Article

CHEMICAL CONSTITUENTS, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF *Pavonia glazioviana* Gurke (Malvaceae)

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Species from *Pavonia* genus (Malvaceae) are traditionally used as antioxidant, n-anti-inflammatory and antimicrobial remedies. Previous studies have revealed their diversity of specialized metabolites widely used in the development of new medicines. This is the first study on *Pavonia glazioviana* Gurke, a Brazilian species known as "Malva-da-Chapada". The research led to identify fifteen compounds: 13^2-(*S*)-hydroxy-pheophytin a; and 13^2(*S*)-hydroxy-17^3-ethoxyphaeophorbide, *n*-decanol, cycloart-23*Z*-ene-3β,25-diol, cycloart-24*S*-25-ene-3β,24-diol, sitosterol-3-*O*-β-D-glucopyranoside, stigmasterol-3-*O*-β-D-glucopyr anoside, quercetin, acacetin, kaempferol, tiliroside, 5-hydroxy-3,7,8,4'-tetramethoxyflavone, 5,7-dihydroxy-3,8,4'-trimethoxyflavone, 5,7-dihydroxy-4'-methoxyflavone, 5,7,4'-trihydroxy-3,8-dimethoxyflavone and 5,7,4'-trihydroxy-3-methoxyflavone. The major flavonoids were tested for their antimicrobial activity, and the compound 5,7-dihydroxy-3,8,4'-trimethoxyflavone showed strong activity against *Escherichia coli* (MIC = 512 μg mL^{-1}), *Pseudomonas aeruginosa* (MIC = 512 μg mL^{-1}), *Candida albicans* (MIC = 512 μg mL^{-1}), *Candida tropicalis* (MIC = 512 μg mL^{-1}), *Candida parapsilosis* (MIC = 512 μg mL^{-1}), *Aspergillus flavus* (MIC = 512 μg mL^{-1}) and *A. fumigantus* (MIC = 512 μg mL^{-1}). The total phenolic content, flavonoid content, and radical scavenging potential were determined showing its great production of phenolics and flavonoids, along with interesting radical scavenging potential (DPPH EC_{50} = 6.36 ± 0.029 mg mL^{-1}). Our findings contributed to characterize *P. glazioviana* as a source of methoxylated flavonoids with antimicrobial potential.

Keywords: *Pavonia glazioviana*; methoxylated flavonoids; antioxidant potential; antimicrobial activity.

INTRODUCTION

The *Pavonia* genus is one of the largest genera of the Malvaceae family, with around 270 species, distributed throughout the world.[1] Previous studies with *Pavonia* species have reported several pharmacological activities such as antioxidant, anti-inflammatory, cytotoxic, hypotensive, anthelmintic, antibacterial, and antifungal effects.[2-6]

Despite the potential of *Pavonia* species, this is the first study aiming to assess the specialized metabolism and biological activities of *Pavonia glazioviana* Gurke. This plant is popularly known as "Malva-da-Chapada" and occurs in the Northeast region of Brazil, at Caatinga biome. Local population use it as cattle feed and as anti-inflammatory agent.[7,8]

The wide medicinal potential of *Pavonia* species is related to its diverse specialized metabolites that are originated by specific enzymatic reactions from primary metabolites, granting environmental adaptive advantages for the plant.[9] Those metabolites have been widely used in the development of new medicines. Phytochemical studies on other *Pavonia* species have reported the production of fatty acids, steroids, terpenoids and a variety of phenolic compounds, such as flavonoids.[10-13]

Phenolic compounds are extensively described in Malvaceae family.[14,15] They play relevant role in plant physiology being related to the modulation of plant growth and reproduction.[16] Phenolics have also demonstrated several relevant pharmacological and biological properties such as antioxidant, leishmanicidal, antimicrobial and anticancer.[14,16-18] Their antioxidant potential has been widely described. They are able to act as radical scavengers in the initiation and propagation of the oxidative process, which is one of the most important events in pathogenesis of diabetes, atherosclerosis, cancer and Alzheimer.[18,19]

Specialized metabolites with antimicrobial activity are produced by the vegetable as phytoalexins for their own protection, and those are also used as prototypes for human antimicrobial drugs. There are relevant indications in the literature regarding the stress activation of vegetable *O*-methyltransferases, the enzymes responsible by transfer methyl groups to hydroxylated substrates, increasing the production of methoxylated flavonoids with antimicrobial activity.[20,21]

Considering the great potential of *Pavonia* genus, this work presents the phytochemical study of *P. glazioviana* and the antimicrobial activity of its major isolated compounds.

