

Antibacterial substances from leaves of *Protium spruceanum* (Burseraceae): *in vitro* and *in silico* evaluation

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Due to the increase of bacterial resistance, the search for new antibiotics is necessary and the medicinal plants represent its most important source. The aim of this study was to evaluate the antibacterial property of extract and fractions from *Protium spruceanum* leaves, against pathogenic bacteria. By means of diffusion and microdilution assays, the crude extract was active against the nine bacteria tested being the hydromethanolic fraction the most active. During phytochemical procedures, procyanidin (1) and catechin (2) were identified as the main antibacterial constituents of this fraction. *In silico* results obtained using PASSonline tool indicated 1 and 2 as having good potential to interact with different targets of currently used antibiotics. These results no indicated potential to none DNA effect and indicated the cell wall as mainly target. Electrophoresis result supported that had no DNA damage. Cell wall damage was confirmed by propidium iodide test that showed increased membrane permeability and by cell surface deformations observed in scanning electronic microscopy. The *in vitro* assays together with the *in silico* prediction results establish the potential of *P. spruceanum* as source of antibacterial compounds that acts on important bacterial targets. These results contribute to the development of natural substances against pathogenic bacteria and to discovery of new antibiotics.

Keywords: *Protium spruceanum*. Burseraceae. Procyanidin. Catechin. Antibacterial activity.

INTRODUCTION

The emergence of antibiotic resistance bacterial strains has hampered the treatment of infections

and represents an important public health problem, being infectious diseases one of the major causes of morbidity and mortality worldwide (Penduka *et al.*, 2018). Therefore, there is a need to developing new antibiotics using natural products, which are widely prescribed worldwide and are an important source of bioactive compounds (Kumar *et al.*, 2017; Moro *et al.*, 2017). Natural compounds such as alkaloids,

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lectins, flavonoids and terpenoids are considered as an adequate strategy against bacterial resistance and an alternative for treatment of infections (Chandra *et al.*, 2017). Thus, plants are important source of biological active compounds and/or of chemical models for the development of new more effective medicines. In the last decades, natural compounds and its derivatives participated into development of more than 58% of the approved antibiotics by regulatory agencies worldwide (Newman, Cragg, 2016).

In this context, species of the Burseraceae family stand out due to their ethnopharmacological applications. The distribution of family Burseraceae is typically pantropical and it occurs in tropical Africa and America. The Burseraceae family has seventeen genera and 500 species and its most important genera are *Bursera*, *Commiphora*, *Protium* and *Canarium*. In Brazil, there are seven genera and about sixty species, mostly native to the Amazon region (Rosalem *et al.*, 2017). Among these genera, species of the genus *Protium* are important sources of bioactive substances and are predominant in South America, being that more than 80% of the Burseraceae species in the Amazon Biome belong to this genus (Siani *et al.*, 2004; Marques *et al.*, 2010).

Protium spruceanum (Benth.) Engler, popularly known in Brazil as breu-branco is a large canopy tree (up to 20 m tall), native of Atlantic and Amazon rain forests and in riparian forests of the Cerrado, a typical tropical savanna region (Vieira *et al.*, 2010; Rodrigues *et al.*, 2013).

In Brazilian traditional medicine *P. spruceanum* is used as analgesic and anti-inflammatory (Rodrigues *et al.*, 2013). The antibacterial property of crude extract and fractions from branches of *P. spruceanum* was previously reported (Amparo *et al.*, 2017). However, bioactive compounds from this specie were not reported yet.

Currently, studies related to pharmacological properties involve the use of laboratory experiments associated with *in silico* studies aiming to optimize the researches. In this context, virtual screening (*in silico*) have been applied to avoiding the excessive use of animal models, minimizing the high cost of biological experiments and allowing to results in a short time. By means of *in silico* studies it's possible to predict more appropriate biological target that adequately directing the laboratorial researches to the medicine discovery (de Oliveira *et al.*, 2014).

Among *in silico* tools currently used to predict pharmacological effects of natural compounds stand out "Prediction of Activity Spectra for Substances" (PASS) software available online. Through these procedures the application of public financial resources are pharmacologic and economically more suitable.

