SUPPLEMENTARY MATERIAL

Antibacterial activity of extract and fractions from branches of *Protium spruceanum* and cytotoxicity on fibroblasts

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Abstract: The crude ethanol extract (CEE) and fractions from branches of *Protium spruceanum* were subjected to antibacterial and cytotoxicity assays. Compounds of the most active fraction were identified by GC-MS and LC-MS. CEE was active against 19 bacteria and the ethyl acetate fraction (EAF) showed the lowest minimum bactericidal concentration (MBC 0.3–80.0 mg/mL). Through time-kill assay was observed that EAF induced rapid bactericidal effect against *Staphylococcus saprophyticus*. The cytotoxicity tests against L929 fibroblasts showed great potential of EAF on the treatment of infections caused by 5 bacteria (MBC < IC_{50}). The results provide *in vitro* scientific support to the possible application of branches of *P. spruceanum* as antimicrobial agent that may contribute for treatment of infections.

Keywords: *Protium spruceanum*; antbacterial activity; cytotoxicity

Experimental

Plant material and obtaining of crude extract and fractions

Thin branches of *P. spruceanum* were collected in Lavras municipality, Minas Gerais, Brazil (Coordinates 21°17’33, 6”S and 44°59’15, 1”W, 21°18’11, 9”S and 44°59’18, 8”W”). A voucher specimen was deposited (No 16399 HESAL) in the Herbario of Universidade Federal de Lavras, Minas Gerais, Brazil. The branches were dried at room temperature and fragmented in a knife mill. The powdered material (500.0 g) was subjected to maceration with 2.0 L of ethanol (40% w/v). This procedure was repeated ten times. The ethanol was recovered in a rotary evaporator, at temperature ≤ 40 ^{0}C yielding the crude ethanol extract (CEE, 48.0 g, 9.6%). A sample of CEE (20.0 g) was dissolved in methanol-water solution (1:1 v/v) and subjected to three sequential liquid-liquid partition, to obtain hexane fraction (HF, 2.8 g, 14.0%), ethyl acetate (EAF, 9.2 g, 46.0%), and hydromethanolic fraction (HMeF, 7.0 g, 35.0%).

Antimicrobial activity

In vitro assays were performed using 19 bacteria, being standard strains and clinic isolates. For the screening of antimicrobial activity were used the Gram-positive bacteria *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* MRSA clinical isolated, *Staphylococcus saprophyticus* ATCC 15305, *Listeria monocytogenes* clinical isolated, *Streptococcus pyogenes* ATCC 19615, *Enterococcus faecalis* ATCC 19433 and *Enterococcus faecium* ATCC 6569) and the Gram-negative bacteria *Enterobacter aerogenes* ATCC 13048, *Proteus mirabilis* ATCC 25933, *Salmonella typhimurium* ATCC 14028, *Salmonella enteritidis* ATCC13076, *Shigella sonnei* ATCC 25931, *Shigella flexneri* ATCC 12022, *Escherichia coli* ATCC 25922, *Escherichia coli* EPEC CDC O11, *Pseudomonas aeruginosa* ATCC 27853, *Providencia rettgeri* ATCC 29944, *Klebsiella pneumoniae* ATCC 13833 and *Klebsiella oxytoca* CDC 29. These bacteria were cultivated in Müeller-Hinton medium (Himedia®) during 24 h/37°C. All inoculums were prepared using direct colony suspension method making a saline suspension (0.9% NaCl) of colonies selected from a 24 h or 48 h agar plate, before each assay. The suspension was adjusted to achieve turbidity equivalent to a 0.5 McFarland standard (about 1 x 10^{8} CFU/mL) (CLSI 2012). The agar diffusion method was used in the screening and the minimum bactericidal concentration (MBC) was determined by microdilution assay.

Agar diffusion method

The screening of antimicrobial activity of extract and fractions from branches of *P. spruceanum* were performed using agar diffusion method (Valgas et al. 2007). The extract and fractions were solubilized (80.0 mg/mL) in dimethylsulfoxide (DMSO) (Vetec®) and this solvent was used as a negative control. Tetracycline (100.0 μg/mL) was used as a positive control, except for *P. aeruginosa*, *P. mirabilis* and *P. rettgeri* for which it was used moxifloxacin (100.0 μg/mL). The Müeller-Hinton medium (50.0 mL) was added at Petri dishes (15 cm diameter). After medium solidification were made wells (6 mm diameter) by means of a glass sterile cylinder. The bacterial inoculums were uniformly spread using sterile cotton swab on agar and 50.0 μL of CEE, fractions and controls solutions were added in the wells. Dishes were incubated for 24 h or 48 h at 37°C and the growth inhibition zone around the well were measured in millimeters. The assays were run in triplicates and samples that generated an inhibition zone (IZ) mean bigger than 10 mm were considered actives (Lima-Filho et al. 2002).