EXPERIMENTAL

General procedures

For the isolation of chemical constituents of *P. glazioviana* it has been used chromatographic glass columns chosen according to the amount of sample to be applied, for separation of larger quantities

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of materials (1.0 g), columns of approximately 50 × 2.5 cm diameter were used, collecting fractions of 20 mL each, while for purification of smaller quantities (< 1.0 g) columns of 30 × 1.0 cm diameter were used and fractions of 5 mL collected. The silica gel or Sephadex LH-20 were used as stationary phase, using 30 times the sample mass as chromatographic support. Thin-layer chromatography (TLC) was performed on Merck silica gel plates and the spots were revealed with diphenyl-boryloxyethamine, sulfuric anisaldehyde and under UV light (254 and 366 nm).

Plant material

The aerial parts of *P. glazioviana* were collected in March 2014 in Serra Branca/Raso da Catarina (Jeremoabo, Bahia, Brazil: 09°53'15.5"; 09°44'34.6"S and 38°49'36,1"; 38°52'20.4"W), and identified by Dr. Adilva de Souza Conceição. A voucher specimen (28709) was deposited in the Herbarium of Universidade do Estado da Bahia (Paulo Afonso Collection). This research has been registered at National System of Genetic Resource Management and Associated Traditional Knowledge (SisGen) under the code ADC0E00.

Extraction

The aerial parts of *P. glazioviana* were dehydrated at 40 °C for 72 h and grounded, yielding 5,400 g of powder. The powder was macerated with 95% ethanol (EtOH) for 72 h. The extraction was repeated to improve the extraction yield. The extractive solution was filtered and concentrated under reduced pressure, resulting in the crude ethanolic extract (CEE) (300.0 g).

Phytochemical screening

An aliquot of the CEE was submitted to a qualitative phytochemical screening to detect the main groups of metabolites in the sample (alkaloids, phenolics, flavonoids, quinones, coumarins, tannins, steroids, triterpenes and saponins).[22]

Fractionation and isolation of the constituents of P. glazioviana

The CEE was submitted to a liquid-liquid extraction using *n*-hexane (Hex), chloroform ($CHCl_3$), ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH), yielding in 88.72 g of the hexane phase (HP), 45.8 g of the $CHCl_3$ phase (CLP), 5.0 g of the EtOAc phase (ACP), 17.0 g of the *n*-butanol phase (BP), and 100.0 g of the hydroalcoholic phase (HAP).

A sample of HP (5.0 g) was chromatographed in silica gel column using Hex, EtOAc and methanol (MeOH), alone or in binary mixtures in ascending order of polarity, resulting in 50 fractions, combined by similarity on TLC. Sub-fractions 30-36 (170.0 mg) were chromatographed using the same methodology to obtain 61 fractions that were analyzed by TLC. The combined fractions 16-27 provided the mixture of the compounds **1** and **2** (10.0 mg).

Another aliquot of the HP (50.0 g) was subjected to a filtration under vacuum, using silica gel as stationary phase and mixtures of Hex, $CHCl_3$, MeOH in gradient wise as mobile phase. After TLC, the obtained samples were combined in four groups of increasing polarity: A (12.8 g), B (4.9 g), C (11.75 g) and D (10.9 g). The combined sample A was submitted to a chromatographic procedure like that used to obtain a mixture of compounds **1** and **2**, resulting in the isolation of compounds **3** (9.0 mg), **4** (9.0 mg) and **5** (5.0 mg).

A sample of CLP (20.0 g) was chromatographed in Sephadex LH-20 column eluted with MeOH and then $CHCl_3$ isocratic wise. The procedure yielded 11 fractions, analyzed by TLC and grouped in two fractions: Fr01-06 (15.0 g) and Fr07-11 (3.7 g). The sample Fr01-06 was chromatographed on Sephadex LH-20 column using MeOH and the sub-fractions 24-27 were later identified as a mixture of compounds **6** and **7** (15.0 mg). The sample Fr07-11 was chromatographed using the same method resulting in the isolation of compounds **8** (18.0 mg) and **9** (30.0 mg).

An aliquot of ACP (2.0 g) was chromatographed on Sephadex LH-20 eluted with MeOH providing 34 fractions, analyzed by TLC. The combined fractions 15-21 (61.0 mg) were chromatographed using the same method yielding two yellow powders identified as compounds **10** (17.0 mg) and **11** (12.0 mg).

