BOLETIN LATINOAMERICANO Y DEL CARIBE DE PLANTAS MEDICINALES Y AROMÁTICAS
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Articulo Original / Original Article

Phytochemical and biological study of phenolic components from *Geoffroea decorticans* stem bark

[Estudio fitoquímico y biológico de los componentes fenólicos de la corteza de *Geoffroea decorticans*]

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Section Biological activity

Received: 2 August 2021
Accepted: 2 November 2021
Accepted corrected: 5 January 2022
Published: 30 January 2023

Citation:
Pastoriza AC, Sgariglia MA, Soberón JR, Sampietro DA
Phytochemical and biological study of phenolic components from *Geoffroea decorticans* stem bark
Bol Latinoam Caribe Plant Med Aromat
22 (1): 115 - 129 (2023).
https://doi.org/10.37360/blacpma.23.22.1.9

Abstract: The stem bark of *Geoffroea decorticans* (Gill.ex Hook. etArn.) Burk. was used medicinally to cure several skin affections; however, phytochemical and biological antecedents were not found. Analyses of purified methanolic extract from *G. decorticans* bark (PFGB), realized by silylation derivatization for GC/MS, C18-CC and HPLC followed by two-dimensional TLC and UV-Vis spectroscopy, allowed to characterize nine phenolic compounds, among these, two methoxy flavonoids. Antibacterial assays of PFGB showed the highest activity (MICs = 125 µg/mL) against *Staphylococcus aureus* (25923) and *Enterococcus faecalis* (29212) ATCC strains. Moreover, PFGB showed the highest intracellular antioxidant activity at low concentration (5 µg/mL), evaluated by using the fluorescent DA-H2DCF probe on lymphocyte culture; cytotoxic effects on lymphocytes activated or not by LPS were not observed, through Trypan Blue Exclusion and MTT colorimetric assays. The results obtained from the ethnomedicinal approach of this work contribute to the scientific validation of the vulnerary medicinal use of *G. decorticans*.

Keywords: *Geoffroea decorticans* bark; Phenolic fraction; Antibacterial; Cytotoxicity; Phytochemical analysis

Resumen: La corteza de *Geoffroea decorticans* (Gill.ex Hook. EtArn.) Burk. se utiliza con fines medicinales para curar diferentes afecciones de la piel; sin embargo, no encontramos antecedentes fitoquímicos y biológicos que validen las propiedades medicinales atribuidas. Analizamos el extracto metanólico purificado de corteza de *G. decorticans* (PFGB), por CG-EM de la muestra derivatizada por sililación, C18-CC y HPLC seguido de CCF bidimensional, y espectroscopia UV-Vis; estos métodos nos permitieron caracterizar nueve compuestos fenólicos, entre estos, dos metoxi-flavonoides. Los ensayos antibacterianos de PFGB mostraron mayor actividad (CIMs = 125 µg/mL) contra las cepas ATCC de *Staphylococcus aureus* (25923) y *Enterococcus faecalis* (29212). Además, PFGB evidenció la mayor actividad antioxidante intracelular a baja concentración (5 µg/mL), evaluada en cultivo de linfocitos, mediante el uso de sonda fluorescente DA-H2DCF; no se observaron efectos citotóxicos sobre linfocitos activados o no por LPS, a través de ensayos colorimétricos con MTT y test de exclusión con azul Tripán. Los resultados obtenidos del abordaje etnomedicinal de este trabajo, contribuyen con la validación científica del uso medicinal vulnerario de *G. decorticans*.

Palabras clave: Corteza de *Geoffroea decorticans*; Fracción fenólica; Anitibacteriano; Citotoxicidad; Análisis fitoquímico.

INTRODUCTION

Since ancient times, man has used plants for medicinal purposes. Currently, traditional medicine plays an important role in health care, both in developed and developing countries (Bungau & Popa, 2015). It is estimated that 80% of the world's population depends on traditional medicine for their primary health care needs (Twarog & Kapoor, 2004).

Geoffroea decorticans (Gill. ex Hook. et Arn.) Burk. (Fabaceae), known as "chañar", is a tree that grows in several regions of Argentina (mainly in the north and center of the country), Bolivia, Southern Peru, Chile, Western Paraguay and Uruguay (Alonso & Desmarchelier, 2015). Traditionally, chañar is used with medicinal purposes as abortifacient, anti-asthmatic, emollient, against snake bites, to treat urinary tract and respiratory tract infections, and as vulnerary (treatment for several skin affections) (Barboza *et al*., 2009; Martinez *et al*., 2019). Most of the scientific works studied the fruit, due to its expectorant, antitussive and antimicrobial properties (Reynoso *et al*., 2012; Jiménez-Aspee *et al*., 2017). We found only two scientific works about chañar's stem bark (Vila *et al*., 1998; Jofré *et al*., 2017), but such antecedents, due to its unconnected approach, did not contribute to validate its traditional use.

Considering the vulnerary medicinal use of *G. decorticans* stem bark, widely extended in our region, as well as the phytochemical background of the plant barks, which were rich in phenolic compounds that presented wide interspecific chemical diversity, and moreover are recognized due to their multiple biological activities (Rice-Evans *et al*., 1997; Puupponen-Pimia *et al*., 2001), the research from traditional use prospect and type of secondary metabolites, could be substantial to know the more relevant biological activities associated with the chemical characterization of its main constituents; accordingly, this work researched for the first time the antibacterial and antioxidant activities, and cytotoxicity, of the purified phenolic fraction of *G. decorticans* stem bark (PFGB), as well as chemical identity of its components, to validate its traditional usage, and to know the medicinal potential of phenolic phytocomplex studied.