The objective of the present work was to analyze the *in vitro* antibacterial property of crude extract and fractions from leaves of *P. spruceanum* and identify secondary metabolites present in the active subtractions. Additionally, the chemical structures of procyanidin and catechin constituents of *P. spruceanum*, were subjected to PASSonline tool, aiming predict mechanisms of antibacterial activity.

MATERIAL AND METHODS

Plant collection and identification

Leaves of *P. spruceanum* were collected in June 2014 at the region of Lavras municipality, Minas Gerais, Brazil. The specie was identified by Prof. Dr. Vivette Appolinário Rodrigues Cabral of Departamento de Ciências Florestais, Universidade Federal de Lavras. After botanical identification, a voucher of *P. spruceanum* was deposited in the Herbarium of Universidade Federal de Lavras, under the code No 16399 HESAL. Leaves were dried at room temperature until a constant weight was achieved and then powdered on a knife mill. Activity was registered in SisGen, the National System of Genetic Resource Management and Associated Traditional Knowledge under number A2B5290.

Extraction and fractions obtainment

Fragmented leaves (220.0 g) were subjected to maceration with ethanol 95 °GL (2 L). After 1 day, the mixture was filtered and the ethanol was recovered in a rotatory evaporator at ≤ 45 °C under reduced pressure. This extraction process was repeated 5 times, resulting in the crude ethanol extract (CEE, 37.2 g, 16.9% yield). A sample of CEE (20.0 g) was dissolved in methanol-water (1:1) and subjected to liquid-liquid partitions, first with hexane and after with ethyl acetate. After three repetitions of partition process and recovery of extractive solvents were obtained the fractions hexane (HF, 3.6 g, 18.0% yield), ethyl acetate (EAF, 6.0 g, 30.0% yield) and hydromethanolic (HMF, 9.3 g, 46.5% yield).

***In vitro* antibacterial evaluation**

The *in vitro* antibacterial property of extract and fractions from leaves of *P. spruceanum* were evaluated against four Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* clinical isolated, *Enterococcus faecalis* ATCC 15325 and *Streptococcus pyogenes* ATCC 19615), and five Gram-negative bacteria (*Salmonella enteritidis* ATCC13076, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13833, *Shigella sonnei* ATCC 25931 and *Escherichia coli* ATCC 25922). Well diffusion method on Mueller-Hinton agar (MHA) was used in the screening for antibacterial activity and the broth microdilution method was to establish the minimum bactericidal concentration (MBC). Bacteria were cultivated in Müller-Hinton agar (MHA) medium at 37 °C, for 24 h. The inoculums were prepared using the direct colony suspension method by means of a saline (0.9% NaCl) suspension of colonies selected from a 24 h agar plate, before each assay. The suspension was adjusted to reach turbidity equivalent to a 0.5 of the McFarland standard scale (1×10^8 CFU ml⁻¹) (CLSI, 2012).

For the agar diffusion method, Petri dishes (15 cm diameter) containing MHA medium (50.0 mL) were punched with a sterile cylinder (6 mm diameter wells) and the microbial inoculum was uniformly spread using sterile cotton swab on agar. The extract and fractions were dissolved (80.0 mg/mL) in dimethylsulfoxide (DMSO). DMSO was used as negative control. As positive control tetracycline (100.0 µg/mL) was used for bacteria and moxifloxacin (100.0 µg/mL) specifically for *P. aeruginosa*. Then, 50.0 µL of the solution of extracts, fractions and of the controls were added in the wells. The dishes were incubated at 37 °C for 24 h. The bacterial growth inhibition zone around the well was determined in millimeters. The assays were realized in triplicates. Those samples that generated inhibition zone ≥ 10 mm were considered actives (Lima-Filho *et al.*, 2002).