A sample of BP (2.2 g) was chromatographed on Sephadex LH-20, eluted with MeOH, and 47 fractions were obtained. The fractions were analyzed by TLC, showing the pure compounds **12** (5.0 mg) and compound **13** (9.0 mg). Another sample from the BP (5.0 g) was submitted to column chromatography using XAD-2 as stationary phase. The following eluents were used: H_2O (100%), H_2O:MeOH (7:3), H_2O:MeOH (1:1), MeOH, Hex, acetone and EtOAc. The fraction obtained from H_2O:MeOH (1:1) elution (1.6 g) was chromatographed (Sephadex LH-20), yielding 25 fractions. The sub-fractions 13-18 (500.0 mg) were again chromatographed (Sephadex LH-20) providing the compounds **14** (8.0 mg) and **15** (5.0 mg).

The isolated compounds were identified by infrared spectroscopy (IR) (WSF-510AFTIR, China) using 1 mg of sample impregnated in KBr disc, with a spectral range from 7800 to 350 cm^{-1} and NMR analysis (^{1}H 500 MHz/^{13}C 125 MHz and ^{1}H 200 MHz/^{13}C 50 MHz in a Varian equipment, and ^{1}H 400 MHz/^{13}C 100 MHz in a Bruker equipment), using deuterated solvents: chloroform ($CDCl_3$), dimethyl sulfoxide (DMSO) or methanol (CD_3OD). Chemical shifts were measured in parts *per* million (δ).

*Compound **4***

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.59 (2H, m, H-23, 24), 3.28 (1H, dd, *J* 11.3, 4.4 Hz, H-3), 1.31 (6H, s, H-26, 27), 0.96 (6H, s, H-18, 29), 0.88 (3H, s, H-30), 0.85 (3H, d, *J* 6.4 Hz, H-21), 0.80 (3H, s, H-28), 0.55, 0.33 (1H, d, *J* 4.2 Hz, H-19); ^{13}C NMR (100 MHz, $CDCl_3$) δ 32.1 (C-1), 30.5 (C-2), 79.0 (C-3), 40.6 (C-4), 47.2 (C-5), 21.2 (C-6), 28.2 (C-7), 48.1 (C-8), 20.1 (C-9), 26.1 (C-10/11), 32.9 (C-12), 45.4 (C-13), 48.9 (C-14), 35.7 (C-15), 26.5 (C-16), 52.0 (C-17), 18.2 (C-18), 30.0 (C-19), 36.5 (C-20), 18.4 (C-21), 38.18 (C-22), 125.8 (C-23), 139.5 (C-24), 70.9 (C-25), 30.1 (C-26), 30.1 (C-27), 19.4 (C-28), 14.1 (C-29), 25.5 (C-30).

*Compound **5***

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.93 and 4.83 (1H, s, H-26), 4.03 (1H, t, *J* 6.0 Hz, H-24), 3.30 (1H, dd, *J* 4.0 and 10.8 Hz, H-3), 1.72 (3H, s, H-27), 0.96 (6H, s, H-29, 18), 0.87 (3H, d, *J* 6.0 Hz, H-21), 0.88 (3H, s, H-28), 0.80 (3H, s, H-30), 0.55, 0.33 (each 1H, d, *J* 4.2 Hz, H-19); ^{13}C NMR (100 MHz, $CDCl_3$) δ 31.9 (C-1), 30.3 (C-2), 78.8 (C-3), 40.4 (C-4), 47.1 (C-5), 21.1 (C-6), 28.02 (C-7), 47.2 (C-8), 20.0 (C-9), 26.02 (C-10), 26.4 (C-11), 32.8 (C-12), 45.2 (C-13), 48.7 (C-14), 35.5 (C-15), 28.1 (C-16), 52.1 (C-17), 18.05 (C-18), 29.9 (C-19), 35.9 (C-20), 18.3 (C-21), 31.9 (C-22), 31.6 (C-23), 76.3 (C-24), 147.7 (C-25), 110.9 (C-26), 17.6 (C-27), 19.2 (C-28), 14.01 (C-29), 25.4 (C-30).

*Compound **8***

^{1}H NMR (500 MHz, $CDCl_3$) δ 6.42 (1H, s, H-6), 8.16 (2H, d, *J* 9.0 Hz, H-2',6'), 7.05 (2H, d, *J* 9.0 Hz, H-3',5'), 3.87 (3H, s, OCH_3-3), 3.95 (3H, s, OCH_3-7), 3.91 (3H, s, OCH_3-8), 3.90 (3H, s, OCH_3-4'), 12.5 (1H, s, OH-5); ^{13}C NMR (125 MHz, $CDCl_3$) δ 154.08 (C-2), 141.24 (C-3), 176.08 (C-4), 157.53 (C-5), 95.59 (C-6), 158.63 (C-7), 128.68 (C-8), 141.24 (C-9), 105.54 (C-10), 123.16 (C-1'),

130.40 (C-2',6'), 114.35 (C-3',5'), 161.90 (C-4'), 60.28 (OCH_3-3), 56.53 (OCH_3-7), 61.78 (OCH_3-8), 55.59 (OCH_3-4').

