₃) were the only identified signals. The ¹H-NMR and ¹³C-NMR (acetone-d₆) of F5 fraction showed characteristic peaks for (-) epicatechin (main compound) and (-) catechin (minor compound) (Table 2).

Evaluation of antibacterial activity

The antibacterial assay was performed against 28 *S. aureus* isolates as mentioned previously (Table 1). The preliminary results showed a pronounced antibacterial activity in EtOAc fraction. It decreased the bacterial growth at 256 µg/mL and inhibited all bacteria tested at 512 µg/mL, including the resistant and the sensitive hospital isolates as well as the ATCC 29213 reference strain (MSSA). Therefore, this result establishes a MIC value of 256-512 µg/mL of the fraction against a variety of bacterial resistant clones, including that prevalent in Brazil. Other plant extracts did not demonstrate any significant effect on the bacterial growth.

DPPH photometric assay

DPPH is a free radical, stable at room temperature, which produces a violet solution in ethanol. It is reduced in the presence of antioxidant under loss of coloration (Mensor et al., 2001). The Table 3 reports the EC50 values for all plant extracts assayed. EC50 values obtained from regression lines showed a good linear correlation coefficient ($r^2 > 0.8$) and statistical treatment (ANOVA) of data from the three separate tests shows that all the experiments are statistically equivalent ($p = 0.05$). These results illustrate that *B. sartorum* extracts have a high antioxidant potential at least *in vitro*. The methanolic crude extract and the EtOAc fraction show EC50 values considerably lower than the Ginkgo-standard EGb 761®. The *n*-hexane and dichloromethane fractions demonstrated a lower antioxidant activity than the other extracts. However, these values are still significant when compared with the standard. Only the *n*-butanol extract did not demonstrate measurable antioxidant capacity for this methodology (Table 3).

Table 2. Most prominent peaks from direct diffusion APCI/MS of the samples eluted from Diaion HP-20 column.

Vegetal samples from Diaion HP-20 column	Substances	Prominent Peaks [M-H] ⁻
F1 and F2	Protocatechuic acid	152.9
F3	Prodelphinidin dimer	609.4
F4	Epicatechin/catechin	289.2
	Procyanidin dimer	577.7
	Prodelphinidin dimer	609.4
F5	Syringic acid	167.1
	Epicatechin/catechin	289.2
	Ellagic acid	301.3
	Procyanidin dimer	577.7
F6	Ellagic acid	301.3
F9 and F10	3- <i>O</i> -methyl ellagic acid	315.1
	3,3'-di- <i>O</i> -methyl ellagic acid	329.4

Table 3. Middle percentage of the antioxidant activities (AA%) of the fractions of *Bumelia sartorum* Mart., Sapotaceae, and values of EC50 comparing with the positive standard *Ginkgo biloba* EGb 761®.

Vegetal samples	AA% 250 µg/mL	AA% 125 µg/mL	AA% 50 µg/mL	AA% 25 µg/mL	AA% 10 µg/mL	AA% 5 µg/mL	EC50 (µg/mL)
Methanolic extract	93.35±0.28	93.25±0.62	93.62±0.61	92.87±0.39	59.53±2.12	30.88±0.69	5.67
<i>n</i> -Hexane fraction	92.62±0.19	79.83±1.69	38.92±0.24	19.46±0.31	7.06±0.28	1.66±1.18	52.94
DCM fraction	93.48±0.1	92.91±0.43	79.68±1.1	48.9±1.55	22.45±0.56	11.23±0.46	25.04
EtOAc fraction	94.60±0.71	93.65±0.09	93.53±0.05	93.38±0.16	62.59±1.05	30.81±0.47	5.30
<i>n</i> -Butanol fraction	—	—	—	—	—	—	—
EGb 761®	95.23±0.43	90.07±0.82	57.02±0.55	28.14±1.37	10.03±3.25	4.57±1.51	38.58

Acute toxicity evaluation

The animals receiving sterile water and the EtOAc fraction at doses of 50, 100 or 150 mg/kg did not show any physical alteration. The three groups with higher doses of 1500, 1000 and 500 mg/kg showed sedation after the administration of the solution and eventually had 100, 80, and 20% death rate, respectively. In accordance with this result, the LD50 by intraperitoneal route calculated for this fraction was 777 (648.4 to 905.9) mg/kg.

Discussion

Epicatechin, catechin, procyanidins B and ellagic acid were the most abundant polyphenolic compounds in EtOAc extract. Such substances are for the first time identified in *B. sartorum* and seem to be correlated with the biological activities attributed to it. This finding is in accordance with the literature (Hamilton-Miller & Shah, 2000; Verdi et al., 2004) that describes polyphenols as potential antibacterial compounds against *S. aureus*. *Vitis vinifera* extracts, which are rich sources of monomeric phenolic compounds, such as (+)-catechins, (-)-epicatechin and (-)-epicatechin-3-*O*-gallate and dimeric, trimeric and tetrameric procyanidins, showed high antibacterial activity against Gram-positive bacteria (Jayaprakasha et al., 2003). Hatano et al. (2005) showed the effect of dimeric procyanidins (B-3 and B-4) on the antibiotic resistance of MRSA. These compounds showed MIC values at 512 and 1024 µg/mL when assayed against four MRSA strains. However, together with oxacillin and penicillin G, they showed a synergistic enhancement of the antimicrobial effect.

Although antioxidant activity is a common property in the plant kingdom, *B. sartorum* extracts were considerably more antioxidative than *G. biloba* extract. Several authors associated the antioxidant capacity of plant extracts with the antibacterial activity. Alviano et al. (2008) reported that several Brazilian plants traditionally used against bacterial infections also present high antioxidant activity. Pattanayak & Sunita (2008) demonstrated that the synergistic effect of both

antimicrobial and antioxidant activity of *Dendrophthoe falcata* accelerated the wound healing process.

The study of the acute toxicity of EtOAc fraction suggests a low toxicity. Almeida et al. (1985) determined an LD50 ethanolic extract of *B. sartorum* in mice of 127 (91-163) mg/kg by intraperitoneal route. The EtOAc fraction is some six times less toxic than the ethanolic extract of the plant, for the route of administration tested.

The activities found suggest that the use of *B. sartorum* extract as a future phytotherapeutic agent can probably represent an alternative source for the treatment of infective diseases. However, further research is required to evaluate the practical values of a therapeutic application.

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