For the extract and fractions from leaves of *P. spruceanum* considered actives in diffusion assay the minimum bactericidal concentration (MBC) was determined through microdilution method (CLSI, 2012; Amparo *et al.*, 2017). In 96-well plates, 50.0 µL of methanol were added from the second well of each line on and 100.0 µL of the samples dissolved in methanol (160.0 mg/mL) were added in the first well. Thus, 50.0 µL from the first well were transferred to the next (serial dilutions 1:2) to obtain extract and fractions

concentrations from 80.00 to 0.15 mg/mL. Then 75.0 µL of Müller-Hinton broth were added in each well. In the negative control were added 50 µL of methanol and 75 µL of broth. As growth control 75 µL of broth were added in a well. To verify the methodology efficacy, tetracycline or moxifloxacin (100.0 µg/mL) were used as positive control. To methanol withdraw opened plates were placed in a vacuum desiccator at 25 °C for one hour. Then, the plates were sterilized by ultraviolet germicidal irradiation for 20 minutes. The inoculum (1×10^8 CFU/mL) were diluted 1:50 in broth in order to obtain a final assay with 5×10^5 CFU/mL. From these diluted inoculum 25 µL were added in the wells (except in the medium control). The plates were closed and incubated at 37 °C, for 24 h. After the incubation period, 30 µL of triphenyl tetrazolium chloride (TTC, 0.25 mg/mL) were added and the plates were incubated for additional 3 h. After TTC addition the wells without visible color was peaked to Petri dishes using inoculation loop (10 µL). These Petri dishes were incubated for 24 h. MBC was established as the smallest concentration in which no bacterial growth was detected.

Cytotoxicity and selectivity index

Cytotoxicity of the samples was evaluated using murine fibroblasts L929, cultivated in RPMI 1640 medium. Extract and fractions were dissolved in 2% DMSO, at concentrations ranging from 4.00 to 0.01 mg/mL. Cells were distributed in 96-well microtiter (1×10^5 cell/well) and were incubated at 37 °C with 5% of CO₂ for 24 h. Cells were treated with the samples and after 24 h incubation, the cell viability was evaluated using the sulforhodamine B assay (SRB) (Skehan *et al.*, 1990). 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 cytotoxic concentration of sample for 50% of the cell (CC₅₀), compared to control. CC₅₀ values were calculated by a polynomial regression equation. Selectivity index (SI) was calculated by the ration CC₅₀/MBC.

Chromatographic fractionation and analysis

The most active fraction, HMF was fractionated aiming to identify the antibacterial compounds. Thus, a sample of HMF was dissolved in methanol reaching 15.0 mg/mL. An aliquot (20 μ L) of this solution was injected in a Shimadzu LC-6AD[®] chromatographer equipped with Phenomenex Luna[®] 5 μ m C18(2) 100 Å, LC Column 250 x 4.6 mm. Milli-Q[®] purified water (A) and methanol (B) were used as mobile phase at flow rate of 0.7 mL/min. The following gradient was used: 5% B at 0 min, 80% B at 20.0 min, 100% B at 30 to 35 min and 5% B at 40.0 min. Injections were repeated 75 times and each run elution was monitored at 210 and 350 nm. The fractions were collected at the interval of 15 to 20 min (F2) and of 20 to 25 min (F3). These retention times were chosen according to the UV spectra profile. To identify the respective constituents, after dried, fraction F2 (X.0 mg) and F3 (Y.0 mg) were analysed by LC-DAD-ESI/MS. 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 in a gradient elution from 5% to 95% ACN in 10 min, held until 11 min. Aliquot of 4.0 μ L of each sample solution (1.0 mg/mL) was injected. ESI-MS/MS analysis was performed in following conditions: 3.5 kV capillary voltage, positive and negative ion mode, 320 °C capillary temperature, 5 kV source voltage, 320 °C vaporizer temperature, 5 mA corona needles current and 27 psi gas and nitrogen pressure. Analyses were run in a full scan mode (100-2000 u). The UV spectra were registered from 190 to 450 nm.

The antibacterial property of collected fractions F2 and F3 were evaluated using *S. aureus*.