Evaluation of the method proposed to determinate the minimum bactericidal concentration (MBC).

Due to the lack of a standard of antimicrobial assays applied to natural products and the difficulty dissolution of extracts and fractions in the liquid medium, some modifications were proposed to the methodology described by *Clinical and Laboratory Standards Institute* (CLSI 2012). The method proposed was evaluated analyzing the microbial viability with the modifications. The inoculums (1 x 10^8 CFU/mL) were diluted 1:50 in Müeller-Hinton broth aiming to obtain a final assay with 5 x 10^5 CFU/mL. The microbial viability was evaluated using 96-well plates to which were added 50 μL of methanol and 75 μL of broth. Only 75.0 μL of broth were added in the grown control. Tetracycline (100.0 μg/mL) was used as positive control and the control of the culture medium was made using only broth. To evaporate methanol, opened plates were placed in a desiccator, under vacuum at 25 °C for one hour. The remaining volume in each well was checked and when it was equals to the volume of culture medium added was proven the evaporation of methanol. Then, the plates were sterilized by means of short-wavelength ultraviolet (UV-C) irradiation, for 20 minutes and 25.0 μL of the diluted inoculums were added (except in the medium control). The plates were closed and incubated for 24 h or 48 h at 37°C. After the incubation period, 30 μL of triphenyl tetrazolium chloride (TTC) (0.25 mg/mL) (Neon®) were added and the plates were again incubated for three hours. The absorbance was read at 650 nm in ELISA plate reader (Molecular Devices®). The results of absorbance were analyzed by One-way analysis of variance (ANOVA) with post hoc Dunnett's test using the software GraphPad Prism (Version 5.0). A value of $P < 0.05$ was considered statistically significant.

Minimum bactericidal concentration (MBC)

MBC of CEE and fractions considered active in diffusion assay was determined using the proposed modified method. An aliquot (100.0 μL) of sample solution at 160.0 mg/mL was added into the first well of the 96-well microtiter plate. Methanol (50.0 μL) was added to the other wells. Serial dilutions 1:2 were made removing 50 μL from the first well and transferring to the next to obtain extract and fractions concentrations from 80 to 0.039 mg/mL. Then 75.0 μL of broth were added in each well, following the same procedure previously described. Due to the precipitation of extracts, it was not possible to read the sample absorbance. Therefore, three hours after addition of TTC, the wells without visible color were peaked to Petri dishes using inoculation loop. The Petri dishes were incubated for 24 or 48 h. The MBC was established as the smallest concentration at which no microbial growth was observed.

Total antimicrobial activity (TAA)

This parameter was determined according with Eloff 2004. For CEE was used the equation: TAA= mg of extract partitioned / MBC). For fractions the TAA was calculated by: mg of fraction / MBC).

Time-kill assay

Time-kill assays of the most sensitive bacteria (*S. saprophyticus*) were performed by the broth microdilution method described by CLSI with modifications. The EAF was incorporated into Mueller Hinton broth in 96-wells plate to obtain the final concentrations: ½ MBC, MBC and 2× MBC. A growth control comprising the broth without the test fraction was included. Inoculum suspension with approximately 10^5 CFU/mL of exponentially growing bacterial cells was used in this assay. After addition of inoculum, the plate was incubated at 37°C for 24h. Samples were removed from the wells at 0, 3, 6, 12 and 24 hours and serial tenfold dilutions were prepared in saline as needed. The determination of CFU/mL was performed by the plate count technique on Mueller Hinton agar plates. The experiments were performed in triplicate. Data were analyzed as killing curves by plotting the $\log_{10}$ CFU/mL versus time (hours). The bactericidal activity is defined as a 3 $\log_{10}$ CFU/mL or greater reduction in the viable colony count, whereas a less than 3 $\log_{10}$ CFU/mL reduction corresponds to a bacteriostatic activity (Petersen et al. 2007). The results also were analyzed by One-way analysis of variance (ANOVA) with post hoc Bonferroni's test using the software GraphPad Prism (Version 5.0).