MATERIALS AND METHODS

Chemical and Reagents

Dimethyl sulfoxide (DMSO), n-hexano, methanol and acetone were purchased from Sintorgan Labs. (Buenos Aires, Argentina), N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), 3-[4.5-dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide (MTT), histopaque 1077, phytohemagglutinin (PHA), lipopolysaccharides (LPS), kaempferol, quercetin, apigenin, gallic acid and methoxyamine were from Sigma-Aldrich (Misuri, USA.); penicillin/streptomycin/amphotericin B (100X), phosphate buffered saline (PBS) and Hank's salt (HBSS) for cell culture, were from MicroVet Labs. (Buenos Aires, Argentina); pyridine and Trypan blue were from Cicarelli Labs. (Santa Fé, Argentina); 2´, 7′- dichlorodihydrofluorescein diacetate (DCFH-DA) was from Calbiochem (California, USA); Ciriax © i.v was from Roemmers Labs. (Buenos Aires, Argentina); Muller Hinton was from Britania Labs. (Buenos Aires, Argentina); fetal bovine serum (FBS) was from Natocor Biotechnology (Córdoba, Argentina).

Culture media used in cell-based assays: RPMI 1640 with L- Glutamine, HEPES and phenol red (serum-free RPMI 1640 medium) was from Biological Industries (Cromwell, USA).

Plant Material

Stem bark from *Geoffroea decorticans* (Gill. ex Hook. et Arn.) Burk. (Fabaceae), was collected in 9 de Julio Department, Chaco, Argentina (27°12'02.4"S 60°58'52.5"W). The species was taxonomically classified by the biologist Lic. Nora Muruaga from Instituto ''Miguel Lillo'', Tucumán, Argentina, and the herborized material was deposited in the Herbarium of the same Institute for future reference (voucher number: LIL 612255). Stem barks were cleaned, dried, and stored as powder in caramel colored, filled and tightly closed flask, at -20ºC.

Preparation of the phenolic fraction from G. decorticans stem bark

Stem bark grounded powder was washed with n-hexane, dried and extracted by Soxhlet with methanol 100% (16% p/v), for 24 h. The extract was dried under reduced pressure using rotary evaporator at 30°C. The dried material obtained represented the total extract. Phenolic constituents were extracted by gently mixing of total extract (1 g) in acetone (25 mL) at room temperature, and centrifuging the mixture at 2000 g for 30 min to separate the soluble fraction; this procedure was repeated three times, and supernatants collected were pooled and represented the purified phenolic fraction from *G. decorticans* stem bark (PFGB).

Phytochemical analysis of PFGB

Silylation derivatization and analysis conditions for GC/MS

PFGB (0.4 mg) was derivatized by the addition of 80 µL of 20 mg/mL methoxyamine dissolved in anhydrous pyridine. After incubation at 37°C for 90 min, 140 µL of N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were incorporated, and then incubated at 37°C for 30 min.

GC/MS analysis of the silylated sample was carried out in a ThermoElectron TraceGcUltra gas chromatograph coupled to a Polaris Q mass detector. The column used was a DB-5 column (30 m x 0.32 mm inner diameter, 0.25 µm; Agilent, California, USA). Helium was the carrier gas at a flow rate of 1 mL/min. Injector temperature was maintained at 280°C, and the injection volume was 1 µL in the splitless mode. The temperature program was as follows: isothermal for 2 min at 45°C, increased at 10°C/min to 150°C, isothermal for 1 min, increased at 10°C/min to 270°C, and isothermal for 1 min. The mass spectrometer was run in the electron ionization mode (70 eV). Mass spectra were acquired by scanning along the m/z 50 - 1000 range. Interpretation on MS spectra was conducted using the NIST MS search software (version 2.2). MS spectra of each detected component on analyzed sample (PFGB) were compared with spectra of the NIST library and Mainlib databases. The search was performed with a *Minimum match factor* of 75 for all components.

Two-dimensional TLC analysis

PFGB was analyzed by two-dimensional TLC on plastic plates coated with cellulose (Merck, Germany). The mobile phase in the first dimension was Forestal (acetic acid- conc. hydrochloric acid-water, 30:3:10, v/v/v); in the second dimension was 15% HOAc (v/v). The developed dried plates were visualized under visible and UV light (254 or 365 nm, UV Lamp Model UV 5L-58 Mineralight Lamp) before and after spraying with either 1% methanolic 2-aminoethyl diphenylborate/Polyethylene glycol (NP/PEG) reagents (Wagner *et al.*, 1996). Results were compared with those obtained by a mixture of assumed standards (Kaempferol, Quercetin, Apigenin and Gallic acid).

UV-VIS Spectroscopic analysis

For UV-VIS spectrophotometer analysis, PFGB was dissolved in methanol (0.5 mg/mL), loaded in 1 cm OPL quartz cuvette, and scanned in the wavelength ranging from 250-600 nm using Beckman DU 650 Spectrophotometer. The characteristic peaks were recorded and compared with characteristic λmax reported on specific literature.

HPLC profile of PFGB

Due to its complexity, PFGB (10 mg) was previously sub fractionated by C-18 cartridge using methanol: water (40:60, v/v) and 100% methanol as eluents. The sub fractions obtained (PFGB1 and PFGB2, respectively) were analyzed by RP- HPLC. The run was performed in analytical conditions on a Gilson HPLC (Villiers Le Bel, Val d′Oise, France) using an IB-SIL 5 C18 column (5 µm, 250×4.6 mm ID) from Phenomenex (Torrance, CA, USA), a UV detector from Gilson and a Rheodyne injector fitted with a 20 µL loop. Elution was carried out with a gradient of water: formic acid; 99.5:0.5; v/v (solvent A) and methanol: acetonitrile: formic acid; 59.75:39.75:0.5; v/v/v (solvent B). The gradient for PFGB1 was applied as follows: 0 - 35 min, 0 - 70% solvent B, 35 - 45 min, 70 - 100% solvent B, and for PFGB2 was as follows: 0 - 25 min, 40-100% solvent B, 25 - 40 min, 100% solvent B. The peaks were detected at 280 nm at a flow rate of 0.65 mL/min. The retention times (Rts) were registered. The samples were filtered through membrane filters (pore size 0.45 µm) prior to analysis. Standards solutions of quercetin, apigenin and kaempferol were developed in the same conditions.