In silico antimicrobial activity evaluation

The identified constituents in the bioactive fraction, procyanidin and catechin, from leaves of *P. spruceanum* were subjected to *in silico* analysis by means of PASSonline tool aiming to predict its antibacterial action mechanisms (available at <http://www.pharmaexpert.ru/passonline/>). The Software Chemdraw 10.0 (ChemBiodraw, Cambridge Soft) was used to draw the chemical structures and they were saved in sdf file. PASS online tool decompose the chemical structure into descriptors and realize a comparison with a database containing more than 250,000 biologically active targets. The results are expressed as the probability of the compound under analysis be active (Pa) or inactive (Pi)

(de Oliveira *et al.*, 2014). For the *in silico* analysis were included four classical targets corresponding to those reached by antibiotics actually used in the medicine, and two considered as new bacterial targets described in the scientific literature. The potential antibacterial action targets of compounds were evaluated based on the difference (Pa-Pi). Thus, the potential was considered as unsatisfactory (Pa < Pi), low [(Pa-Pi) < 0.2], moderate [(Pa - Pi) \geq 0.2 and < 0.5] or high [(Pa - Pi) \geq 0.5].

DNA damage

HMF was dissolved in broth with 2% DMSO to obtain a final concentration equivalent to MBC. Then 5.0 mL of *S. aureus* inoculum (5×10^7 CFU/mL) and 5.0 mL of sample were added in tubes and incubated for 24 h at 37 °C. For the control, were added broth and inoculum. The tubes were centrifuged for 5 minutes at 15000 x g, The pellet were resuspended in 300 μ L of TE buffer (50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8,0), 5 μ L of proteinase k (2 mg/mL) and 30 μ L sodium dodecyl sulfate (SDS) 10%. After incubation 37 °C for 2 h, and two extractions were made with 300 μ L of chloroform. The aqueous phase were combined with 0.1 volume of NaCl 3 M and equal volume of isopropanol and the samples were freezer incubated for 1 h. After centrifugation the pellet were washed with ethanol 70%, dried and resuspended in 25 μ L TE buffer. Test samples and DNA marker were analyzed by electrophoresis in TAE buffer (4.84 g Tris base, pH 8.0, 0.5M EDTA/1 L) using 1.8% agarose gel containing ethidium bromide, subjected to 100 V for 90 min (Jin *et al.*, 2006).

Membrane permeability test

The inoculum (1×10^8 CFU/mL) was diluted in Mueller-Hinton broth in order to obtain a final assay with 1×10^6 CFU/mL and the HMF was dissolved in broth with 2% DMSO to obtain concentrations equivalent to 2x MBC, MBC and $\frac{1}{2}$ MBC. Then into a 96-well plate were added 100 μ L of inoculum and 100 μ L of sample and for the control were added 100 μ L of inoculum and 100 μ L of Mueller-Hinton broth. This assay was performed in triplicate for each of the samples and the plate was incubated for 24 h at 37 °C. After incubation, samples were taken from the wells and were added in tubes, washed with 2 mL of phosphate buffered saline (PBS) and centrifuged for 10 minutes at 2500 xg. The supernatant was discarded and the sample were

resuspended in 100 μ L of PBS and incubated with 1 μ L propidium iodide solution (1 mg/mL). The samples were incubated at room temperature and protected from light for 30 min. Flow cytometry analysis were conducted using BD FACS Calibur (BD Bioscience[®]) and data were analyzed by Flow Jo v.10[®] (Chopra *et al.*, 2015). Results are presented as mean \pm standard deviation (SD) and data were evaluated by one-way analysis of variance (ANOVA) followed by Dunn's test, using GraphPad Prism software. P values less than 0.05 ($p < 0.05$) were considered as indicative of significance.

Scanning electronic microscopy (SEM)

HMF was dissolved in broth with 2% DMSO to obtain a final concentration equivalent to MBC. Then 500 μ L of *S. aureus* inoculum (1×10^8 CFU/mL) and 500 μ L of sample were added in microtubes and incubated for 24 h at 37 °C. For the control, were added 500 μ L of broth and 500 μ L of inoculum. After incubation, the microtubes were centrifuged for 10 minutes at 2500 xg and the supernatant was discarded. Glutaraldehyde fixative 3% in 0.1M phosphate buffer pH 7.3 was added (1 mL) and incubated for 1 h at room temperature. The samples were centrifuged again and resuspended with 100 μ L PBS. The suspension (50 μ L) was spread in a coverslip and dried at 50 °C for 1 h. Then, samples serially dehydrated in ethanol (70%, 95% and 100%, 2 min in each) and dried again at 50 °C (Brudzynski,

Sjaarda, 2014). A small amount of carbon was sputtered on the samples and the coverslips observed in scanning electron microscope (JSM – 6010LA[®]). Secondary electron images were taken at 5.0 kV.