*Compound **9***

^{1}H NMR (500 MHz, $CDCl_3$) δ 6.42 (1H, s, H-6), 8.11 (2H, d, *J* 10.0 Hz, H-2',6'), 7.05 (2H, d, *J* 9.0 Hz, H-3',5'), 3.86 (3H, s, OCH_3-3), 3.99 (3H, s, OCH_3-8), 3.90 (3H, s, OCH_3-4'), 12.43 (1H, s, OH-5); ^{13}C NMR (125 MHz, $CDCl_3$) δ 155.08 (C-2), 138.95 (C-3), 179.04 (C-4), 157.68 (C-5), 98.55 (C-6), 155.74 (C-7), 126.81 (C-8), 148.06 (C-9), 105.81 (C-10), 122.92 (C-1'), 130.21 (C-2',6'), 114.39 (C-3',5'), 161.93 (C-4'), 60.34 (OCH_3-3), 62.10 (OCH_3-8), 55.61 (OCH_3-4').

*Compound **10***

^{1}H NMR (200 MHz, CD_3OD) δ 6.11 (1H, d, *J* 2.0 Hz, H-6), 6.27 (1H, d, *J* 2.0 Hz, H-8), 7.96 (2H, d, *J* 9.0 Hz, H-2'/6'), 6.79 (2H, d, *J* 9.0 Hz, H-3'/5'), 5.23 (1H, d, *J* 7.6 Hz, H-1''), 3.38-3.34 (m, H-2'',3'',4''), 3.25-3.16 (m, H-5''), 4.19 (1H, dd, *J* 11.8 and 2.2 Hz, H-6''), 4.06 (1H, dd, *J* 11.6 and *J* 6.4 Hz, H-6''), 6.05 (1H, d, *J* 15.9 Hz, H-α), 7.38 (1H, d, *J* 15.9 Hz, H-β), 7.25 (2H, d, *J* 8.6 Hz, H-2'''/6'''), 6.77 (2H, d, *J* 8.6 Hz, H-3'''/5'''); ^{13}C NMR (50 MHz, CD_3OD) δ 179.3 (C-4), 168.81 (COO), 165.7 (C-7), 162.8 (C-5), 161.4 (C-4'), 161.0 (C-4'''), 159.2 (C-2), 158.2 (C-9), 146.5 (CH-β), 135.2 (C-3), 132.1 (CH-2'/CH-6'), 131.1 (CH-2'''/6'''), 127.0 (C-1'''), 122.6 (C-1'), 116.7 (CH-3'''/5'''), 115.9 (CH-3'/5'), 114.7 (CH-α), 105.5 (C-10), 104.0 (CH-1''), 99.9 (CH-6), 94.8 (CH-8), 77.9 (CH-3''), 75.7 (CH-2'', CH-5''), 71.6 (CH-4''), 64.3 (CH_2-6'').

*Compound **12***

IR (KBr) *v* / cm^{-1} 3458, 1609, 1510, 2938, 2840; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.43 (1H, s, H-3), 6.15 (1H, d, *J* 2.0 Hz, H-6), 6.32 (1H, d, *J* 2.0 Hz, H-8), 7.72 (2H, d, *J* 2.0 Hz, H-2'/6'), 6.89 (2H, d, *J* 9.0 Hz, H-3'/5'), 3.76 (3H, s, OCH_3-4'); ^{13}C NMR (125 MHz, $CDCl_3$) δ 164.5 (C-2), 103.3 (C-3), 182.5 (C-4), 161.50 (C-5), 99.13 (C-6), 163.9 (C-7), 94.18 (C-8), 157.5 (C-9), 104.4 (C-10), 123.5 (C-1'), 127.0 (C-2'), 114.0 (C-3'), 162.7 (C-4'), 114.0 (C-5'), 127.0 (C-6'), 55.9 (OCH_3-4').