Bioactivity assays of PFGB

Antibacterial assays

Antibacterial activity of the PFGB was assayed against *Staphylococcus aureus* (ATCC 29213 and ATCC 25923), *Escherichia coli* (ATCC 25922) and *Enterococcus faecalis* (ATCC 29212), pathogenic species that cause skin and soft tissue infections (Lowy, 1998; Rhoads *et al*., 2012). These strains were cultured on solid Mueller Hinton (MH) medium, from cultures kept at -20°C previously activated at 37°C for 2 h.

Dot-blot bioautography test: Briefly, 62.5 - 750 µg of PFGB were sown on Silica gel plates (4 x 7 cm), and ciprofloxacin was used as control. 4 mL of semi-solid MH (0.8% agar), molten at 50°C and inoculated with each strain (1 x 10^6 CFU/ mL) were distributed on pre-seeded and labeled plates; these were incubated at 37°C, and after 24 h were sprayed with MTT solution (2.5% p/v) and incubated again for 30 min to observe the inhibition halos around sown dots. Diameters of inhibition halos were

measured with caliber in three directions.

Broth microdilution method: This assay was applied to determine the minimal inhibitory concentrations (MIC). Briefly, PFGB dilutions were prepared according to Andrews *et al.* (2001), and were tested between 100 - 1000 µg/mL against *E. coli* and *E. faecalis*, and 50 - 1000 µg/mL against two strains of *S. aureus* (ATCC 29213 and ATCC 25923). Ciprofloxacin was used as positive control. Decreasing concentrations of sample or antibiotic and bacterial suspension ($2.5x10^5$ CFU/mL), in MH broth medium supplemented with $CaCl_2$ (25 mg/L) and $MgCl_2$ 12.5 (mg/L) (Patel, 2017), were added to sterile 96-well polystyrene flat bottom micro-plates and incubated at 37°C for 24 h. Both, bacterial growth and sterility controls were performed. Color controls were included to avoid interferences due to color of sample (Sgariglia *et al*., 2009). Absorbance at 630 nm was measured at the start and after 24 h of incubation. MIC was defined as the lowest concentration of sample or antibacterial substance that inhibits 99.9% bacterial growth compared with growth control on experimental conditions. Aliquots from micro-dilution assays, where bacterial growth was not detected, were sub-cultured on MH agar plates to determine if the sample had bactericidal action (MBC). MBC was defined as the lowest concentration of antibacterial substance able to kill most of the microorganisms with 99.9% effectiveness compared with growth control.

Cell-based assays

The tests were carried out with non-activated and lipopolysaccharides (LPS)-activated human lymphocytes; which were isolated from fresh whole blood from healthy volunteers in the age range of 25 - 35 years old (due to the fact that the file was pending approval by "*Comité de Ética en Investigación de la Facultad de Medicina de la Universidad Nacional de Tucumán*, Expte. 2125/412-D-2019, we have used blood from researchers from our laboratory) using Histopaque 1077. Briefly, anticoagulated blood (K_2EDTA) was diluted with an equal volume of RPMI 1640 medium supplemented with glutamine, containing 10% fetal bovine serum (FBS), underlying it with Histopaque 1077 (2:1 ratio, respectively) and centrifuging at 200 g for 30 min. Mononuclear cells were separated as a white layer at the top of the Histopaque (Noroozi *et al*., 1998). These cells were washed with Hank's Salt (HBSS) and centrifuged at 200 g for 5 min (two times). Cells were rinsed with PBS and diluted to 5 10^6 cells/mL, and cultured in complete RPMI 1640 medium (10% FBS, 100 IU/mL of penicillin, 100 ng/mL of streptomycin and 0.25 µg/mL of amphotericin B) during 24 h. No adherent peripheral blood lymphocytes (PBL) were separated from adherent cells by aspiration, and the number of viable cells was determined by Trypan blue exclusion test, on a hematological counter (Neubauer Chamber).

Harvested lymphocytes were cultured in complete RPMI 1640 medium supplemented with Concanavalin A (4 µg/mL) as mitogen agent, on 5% CO_2 atmosphere at 37°C, during 24 h.

Measurement of oxidative stress: cell-based assay using DCFH oxidation

Intracellular antioxidant activity of PFGB was evaluated by using the 2, 7′-dichlorodihydrofluorescein diacetate (DA-H_2DCF) probe (Dikalov & Harrison, 2014). Lymphocytes were treated with 5 µM of DA-H_2DCF in serum-free RPMI 1640 medium for 30 min at 37°C. Then, cells were washed with HBBS by centrifugation 200 g for 5 min (two times) and resuspended in RPMI 1640 medium with LPS (2% FBS, 2 µg/mL LPS) at 10^5 cells/mL. Suspension of treated cells (10^4 cells/well) were exposed to PFGB concentrations (5 - 250 µg/mL) on black 96-well plates, and incubated at 37°C for 1 h. Next, oxidative stress was induced with 100 µM of hydrogen peroxide (LeBel *et al*., 1992). The fluorescence intensity at 495 nm excitation and 530 nm emission was measured by spectrofluorometer (Biotek FLx800, Vermont, USA), every 10 min for 90 min. Ellagic acid was used as positive control.

Analysis of cytotoxicity

Trypan Blue Exclusion Test (TBET)

This assay was used to determine the number of viable cells according to its membrane integrity (Strober, 2001), in a cell suspension, exposed to sample or their solvent. PFGB concentrations to be tested (10 - 100 µg/mL) and aliquots of cell suspension were loaded in 24-well plate ($1x10^5$ cells/mL; final volume: 1mL/well). The plate was incubated for 24 h at 37°C and 5% CO_2. After incubation, the contents of each well were transferred to 2 mL conical tubes and centrifuged at 100 g, 5 minutes, supernatants were removed and cells were resuspended in 300 µL serum-free RPMI 1640 medium. For cell count, 1 part of 0.4% Trypan blue and 1 part of cell suspension were mixed and loaded in a Neubauer chamber and immediately counted

using optical microscope (20X).