GC-MS analysis

Volatile compounds of most active and less toxic fraction (EAF) were identified by gas-chromatography coupled to mass spectrometry (GC-MS) on a GC-MS D5975 Agilent appáratus, equipped with Agilent J&W HP-5MS advanced GC column (30 m x 250 μm x 0.25 μm particle size). Helium was used as carrier gas (1.4 ml/min). The sample was dissolved in methanol (1.0 mg/mL) and 1.0 µL was injected on split less mode. The injector and detector were set at 290 °C. Column temperature, initially at 100 °C (1 min), was increased to 200 °C (5°C/min), following an enhancement of 10 °C/min until 290 °C, keeping at isothermal condition for ten minutes. Compound identification was performed based on relative retention time and comparison of mass spectra with NIST/2.0 library data.

LC-MS analysis

High-performance liquid chromatography coupled to electrospray ionization (ESI) tandem mass spectrometry and photodiode array detection was developed to identify phenolic compounds in EAF. The analysis was performed on a UPLC Acquity chromatographer (Waters) equipped with a CSH 130 C18 column (50 x 10 mm x 1.7 μm particle size) (Waters). Milli-Q purified water with 0.1% methanoic acid and acetonitrile (ACN) with 0.1% methanoic acid were used as mobile phase. The flow rate was 0.3 ml/min and 4 μL of samples (1.0 mg/mL) were injected; with a linear gradient from 5% to 95% ACN in 10 min, held until 11 min. ESI ionization in negative and positive-ion mode was performed under the following conditions: Capillary voltage 3.5 kV, positive and negative ion mode, capillary temperature 320 °C, source voltage 5 kV, vaporizer temperature 320 °C, corona needle current 5 mA and sheath gas and nitrogen 27 psi. Analyses were run in full scan mode (100-2000 u). ESI-mass spectrometry (MS/MS) analyses were performed with a UPLC Acquity (Waters) with argon as the collision gas, with collision energy set to 30 eV. The UV spectra were recorded from 190 to 450 nm. The flavonoids and tannins were identified by UV and mass spectrometric data.

Cytotoxicity

Murine fibroblasts L929 cells, cultivated in RPMI 1640 medium (Sigma®), were distributed in 96-well microtiter plate using a density of 1 x 10^5 cell/well and after they were incubated at 37°C with 5% of CO_2 for 24 h. Cells were treated with EAF dissolved in 2% DMSO, at concentrations ranging from 3.75 to 0.01 mg/mL. Cell viability was evaluated using the sulforhodamine B assay (SRB) (Skehan et al. 1990). After 24 h incubation the media was removed and cells were fixed with cold 20% trichloroacetic acid for 1 h at 4°C. The microtiter plate was washed with distilled water and dried. Thereafter, fixed cells were stained for 30 min with 0.1% SRB dissolved in 1% acetic acid. The plate washed again with 1% acetic acid, again allowed to dry and 200 µL of 10 mM TRIS buffer (pH 10.5) were added to stain solubilization at room temperature for ~30 min. Samples absorbance was read in the spectrophotometer (490 nm) and the values were expressed as the concentration of sample that inhibit 50% of the cell growth (IC_{50}), compared to control. IC_{50} values were calculated by a polynomial regression equation.

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Table S1. Antibacterial activity of ethanol extract and fractions of branches of *P. spruceanum*