*Compound **14***

^{1}H NMR (400 MHz, CD_3OD) δ 6.08 (1H, s, H-6), 8.03 (2H, d, *J* 9.0 Hz, H-2',6'), 6.91 (2H, d, *J* 9.0 Hz, H-3',5'), 3.84 (3H, s, OCH_3-8), 3.74 (3H, s, OCH_3-3); ^{13}C NMR (100 MHz, CD_3OD) δ 156.5 (C-2), 138.72 (C-3), 178.89 (C-4), 168.9 (C-5), 103.56 (C-6), 158.13 (C-7), 131.3 (C-8), 158.23 (C-9), 122.72 (C-1'), 131.1 (C-2'/6'), 116.88 (C-3'/5'), 162.41 (C-4'), 61.31 (OCH_3-8), 3.74 (OCH_3-3).

*Compound **15***

^{1}H NMR (500 MHz, CD_3OD) δ 8.01 (2H, d, *J* 8.8 Hz, H-2',6'), 7.01 (2H, d, *J* 8.8 Hz, H-3',5'), 6.48 (1H, d, *J* 1.95 Hz, H-6), 6.25 (1H, d, *J* 1.95 Hz, H-8), 3.86 (3H, s, OCH_3-3); ^{13}C NMR (100 MHz, CD_3OD) δ 155.7 (C-2), 138.22 (C-3), 162.9 (C-5), 92.6 (C-6), 164.6 (C-7), 97.7 (C-8), 157.12 (C-9), 104.9 (C-10), 121.58 (C-1'), 129.5 (C-2'/6'), 114.7 (C-3'/5'), 160.00 (C-4').

Total phenol content

The total phenolics content of *P. glazioviana* CEE was determined by the spectrophotometric method of Folin-Ciocalteu, using gallic acid as standard and spectrophotometer Cirrus 80MB (FEMTO, city, country).[23] The CEE was solubilized in MeOH to a final concentration of 1000 μg mL^{-1}. The test solution was prepared adding 100 μL of the CEE solution, 50 μL of the Folin-Ciocalteu reagent, 6.0 mL of distilled H_2O and 2.0 mL of a sodium carbonate solution (15%). The experiment was performed in triplicate. The concentration of the phenolic compounds was determined as milligrams of gallic acid equivalent *per* gram of CEE (mg GAE g^{-1} of CEE), from the calibration curve constructed with gallic acid solutions (7.5625 to 125 μg mL^{-1}).

Total flavonoids content

The total flavonoid content was determined using quercetin as standard.[24] The CEE was solubilized in MeOH to obtain a 1000 μg mL^{-1} CEE solution. To prepare the test solution, 400 μL of CEE solution was added to 200 μL of aluminum chloride (2%) in a volumetric flask. The final volume was adjusted to 10 mL. The reaction occurred for 30 min in the dark. The absorbance was read against a blank sample in spectrophotometer Cirrus 80MB (FEMTO, São Paulo, Brazil) at wavelength of 425 nm. The analysis was evaluated in triplicate and the total flavonoid content was determined from the calibration curve constructed with quercetin solutions (1.25 to 40.0 μg mL^{-1}). The result was expressed in milligrams of quercetin equivalents *per* gram of CEE (mg EQ g^{-1} of CEE).

DPPH· radical scavenging activity assay

The antioxidant activity of *P. glazioviana* CEE was evaluated by the DPPH· (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method described by Maciel *et al*.[18] The DPPH· solutions were prepared in ethanol at 60, 50, 30, 15 and 7.5 μM. After 30 min the absorbance of each solution was measured at 517 nm to construct a calibration curve. The values of absorbance *versus* DPPH· concentration were plotted and the graphic was used to calculate the absorbance corresponding to reduction of 50% in DPPH· concentration (EC_{50}). In dark room, 0.1 mL of *P. glazioviana* CEE solution in crescent concentrations was added to 3.9 mL of the DPPH· solution (60 μM). The experiment was performed in triplicate. After 30 min the absorbance was read in spectrophotometer (Cirrus 80MB, FEMTO, São Paulo, Brazil) at 517 nm against a blank sample. From the equation of the obtained straight line, it was calculated the concentration of CEE corresponding to reduction of 50% in DPPH· concentration (EC_{50}).

Evaluation of antimicrobial activity

The used strains (bacteria and yeasts) were obtained from Micoteca of the Laboratório de Micologia, Departamento de Ciências Farmacêuticas (DCF), Centro de Ciências da Saúde (CCS) of the Universidade Federal da Paraíba (UFPB): *Staphylococcus aureus* ATCC-25923, *Escherichia coli* ATCC-18739, *Pseudomonas aeruginosa* ATCC-25853, *Candida albicans* ATCC-60193, *Candida tropicalis* ATCC-13803, *Candida parapsilosis* ATCC-60193, *Aspergillus flavus* LM-248, *Aspergillus fumigatus* ATCC-40640.