MTT colorimetric assay: after 24 h incubation and centrifugation, as explained above (TBET), supernatants, corresponding to PFGB treated cell cultures and controls, were replaced by a solution of MTT (0.25 mg/mL) in serum-free RPMI 1640 medium. After 3 h of incubation at 37°C and 5% CO_2, these solutions were centrifuged at 900 g, 10 min; the supernatant was removed, and cells were washed with HBSS. At the final, the washed supernatants were replaced by DMSO to dissolve formazan. Absorbance was measured at 590 nm in ELISA reader (Bio-rad. California, USA). Complete RPMI 1640 medium was used as blank solution.

Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using Student's t test; $p<0.05$ was considered statistically significant.

RESULTS

Phytochemical analysis of PFGB

GC/MS analysis

Several peaks with distinct mass fragmentation patterns were detected by analysis of the total ion chromatogram (TIC) of silylated PFGB (Figure No. 1). Comparison of spectral data acquired with MS Libraries (NIST and Mainlib) led to the identification of 5 phenolic compounds: Taxifolin (**1**); 7,3',4',5'-Tetramethoxyflavanone (**2**); 2'-Hydroxy-3,4,4',5-tetramethoxychalcone (**3**); Protocatechuic acid, 3-tert-butyl(dimethyl)silyl (TBDMS) derivative (**4**) and 4-Hydroxybenzoic acid, 2-trimethylsilyl (TMS) derivative (**5**), whose structures are shown in Figure No. 2 and fragmentation patterns are listed in Table No. 1.

Table No. 1
Compounds identified from silylated phenolic fraction of *G. decorticans* stem bark (PFGB) by GC/MS analysis

Peak	Rt, min	Compound	Molecular formula	MW	Reference ions[a] (%BPI)
1	09.61	Taxifolin	$C_{15}H_{12}O_7$	304	231 (100), 149 (66), 241(6)
2	13.26	7,3',4',5'-Tetramethoxyflavanone	$C_{19}H_{20}O_6$	344	149 (100), 133 (31), 148 (20), 162 (18), 121 (12), 159 (8)
3	15.95	2'-Hydroxy-3,4,4',5-tetramethoxychalcone	$C_{19}H_{20}O_6$	344	149 (100), 163 (48), 150 (16), 121 (14), 133 (14), 159 (8)
4	26.23	Protocatechuic acid, 3 TBDMS derivative	$C_{25}H_{48}O_4Si_3$	496	439 (100), 73 (67), 440 (41), 441 (19), 193 (18), 223 (8)
5	26.24	4-Hydroxybenzoic acid, 2TMS derivative	$C_{12}H_{22}O_3Si_2$	282	73 (100), 267 (71), 193 (63), 223 (45), 45 (18), 268 (16), 282, M+ (14), 75 (13), 126 (12), 194 (10)

References: Rt, retention time; MW, molecular weight; [a]Mass spectrometry libraries (NIST, 2014 and Mainlib); M+, molecular ion; BPI, base peak intensity

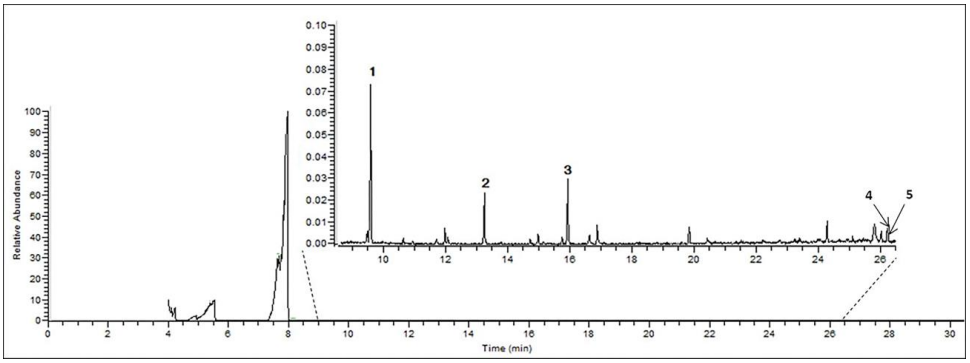

Figure No. 1
Total ion chromatogram (TIC) of silylated phenolic fraction of *G. decorticans* stem bark (PFGB). Numbers refer to compounds in Table No. 1

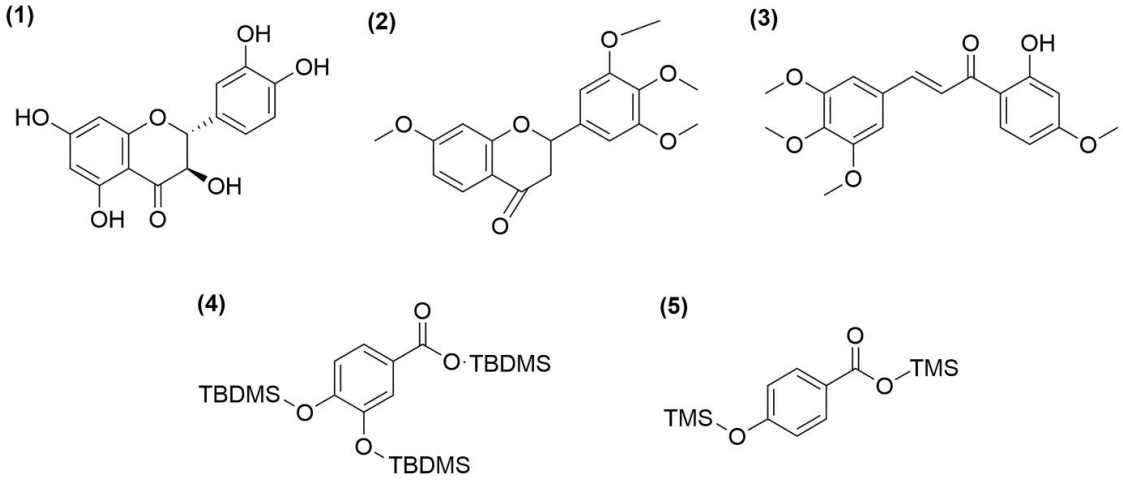

Figure No. 2
Structures of phenolic compounds identified from silylated phenolic fraction of *G. decorticans* stem bark (PFGB), by GC/MS analysis. Taxifolin (1), 7,3',4',5'-Tetramethoxyflavanone (2), 2'-Hydroxy-3,4,4',5-tetramethoxychalcone (3), Protocatechuic acid, 3 TBDMS derivative (4) and 4-Hydroxybenzoic acid, 2TMS derivative (5). Numbers refer to compounds in Table No. 1