Bacterium	Ethanol extract and fractions							
	CEE		HF		EAF		HMeF	
	IZ (mm)	MBC (mg/mL)	IZ (mm)	MBC (mg/mL)	IZ (mm)	MBC (mg/mL)	IZ (mm)	MBC (mg/mL)
Staphylococcus aureus	21.0	2.5	-	-	17.6	1.2	16.9	1.2
S. aureus MRSA	21.6	5.0	-	-	17.5	0.6	15.8	1.2
S. saprophyticus	24.1	5.0	10.2	20.0	16.1	0.3	14.1	0.3
Listeria monocytogenes	11.8	40.0	11.2	80.0	12.0	5.0	11.4	20.0
Streptococcus pyogenes	14.5	80.0	-	-	12.9	20.0	13.7	80.0
Enterococcus faecalis	17.4	5.0	-	-	11.6	1.2	12.1	1.25
E. faecium	10.6	40.0	-	-	13.2	40.0	11.8	80.0
Enterobacter aerogenes	13.5	20.0	-	-	10.3	40.0	7.0	-
Proteus mirabilis	13.7	40.0	-	-	-	80.0	13.6	80.0
Salmonella enteritidis	13.5	10.0	-	-	10.4	1.2	10.2	1.2
S. typhimurium	10.7	40.0	-	-	-	-	10.4	80.0
Shigella sonnei	10.8	80.0	-	-	11.1	20.0	11.7	20.0
S. flexneri	11.0	20.0	-	-	12.2	10.0	8.0	-
Escherichia coli	10.1	20.0	-	-	11.5	10.0	10.1	80.0
E. coli EPEC	11.1	10.0	-	-	11.9	20.0	11.0	80.0
Pseudomonas aeruginosa	12.3	10.0	-	-	10.2	10.0	10.5	20.0
Providencia rettgeri	14.4	20.0	10.9	40.0	10.9	10.0	12.6	20.0
Klebsiella pneumoniae	10.4	40.0	-	-	10.0	10.0	-	-
K. oxytoca	11.0	40.0	-	-	10.3	5.0	-	-

All experiments were conducted in triplicate, and the mean values are presented.
- : not active.
IZ: Inhibition zone. MBC: Minimal bactericidal concentration. CEE: Crude ethanol extract. HF: Hexane fraction. EAF: Ethyl acetate fraction. HMeF: Hydromethanolic fraction.

Table S2. Microbial viability obtained through the modified CLSI method with methanol evaporation

Bacterium	Microbial viability*
Staphylococcus aureus	96.5 ± 7.3
S. aureus MRSA	91.7 ± 19.1
S. saprophyticus	90.7 ± 18.2
Listeria monocytogenes	102.3 ± 4.8
Streptococcus pyogenes	97.6 ± 0.1
Enterococcus faecalis	96.7 ± 0.5
E. faecium	96.1 ± 2.6
Enterobacter aerogenes	109.7 ± 11.7
Proteus mirabilis	100.4 ± 0.3
Salmonella enteritidis	96.0 ± 1.7
S. typhimurium	94.2 ± 6.0
Shigella sonnei	103.4 ± 8.0
S. flexneri	97.6 ± 9.3
Escherichia coli	91.7 ± 6.4
E. coli EPEC	90.3 ± 11.0
Pseudomonas aeruginosa	107.0 ± 8.3
Providencia rettgeri	88.0 ± 1.3
Klebsiella pneumoniae	105.6 ± 6.1
K. oxytoca	86.4 ± 5.8

*Microbial growth of the test relative to growth control.
Results expressed as % of microbial growth ± standard deviation.

Table S3. Total antimicrobial activity (TAA) of extract and fractions of branches of *P. spruceanum*

Ethanol extract and fractions	TAA (mL^{-1})/ bacteria group	
	Gram-positive	Gram-negative
CEE	3035.7	854.2
HF	25.0	5.8
EAF	10889.2	1217.1
HMeF	5680.1	583.3

CEE: Crude ethanol extract. HF: Hexane fraction. EAF: Ethyl acetate fraction. HMeF: Hydromethanolic fraction.

Table S4. Compounds identified in ethyl acetate fraction (EAF) of branches of *P. spruceanum* by CG-MS

Compound	RT (min)	%
Methyl palmitate	13.79	19.22
Methyl linoleate	22.784	18.82
Methyl linolenate	22.880	19.98
β-sitosterol	34.845	8.06
β-amyrin	35.402	13.76
α-amyrin	36.127	16.60

RT: Retention time

Table S5. Phenolic compounds identified in ethyl acetate fraction (EAF) of branches of *P. spruceanum* by LC-MS

Compound	RT (min)	UV (nm)	LC-MS	
			$[M – H]^{-1}$ (*m/z*)	$[M + H]^{+1}$ (*m/z*)
Procyanidin	1.89	279.1	577.27 (407.12; 289.21; 245.04; 205.14; 137.09; 124.90)	579.36 (291.37)
Catechin	2.01	279.1	289.15 (245.14; 176.99; 165.04; 137.04; 124.89; 109.08)	291.37
Quecetin-3-*O*-glicuronide	2.77	268.1; 352.1	477.24 (273.12; 257.25; 178.63; 150.63)	479.34 (303.19)
Quercitrin	3.07	264.1; 348.1	447.32 (301.76; 273.23; 257.12; 151.20)	449.41 (303.26)

RT: Retention time