Determination of minimum inhibitory concentration (MIC)

The antimicrobial activities of three major flavonoids were evaluated: 5-hydroxy-3,7,8,4'-tetramethoxyflavone (**8**), 5,7-dihydroxi-3,8,4'-trimethoxyflavona (**9**), tiliroside (**10**). The compounds were weighed (4.0 mg) solubilized in 250 μL (5%) dimethylsulfoxide (DMSO) and 100 μL (2%) of tween 80 and the final volume was completed with sterile distilled water until 5 mL. In this way, the initial concentration obtained was 1024 μg mL^{-1}, then it was serially diluted to 16 μg mL^{-1}.[25]

Antifungal and antibacterial activities were determined using microbroth dilution assays in duplicate 96-well microplates and amphotericin B (32 μg mL^{-1}) and gentamicin (64 μg mL^{-1}) were used as control to antifungal and antibacterial activities.

For inoculum preparation, colonies were obtained from cultures of bacterial strains in brain heart infusion (BHI) medium and fungi from sabouraud agar dextrose (ASD) medium (Difco Laboratories Ltd,

New Jersey, USA). For the biological activity assays, BHI broth and RPMI 1640 medium with L-glutamine and without bicarbonate were used. The inoculum of the microorganisms was prepared with sterile 0.9% physiological solution and adjusted according to the 0.5 μm tube of the standard Mc Farland scale to obtain 10^6 colony forming units *per* mL (CFU mL^{-1}) for bacteria and fungi.

Initially, 100 μL of doubly concentrated RPMI/BHI broth was distributed into the wells of the 96-well microdilution plates. Then, 100 μL of the emulsion of the prepared substances were dispensed into the wells of the first row of the plate. Then, by serial dilution, were obtained the concentrations from 1024 to 16 μg mL^{-1}. Finally, 10 μL of the suspensions of the bacterial and fungal strains were added and incubated at temperature of 35 ± 2 °C for 24-48 h for bacterial and yeast assays.

In the biological assay with the bacteria, after 24 h of incubation, 20 μL of 0.01% resazurin dye solution (INLAB, São Paulo, Brazil) was added. It is considered an indicator of microbial growth, the color change from blue to red, thus the absence of microbial growth is indicated by blue color. The MIC for each product was defined as the lowest concentration able to be inhibiting microbial growth.

The tested products were considered as active or inactive, according to the following criteria: up to 600 μg mL^{-1} = strong activity; 600 to 1500 μg mL^{-1} = moderate activity; higher than 1500 μg mL^{-1} = poor activity or inactive product.[26,27]

Determination of minimum bactericidal concentration (MBC) and minimum fungicide concentration (MFC)

After reading the MIC, aliquots of 10 μL of the supernatant from the wells where complete inhibition of bacterial and fungal growth (MIC × 2, MIC × 4 and MIC × 8) was observed were transferred to a 96-well microdilution plates containing 100 μL of the liquid culture medium suitable for each group of microorganisms. Plates were incubated at 35 °C for 24-48 h. The MBC and MFC were considered as the lowest concentration of the product that was able to inhibit the growth of the microorganisms (approximately 99 to 99.5% of death activity) by observing the visual absence of growth in the liquid medium. The assays were performed in duplicate and the result expressed by the arithmetic mean of the MBC and MFC obtained in the two assays.[28]

RESULTS AND DISCUSSION

Phytochemical study

The phytochemical screening is a set of reactions based on colorimetry and precipitation that is usually carried out to detect the presence of secondary metabolites in natural extracts. This preliminary information is important to guide the research to select specific chromatographic procedures to certain metabolites detected.[21] For *P. glazioviana* extract, the screening showed the presence of phenolics, flavonoids, tannins, coumarins, alkaloids, steroids, and triterpenes. It is in accordance with previous phytochemical studies in *Pavonia* genus, showing the chemotaxonomic similarity among studied species in the genus.[12,13] In fact, species from Malvaceae family are known to produce large amount of phenolics, including phenolic acids, flavonoids, coumarins and triterpene.[11,14,19,29]

The chromatographic techniques, followed by spectroscopic methods (IR, ^1H and ^{13}C NMR) led to identification of fifteen compounds, two chlorophyll derivatives, a mixture of two glucosyl steroids, an aliphatic alcohol, two triterpenes, and eight flavonoids.