TLC and UV-VIS analysis

The two-dimensional chromatogram obtained on cellulose for PFGB is shown in Figure No. 3. The sample showed spots that matched in R_f and color with gallic acid, quercetin, kaempferol and apigenin standards, when were observed under 365 nm UV lamp, after sprayed with NP/PEG reagent. Protocatechuic acid was recognized by comparison of its R_f and color with data available in literature (Tanchev & Ioncheva, 1976; Kowalski & Wolski, 2003). Furthemore, the brown spots (R_f: 84 with Forestal system and 0 with HOAc 15%), according to Harborne (1984), were consistent with the presence of methoxy-flavonoids, which were detected by GC/MS (Peaks 2 and 3, Table No. 1).

Taxifolin and 4-Hydroxybenzoic acid were detected by GC/MS, but not visualized in two-dimensional TLC or UV spectrum (Table No. 2), this could be due to their low concentration in PFGB. However, the derivatization and high sensitivity of the GC/MS technique could have allowed its detection by this method.

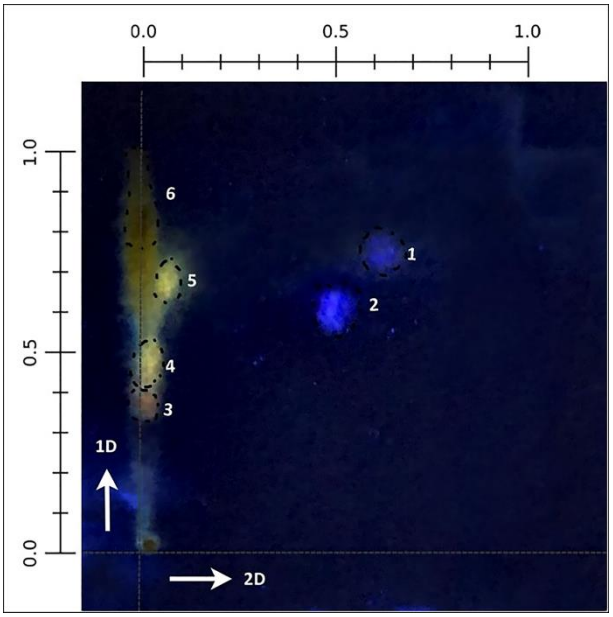

Figure No. 3
Two-dimensional thin-layer chromatogram on cellulose of the phenolic fraction of *G. decorticans* stem bark (PFGB). The mobile phase in the first dimension (1D) was Forestal (acetic acid- conc. HCl- water; 30:3:10; v/v/v), that in the second dimension (2D) was 15% HOAc (15% aqueous acetic acid, v/v). Detection was by observation under 365 nm UV lamp, after sprayed with NP/PEG reagent. Phenolic compounds: Protocatechuic acid (1), Gallic acid (2), Quercetin (3), Kaempferol (4), Apigenin (5), Chalcones and methoxylated flavanones (6)

Table No. 2
Spectral and R_f data of phenolic compounds present in PFGB

	Compound	UV λ_{max} MeOH, nm	TLC, R_f (x100) in		Colour	
			Forestal	15 % HOAc	UV (365 nm)	UV + NP/PEG
1	Protocatechuic acid[a,b]	256, 294	79	60	Dark blue	Blue
2	Gallic acid[c]	270	60	50	Dark	Blue
3	Quercetin[c,d]	255, 374	38	0	Yellow	Orange
4	Kaempferol[c,d]	266, 368	49	0	Yellow	Yellow
5	Apigenin[c,d]	267, 336	70	6	Dark Brown	Yellow
6	2'-Hydroxy-3,4,4',5-tetramethoxychalcone[c,d]	317	84	0	Dark Brown	Brown
7	7,3',4',5'-Tetramethoxyflavanone[c,d]	270-295	84	0	-	-
8	Taxifolin[d]	290	-	-	-	-
9	4-Hydroxybenzoic acid	272	-	-	-	-

References: (PFGB) phenolic fraction of *G. decorticans* stem bark. The obtained data were compared with those of the bibliography: [a]Tanchev & Loncheva, 1986; [b]Kowalski & Wolski, 2003; [c]Harborne (1984); [d]Mabry *et al*., (1970). (-): Reference data not found in scientific literature

HPLC profile of PFGB

The main peaks and their Rts at 280 nm, for PFGB1 and PFGB2, are shown in Figures No. 4 and Figure No. 5, respectively. PFGB2 showed peaks that matched in Rt with quercetin, kaempferol and apigenin standards (Figure No. 5 at 14.8, 17.35 and 17.60 min, respectively), corroborating in this way, the data obtained with two-dimensional TLC and UV spectrum about the presence of these compounds in PFGB. The retention times of the peaks of both samples are summarized in Table No. 3.