RESULTS AND DISCUSSION

The antibacterial property against nine pathogenic bacteria was observed for the extract CEE and its fractions EAF and HMF (Table I). HF not presented antibacterial activity.

Greater growth inhibition activity was observed against *S. aureus* (MBC 0.6 mg/mL). Species of the *Staphylococcus* genus have been reported as cause of skin infections, sepsis, pneumonia, toxic shock syndrome, endocarditis and urinary tract infections (Otto, 2010). Studies involving these bacteria are important also because of resistant strains, that difficult the treatment, as *S. aureus* MRSA (Lopez *et al.*, 2015).

Among fractions from CEE, the HMF showed the lowest MBC values indicating that the mainly antimicrobial compounds responsible for antibacterial activity are polar metabolites. HMF also showed the lowest cytotoxicity (CC_{50} 3.7 mg/mL) and the greater selectivity (SI 6.17), considered satisfactory (Makhafola *et al.*, 2012; Elisha *et al.*, 2017). Thus, HMF was fractionated obtaining the sub-fractions F2 and F3. One tannin and one flavan-3-ol were identified in F2 and three flavonols glycosides were identified in F3 (Table II).

TABLE I - Cytotoxicity and antibacterial activity of ethanol extract (CEE) from leaves of *P. spruceanum* and its fractions

Fibroblasts L929	CEE			Fractions from CEE					
				EAF			HMF		
	$CC_{50} = 1.1$ mg/mL			$CC_{50} = 1.3$ mg/mL			$CC_{50} = 3.7$ mg/mL		
Bacterium	IZ	MBC	SI	IZ	MBC	SI	IZ	MBC	SI
<i>S. aureus</i> ^a	18.5	5.0	0.22	18.0	2.5	0.52	18.6	0.6	6.17
<i>L. monocytogenes</i> ^a	10.2	40.0	0.03	11.0	40.0	0.03	11.7	40.0	0.09
<i>S. pyogene</i> ^a	24.2	80.0	0.01	13.8	80.0	0.02	13.9	10.0	0.37
<i>E. faecalis</i> ^a	13.9	10.0	0.11	11.2	2.5	0.52	12.8	2.5	1.48

(continuing)

TABLE I - Cytotoxicity and antibacterial activity of ethanol extract (CEE) from leaves of *P. spruceanum* and its fractions

Fibroblasts L929	CEE			Fractions from CEE					
				EAF			HMF		
	CC ₅₀ = 1.1 mg/mL			CC ₅₀ = 1.3 mg/mL			CC ₅₀ = 3.7 mg/mL		
<i>S. enteritidis</i> ^b	11.1	10.0	0.11	10.1	1.2	1.08	10.6	5.0	0.74
<i>S. sonnei</i> ^b	13.9	20.0	0.06	10.1	80.0	1.02	10.3	20.0	0.19
<i>E. coli</i> ^b	10.1	5.0	0.22	-	-	-	10.3	80.0	0.05
<i>P. aeruginosa</i> ^b	17.8	10.0	0.22	10.4	20.0	0.07	10.9	20.0	0.19
<i>K. pneumoniae</i> ^b	11.8	40.0	0.03	10.2	80.0	0.02	10.7	10.0	0.37

CC₅₀ = 50% cytotoxic concentration, SI = selectivity index, IZ = Inhibition zone diameter (mm), MBC = minimal bactericidal concentration (mg/mL), CEE = crude ethanol extract, EAF = ethyl acetate fraction, HMF = hydromethanolic fraction, a = Gram positive, b = Gram negative, - = not detected in the assay conditions. All experiments were conducted in triplicate, and the mean values are presented.