The compound **1** was isolated as a green amorphous solid, soluble in chloroform. On TLC it showed very similar spots to chlorophyll derivatives. By analyzing its NMR spectral data and comparing with those from literature, it was identified as a mixture of the compounds: 13^2-(*S*)-hydroxy-pheophytin a (**1**) and 13^2(*S*)-hydroxy-17^3-ethoxyphaeophorbide (**2**), chlorophyll-derivative structures previously isolated from several Malvaceae species (Figure 1).[14-21,29-31] The pheophytins are produced by enzymatic reaction with the exchange of the magnesium of chlorophyll by two hydrogen atoms. Their biological and pharmacological activities have been demonstrated, arising interest in these compounds.[14]

The compound **3** was identified as the aliphatic alcohol *n*-decanol by analyzing its spectral data and comparison with literature. The length of the compound chain was proposed by NMR data, considering the signals at ^{13}C NMR. The compound *n*-decanol has great ecological relevance, since it acts as a growth regulator, and it is commercially used as a pesticide.[32] Compounds **4** and **5** were analyzed and identified as the cycloeucalenol-type triterpenes: cycloart-23*Z*-ene-3β,25-diol (**4**) and cycloart-24*S*-25-ene-3β,24-diol (**5**).[33] Cytotoxic activity on tumor cells have been reported for cycloeucalenol-type triterpenes.[34] Compounds **6** and **7** were obtained as a white powder, identified as a steroid as sitosterol-3-*O*-β-D-glucopyranoside (**6**) and stigmasterol-3-*O*-β-D-glucopyranoside (**7**), a steroid mixture present in plant cell membranes, widely reported including from Malvaceae species.[14]

Compounds **8** to **15** were isolated as yellow powder. The ^1H NMR spectrum of **8** showed a singlet at δ 12.48 indicating the presence of a hydroxyl in an intramolecular hydrogen bond, as found in flavonoids. A singlet at δ 6.42 (s, H-6, 1H) attributed to hydrogen at the 6-position of flavonoid A ring, and two doublets at δ 8.17 (d, *J* 9.0 Hz, 2H) and 7.04 (d, *J* 9.0 Hz, 2H) characteristics of *ortho* coupling, suggested a *para* substituted B ring. The spectrum also showed the presence of four methoxyls by showing four singlets with integration for 3 protons each one (δ 3.87, 3.90, 3.91 and 3.95). The ^{13}C-APT NMR spectrum confirmed the presence of a *para* substituted ring B by the presence of a couple of high intensity signals at δ 130.4 (C-2'/6') and 114.35 (C-3'/5').

A set of carbons also confirmed the presence of four methoxyls in the compound (δ 55.59, 56.53, 60.28 and 61.78). To determine the position of the singlets at carbon 6 of ring A of the flavonoids it was observed the correlations at HMBC spectra. For hydrogen attached to C-6, we find correlations to C-5, C-7, C-10 and C-8, but not with C-9. Analyzing the spectral data and the literature, it was possible to achieve the complete assignment of the NMR data, and the compound **8** was identified as 5-hydroxy-3,7,8,4'-tetramethoxyflavone, previously reported from Capparaceae family.[35]

The ^1H NMR spectrum of compound **9** was quite like compound **8**, except for the absence of one methoxyl signal. Analyzing the ^1H, ^{13}C and 2D NMR spectra, the compound **9** was identified as 5,7-dihydroxy-3,8,4'-trimethoxyflavone, previously isolated in other families, for example in Rutaceae.[22]

The structural assignments and literature data of the compound **10** allowed us to identify it as kaempferol-3-*O*-β-D-(6*"*-*E*-*p*-coumar oyl) glucopyranoside (tiliroside). Tiliroside have been assigned as the most prevalent compound among species from the Malvaceae family, being an important compound for the chemotaxonomy of this family. The compound has showed many biological activities such as vasorelaxant, antimicrobial, antioxidant, and anti-inflammatory.[25]

The spectral data of compounds **11**, **12** and **13** also showed flavonoid characteristics. They have been identified as 3,5,7,3',4'-pentahydroxyflavonol (quercetin) (**11**), 5,7-dihydroxy-4'-methoxy-flavone (acacetin) (**12**), 3,5,7,4'-tetrahydroxyflavonol (kaempferol) (**13**), reported from Malvaceae family.[15,29]

The NMR spectra for compounds **14** and **15** allowed their identification as the methoxylated flavonoids: 5,7,4'-trihydroxy-3,8-dimethoxyflavone (**14**) and 5,7,4'-trihydroxy-3-methoxyflavone (isokaempferide) (**15**). Previous studies showed antimitotic