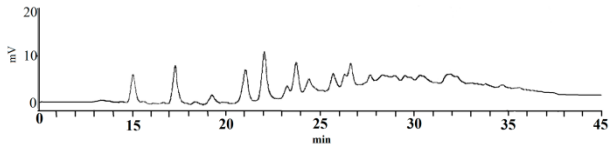

Figure No. 4
HPLC profile of the phenolic fraction of *G. decorticans* stem bark, sub fraction 1 (PFGB1). Elution was carried out with a gradient of water: formic acid; 99.5:0.5; v/v (solvent A) and methanol: acetonitrile: formic acid; 59.75:39.75:0.5; v/v/v (solvent B). The gradient was applied as follows: 0- 35 min, 0 - 70% solvent B, 35 - 45 min, 70 - 100% solvent B. The peaks were detected at 280 nm at a flow rate of 0.65 mL/min

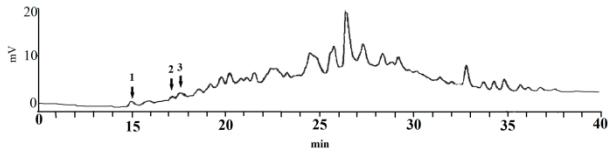

Figure No 5
HPLC profile of the phenolic fraction of *G. decorticans* stem bark, sub fraction 2 (PFGB2). Elution was carried out with a gradient of water: formic acid; 99.5:0.5; v/v (solvent A) and methanol: acetonitrile: formic acid; 59.75:39.75:0.5; v/v/v (solvent B). The gradient was applied as follows: 0 - 25 min, 40 - 100% solvent B, 25 - 40 min, 100% solvent B. The peaks were detected at 280 nm at a flow rate of 0.65 mL/min.
Phenolic compounds: Quercetin (1), Kaempferol (2), Apigenin (3)

Table No. 3
Retention times of PFGB1 and PFGB2 peaks in HPLC analysis

PFGB1		PFGB2	
Peak	**Rt**	**Peak**	**Rt**
1	15	1	14.8
2	17.45	2	15.45
3	18.52	3	17.35
4	19.31	4	17.6
5	20.97	5	18.9
6	21.91	6	19.7
7	23.51	7	20.33
8	24.16	8	20.75
9	25.38	9	21.63
10	26.27	10	22.01
11	27.95	11	23.10
12	28.56	12	24.87
13	29.10	13	26.06
14	29.89	14	26.70
15	31.48	15	27.58
16	34.43	16	28.55
		17	29.02
		18	29.34
		19	31.47
		20	32.83
		21	33.73
		22	34.27
		23	34.83
		24	35.71
		25	36.16
		26	36.83

References: (PFGB1 and PFGB2) phenolic fraction of *G. decorticans* stem bark, subfraction 1 and 2, respectively; (Rt) Retention time

Antibacterial activity of PFGB

PFGB exhibited inhibition halos against all assayed species by dot-blot bioautography test (Table No. 4), indicating the presence of antibacterial compounds. MIC values of Ciprofloxacin on *S. aureus* (ATCC 29213) and *E. coli* (ATCC 25922) were agreed with those reported by CLSI (Patel, 2017) for these strains, validating the experimental procedures carried out. The highest antibacterial activity was observed against *S. aureus* ATCC 25923 and *E. faecalis* ATCC 29212 (MIC: 125 μg/mL); less activity was observed against *S. aureus* ATCC 29213 (MIC: 250 μg/mL) (Table No. 4). Although, *E. coli* (ATCC 25922) was sensitive to PFGB by dot-blot bioautography test, it did not reach the MIC at ≤1000 μg/mL concentrations. Bactericidal effects were not observed against the strains assayed.

Table No. 4
Antibacterial activities of the phenolic fraction of *G. decorticans* stem bark (PFGB)

Microorganism	Dot-blot bioautography[a] PFGB, µg; IH, mm						Broth Microdilution[b]
	62.5	93.75	125	250	500	750	MIC, µg/mL
S. aureus (ATCC 29213)	6.0±0.7	6.5±0.5	8.5±0.4	11.0±0.8	/	/	250
S. aureus (ATCC 25923)	/	7.5±0.3	9.2±0.2	11.7±0.3	13.0±0.1	14.5±0.2	125
E. faecalis (ATCC 29212)	-	-	6.0±0.2	6.2 ±0.2	8.5±0.3	9.5±0.2	125
E. coli (ATCC 25922)	-	-	-	-	8.5±0.2	9.0±0.1	-

References: (/): untested concentrations; (-): no activity was observed at the concentrations assayed; (IH): inhibition halo. Inocula [a]: $1x10^6$ CFU/mL; [b]: $2.5x10^5$ CFU/mL

Cell-based assays

Intracellular antioxidant activity of PFGB

PFGB showed intracellular antioxidant activity in concentration-dependent mode (Figure No. 6), being more efficient at 5 µg/mL, where reached 0.92% intracellular fluorescence after 60 min (≥ 99.05% inhibition of DCFH oxidation). Antioxidant effects less efficient were observed at higher concentrations of PFGB, (Figure No. 6 at 60 min: 40.8% and 60.4% of fluorescence for 50 and 250 µg/mL, respectively).