TABLE II - Compounds identified by means of UV and LC-DAD-ESI/MS analysis of sub-fraction F2 and F3 from hydromethanolic fraction of *P. spruceanum* leaves

Sub-fraction	Compound	Rt	UV	[M - H] ⁻¹ and main peaks (m/z)	Reference
F2	Procyanidin	1.93	279	577.27 (407.12; 289.21; 245.04; 205.14; 137.09; 124.90)	(Callemien, Collin, 2008)
	Catechin	2.04	279	289.15 (245.14; 176.99; 165.04; 137.04; 124.89; 109.08)	(Callemien, Collin, 2008)
F3	Quercetin-3-O-glucuronide	2.81	268; 352	477.24 (301.11; 273.12; 257.25; 178.63; 150.63)	(Fabre <i>et al.</i> , 2001; Martucci <i>et al.</i> , 2014)
	Quercitrin	3.10	264; 348	447.32 (301.76; 273.23; 257.12; 151.20)	(Fabre <i>et al.</i> , 2001; Tiberti <i>et al.</i> , 2007)
	Kaempferol-3-O-rhamnoside	3.41	263; 342	431.31 (285.04; 255.21; 238.87; 227.08; 212.77; 178.88; 163.18; 151.01)	(Fabre <i>et al.</i> , 2001; March, Miao, 2004)

Rt = retention time (min), UV = ultraviolet max absorption band (nm).

The fraction F2, where identified the tannin procyanidin and the flavan-3-ol catechin, presented antibacterial activity against *S. aureus* (MBC 0.6 mg/mL) and for fraction F3 was not observed any effect in the assay conditions. Antibacterial activity of catechin (Bais *et al.*, 2002) and procyanidin (Wang *et al.*, 2015) was already described.

Therefore, it was possible to consider that procyanidin (**1**) and catechin (**2**) (Figure 1) as the main constituents responsible for the antibacterial activity of *P. spruceanum*. For this reason, the chemical structure of these compounds was subjected to PASSonline analysis.

Through PASSonline it was possible to predict pharmacological effects, mechanisms of action and specific toxicity of the studied compounds by comparison with similar structure regions of biologically active substances found in its data bank, with 95% accuracy. Based on the results, *in vitro* and *in vivo* assays are selected, realized and the pharmacologic effect can be validated (Goel *et al.*, 2011).

For the *in silico* activity prediction linked to classical targets reached by the antibiotics currently

prescribed by physicians, compound **1** and **2** were considered as inadequate ($P_a < P_i$) or of low potential ($P_a - P_i < 0.2$). However, in relation to the considered new bacterial targets, these compounds were classified as having moderate [$P_a - P_i \geq 0.2$ and < 0.5] or high [$P_a - P_i \geq 0.5$] potential activity (Table III).

An important target for blocking the development of bacteria is your cell wall. The majority of compounds that act on the cellular wall inhibit the peptidoglycan synthesis involving transpeptidase and glycosyltransferase. Transpeptidase is a target of β -lactam antibiotics, but inhibitors of glycosyltransferase remains to be developed (Derouaux, Sauvage, Terrak, 2013). Other new target involved on the cellular wall integrity is the CDP-glycerol glycerophosphotransferase that catalyzes the teichoic acid synthesis, an essential cell wall component (Fitzgerald, Foster, 2000).

The *in silico* results showed no potential to action mechanisms involved with DNA damage and indicated the bacterial cell wall as a potential action target (Table III). The absence DNA effect was supported by the electrophoresis result (Figure 2A), that showed no changes in relation to the control (Soyingbe *et al.*, 2013).