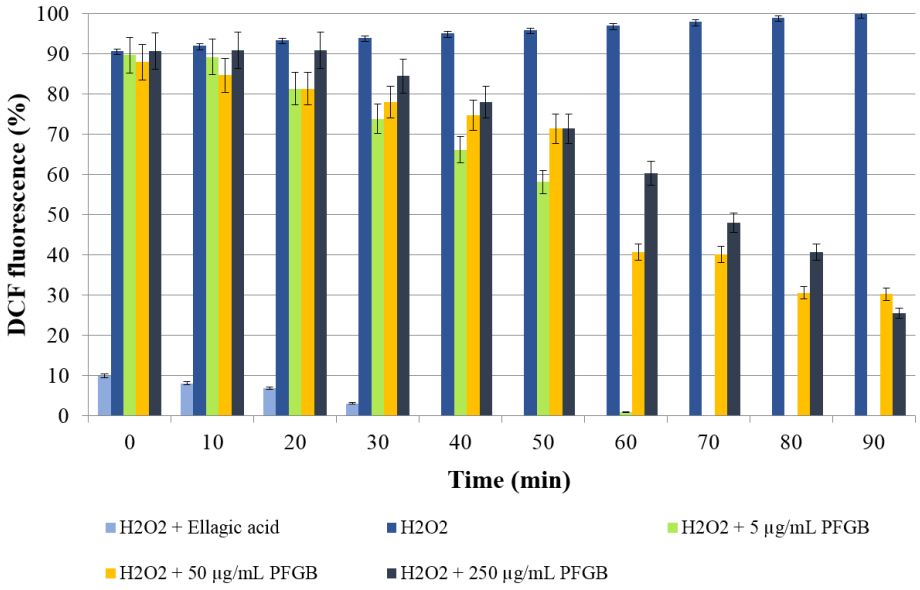

Figure No. 6
Intracellular antioxidant activity of phenolic fraction of *G. decorticans* stem bark (PFGB), was evaluated by using the DCFH-DA probe. LPS- activated lymphocytes, previously treated with DCFH-DA and incubated with different concentrations of PFGB (5 - 250 µg/mL), were induced oxidative stress with 100 µM H_2O_2. The fluorescence intensity was measured by fluorescence spectrophotometer at 495 nm excitation and 530 nm emission ($p<0.05$)

Analysis of cytotoxicity

Cytotoxic effects were not observed for PFGB up to 100 µg/mL, since evaluating membrane integrity and metabolic activity, the cell viability obtained was greater than 75%, limit tolerated for human lymphocytes (Figure No. 7). Significant differences between non-activated and LPS-activated cells were not observed under the assayed conditions.

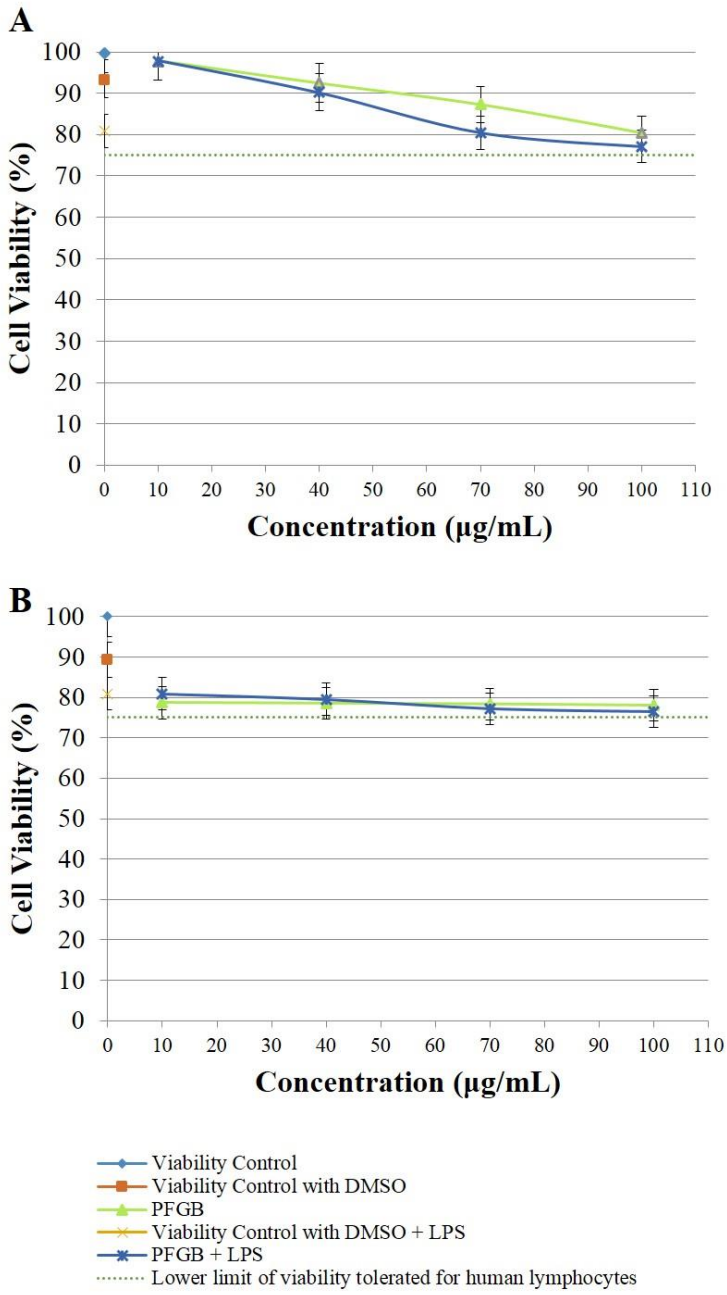

Figure No. 7

A) Cytotoxicity of phenolic fraction of *G. decorticans* stem bark (PFGB) determined by Trypan blue exclusion test on non-activated and LPS-activated human lymphocytes. Cells were treated with PFGB at different concentrations (10–100 µg/mL) for 24 h. The results were expressed as % viability ($p < 0.05$). B) Cytotoxicity of phenolic fraction of *G. decorticans* stem bark (PFGB) determined by MTT colorimetric assay on non-activated and LPS-activated human lymphocytes. Cells were treated with PFGB at different concentrations (10–100 µg/mL) for 24 h. The results were expressed as % viability ($p < 0.05$)

DISCUSSION

Although the perspective of traditional use considers both the use of the vegetable part, to which the medicinal properties were attributed, as well as the way of consumption (usually infusion or tincture); in this work, when considering the perspective of the type of secondary metabolite to be studied (phenolics), the extraction methods focused on obtaining this type of compound. Vila *et al*. (1998) carried out the exploratory phytochemical analysis of the *G. decorticans* stem bark, from which reported the isolation and identification of three prenylisoflavanones. These compounds were not detected in the fraction analyzed in our work, probably due to a lower solubility in methanol than ethanol, and/or a deficient silylation of this type of flavonoid.

This study demonstrated that PFGB is capable of inhibiting the growth of strains involved in skin infections; previous studies demonstrated that certain phenolic compounds such as apigenin, quercetin, gallic acid and protocatechuic acid, isolated from others species, showed antibacterial activity against *S. aureus* ATCC 25923 and 29213, and *E. faecalis* ATCC 29212 (Özçelik *et al*., 2011; Alves *et al*., 2013; Wang *et al*., 2019); these data justified partially the activity found for PFGB. Apigenin and kaempferol, flavonoids present in PFGB, have already been reported to have moderate antibacterial activity against *E. coli* ATCC 25922 (Adamczak *et al*., 2019), however these would contribute in a lesser proportion to the antibacterial activity of PFGB, since the MIC was not reached at the concentrations tested on said strain. This was consistent with the low proportion observed for such flavonoids in PFGB2 analyzed by RP-HPLC (Figure No. 5).

We also found that PFGB possesses intracellular antioxidant activity, being highest at the lowest concentration tested (5 µg/mL). Matsuo *et al*. (2005) showed that the entry of flavonoids such as apigenin and quercetin, into the cell, depends on their concentration, being more effective at lower concentrations; furthermore, apigenin and taxifolin become pro-oxidants at high concentrations. These observations agree with the results obtained in our experiments.

Cytotoxic effects were not observed for PFGB up to 100 µg/mL; apigenin, kaempferol, quercetin and taxifolin showed cytotoxic activity on tumor and normal cell lines, when were individually evaluated (Mori *et al*., 1988; Matsuo *et al*., 2005; Li *et al*., 2008); therefore, our results suggested that these bioactive compounds could be safer for it application into PFGB phytocomplex.

Information available about structure–activity relationships and mechanisms of antibacterial action of flavonoid compounds indicated that hydroxylation at position 5 and 7 of the A ring of kaempferol, quercetin and taxifolin are important on this activity; in addition, hydroxylation on the B and C rings increased the antimicrobial activity of these compounds (Woźnicka *et al*., 2013); taking into account that apigenin possess the structural characteristics detailed, could be the main responsible for the antibacterial activity observed, without avoiding the contribution of other constituents (Hung *et al*., 2008; Xiao *et al*., 2011; Novak *et al*., 2012; Wu *et al*., 2013).

Glevitzky *et al*. (2019), established the relationship between the molecular structure of a series of structurally related flavonoids and their antioxidant activity, using different methods of statistical analysis. They found that the most effective radical scavengers were flavonoids with the 3', 4'-dihydroxy substitution pattern on the B-ring and/or hydroxyl group at the C-3 position (as taxifolin and quercetin); and that flavonoids that lack catechol -OH groups on B ring but possess a 3-OH next to the 4-keto group, possess a high scavenging activity (as kaempferol). However, it would be interesting to determine the contribution of methoxyflavonoids, since due to their lipophilic properties, they could be more efficient crossing biological membranes and exerting their effects at the intracellular level. Likewise, the antioxidant properties of phenolic acids are related to the phenolic hydroxyl groups attached to the ring structures (Heleno *et al*., 2015).

The different classes of phenolic compounds, found in PFGB, showed diverse pharmacological properties beneficial for the treatment and prevention of various pathological conditions (Table No. 5). Among these properties we can highlight the important anti-inflammatory activity of flavones, flavonols and methoxylated flavonoids, which would contribute to the vulnerary activity described. Moreover, this graphic allowed to observe others possible applications, that would have PFGB, and *G. decorticans* stem bark, based on analysis of their phytochemical composition reported in this work.

Table No. 5
Pharmacological properties of phenolic compounds

classes/subclasses of phenolic compounds	Pharmacological properties										Reference
	AIn	AO	AI	E	AD	AC	IPDD	HP	CP	AA	
Chalcones											Manner *et al*., 2013; Kumar & Pandey, 2013; Bose *et al*., 2018
Flavanone											Kumar & Pandey, 2013; Panche *et al*., 2016
Flavone											Heijnen *et al*., 2001; Manner *et al*., 2013; Panche *et al*., 2016; Wang *et al*., 2017
Flavanonol											Sunil & Xu, 2019
Flavonol											Amic *et al*., 2007; Fang *et al*., 2016; Wang *et al*., 2017
Methoxylated flavonoids											Wen *et al*., 2017; Bose *et al*., 2018
Phenolic acids											Kakkar & Bais, 2014; Heleno *et al*., 2015; Badhani *et al*., 2015

References: AIn: anti-infective (antiviral, antibacterial, antifungal); AO: antioxidant; AI: anti-inflammatory/anti-immune; E: estrogenic; AD: antidiabetic; AC: anticancer; IPDD: improvement and prevention of degenerative diseases; HP: hepatoprotective; CP: cardioprotective; AA: antiallergic.

Heat map:

7x	6x	5x	4x	3x	2x	1x	0x

Jofre *et al*. (2017), evidenced the anti-inflammatory activity of polar extracts of *G. decorticans* stem bark; considering that the vulnerary property can include this type of effects, and also taking into account the analysis of pharmacological activities reported for the compounds detected in PFGB, it would be important to investigate the anti-inflammatory activity of PFGB oriented to cutaneous processes, and to delve into the phytochemical composition that explains the possible effects.

CONCLUSIONS

This work report, for the first time, the phytochemical characterization of phenolic fraction from *G. decorticans* stem bark, related to their antibacterial and antioxidant activities, and cytotoxicity; some of the compounds we reported, exhibit a wide range of biological activities described in the literature, among them antioxidant and antibacterial, which were consistent with the activities found in PFGB, where were detected, contributing to the scientific validation of the traditional use of this species as vulnerary, and showing the potential applications of the phenolic phytocomplex obtained from this. Purification procedures and phytochemical analyzes are being carried out to deepen the structural identification of the components detected in PFGB, especially methoxy-flavonoids, which are interesting for the species under study. At the same time, we are conducting anti-inflammatory activity studies and antimicrobial activity against other microorganisms, to delve about their vulnerary property.

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