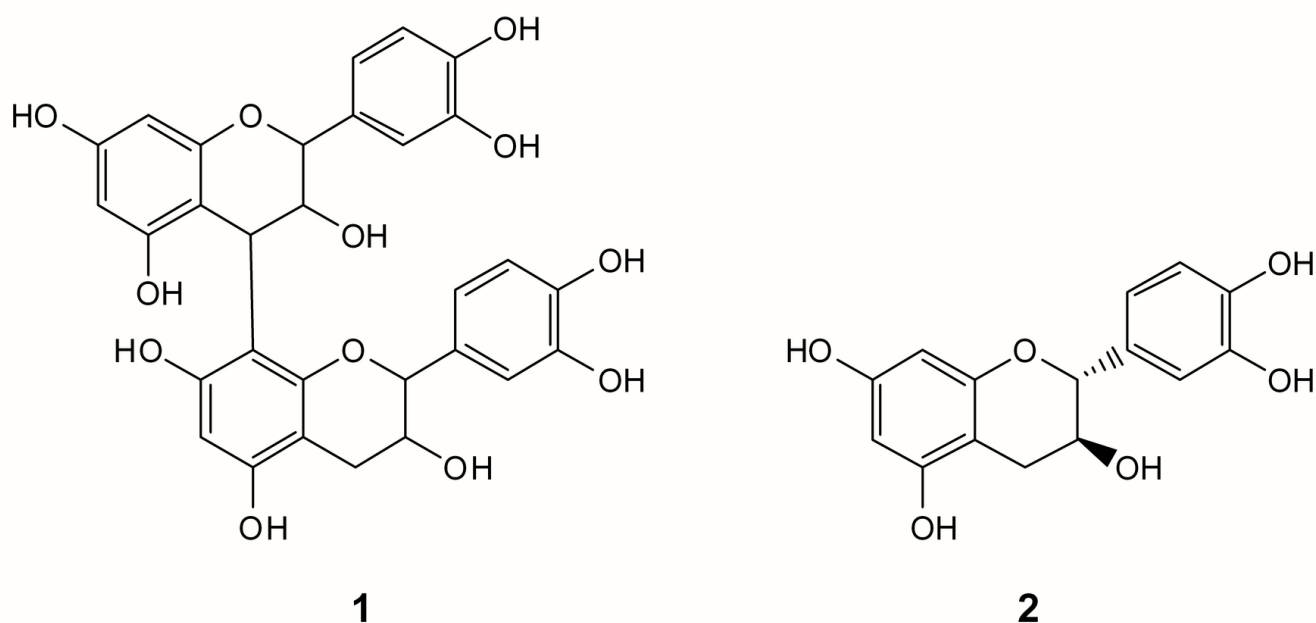


FIGURE 1 - Chemical structures of procyanidin (**1**) and catechin (**2**) identified in antibacterial fraction (F2) from leaves of *P. spruceanum* and subjected to *in silico* PASS online tool, to predict its action mechanisms.

TABLE III - *In silico* test prediction of potential bacterial targets of procyanidin and catechin using PASSonline tool

Potencial bacterial targets		Procyanidin*	Catechin*
Classical targets	DNA gyrase inhibitor	0.1	--
	DNA topoisomerase IV inhibitor	--	--
	Protein ribosomal subunit inhibitor	--	--
	DNA directed RNA polymerase inhibitor	0.1	--
New targets	Peptidoglycan glycosyltransferase inhibitor	--	0.3
	CDP-glycerol glycerophosphotransferase inhibitor	0.5	--

*Values of difference (Pa–Pi). -- = Unsatisfactory: Pa < Pi.

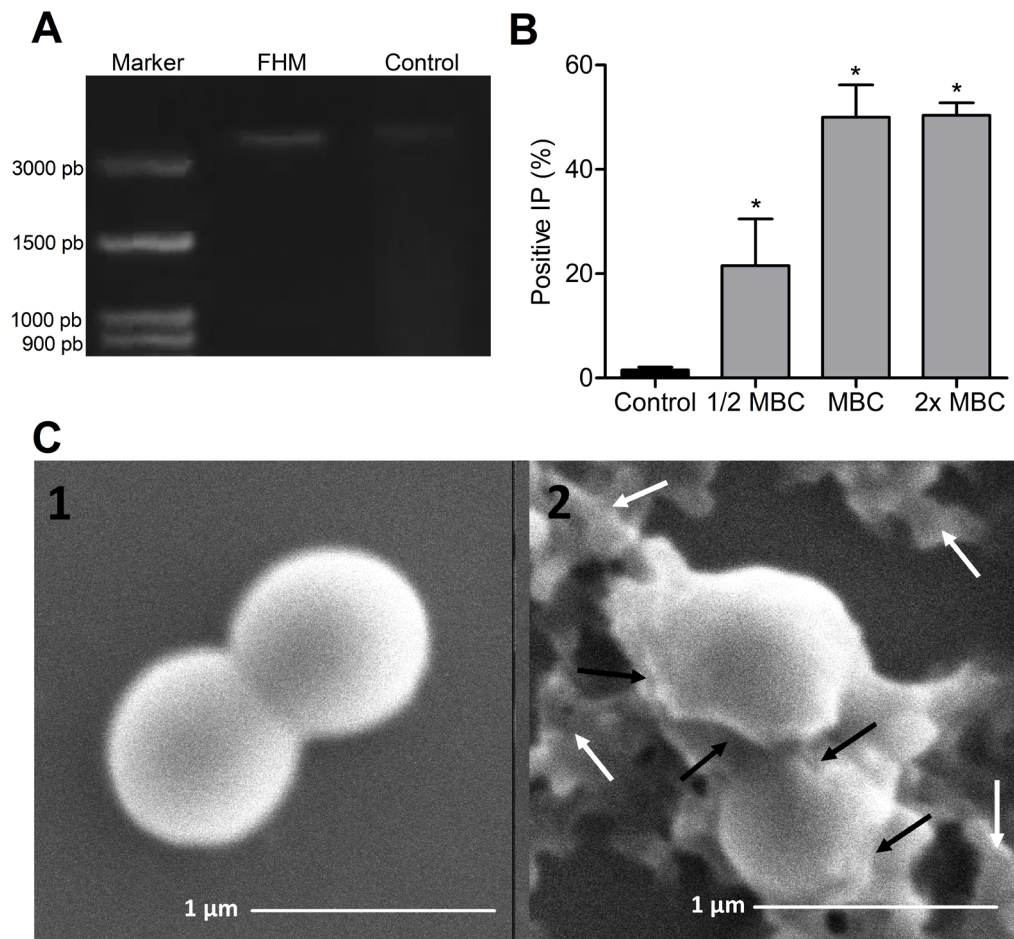


FIGURE 2 – Effects caused in *S. aureus* treated with hidromethanolic fraction (FHM) from *P. spruceanum* leaves. Analysis of bacterial DNA using 1.8% agarose gel electrophoresis (A), membrane permeability by propidium iodide, where * $p < 0.05$ versus control (B) and cell shape by scanning electronic microscopy (20000x, SS20), where white arrows indicate FHM precipitated and black arrows indicate cell surface deformations (C).

The loss of the bacterial cell wall integrity and the inhibition of peptidoglycan turnover enhance membrane permeability, since the outer membrane is cross-bridged to the peptidoglycan of the cell wall (Brudzynski, Sjaarda, 2014; Kim *et al.*, 2015; Hoerr *et al.*, 2016). To investigate bacterial cell membrane damage, propidium iodide uptake was measured. Propidium iodide only pass through damaged membranes, binds non-specifically to DNA and emits fluorescence. Thus greater ratio of propidium iodide uptake cells (% positive IP) indicates membrane damage. FHM treatment increased the fluorescent *S. aureus* cells (Figure 2B), showing that this fraction damage the cell membrane of this bacteria. Bacterial wall cell damage by FHM was also visually confirmed by SEM (Figure 2C). *S. aureus* treated with FHM (Figure 2C2) showed cell surface deformations in comparison to the control (Figure 2C1).

In conclusion, antibacterial property against nine pathogenic bacteria was observed for the extract CEE from leaves of *P. spruceanum* and its fractions EAF and HMF. Catechin and procyanidin were considered as antibacterial constituents of HMF. *In silico* analyses indicated the potential of these compounds to act in new antimicrobial targets and indicated no DNA effect and cell wall as mainly target. The *in silico* results were supported by *in vitro* tests that showed no DNA damage and showed cell wall damage, with increase in membrane permeability and changes in cell shape. Substances from *P. spruceanum* may contribute to treatment of infections caused by resistant bacteria. The results open perspectives to new studies using HMF or F2 as components of medicines against bacteria.

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