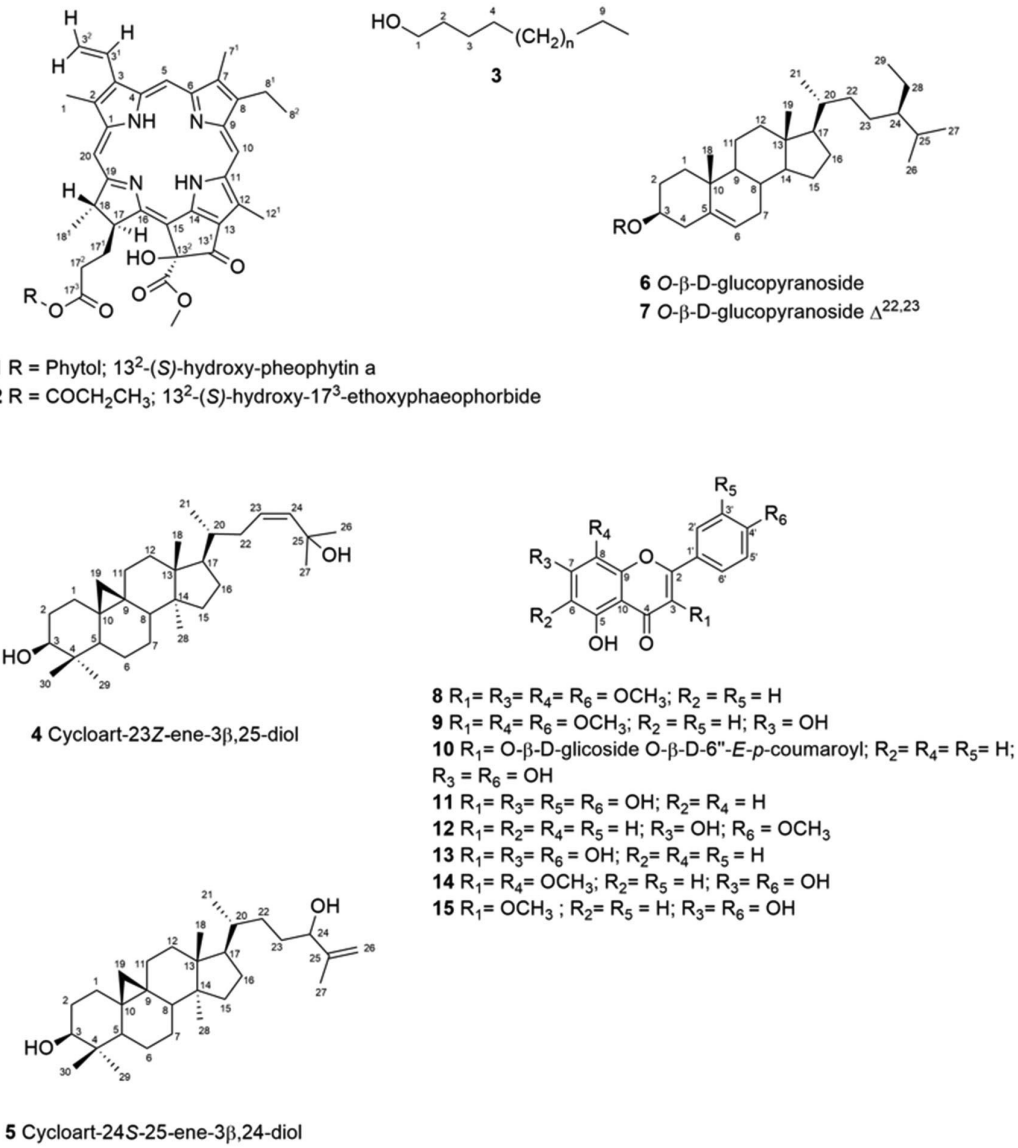

Figure 1. *Structure of compounds obtained from aerial parts of Pavonia glazoviana*

effects for compound **14** and bronchodilator activities for isokaempferide (**15**).[36,37]

It is interesting to observe the production of highly methoxylated flavonoids from *P. glazioviana*. Polymethoxylated flavonoids have been reported from other species of Malvaceae *sensu lato*, such as from *Whalteria* and *Sidastrum* genera.[25] The enzymes responsible by the production of methoxylated flavonoids are the *O*-methyltransferases (OMTs) that depends on the methyl donor *S*-adenosyl-methionine.[38] The OMTs have been showed to be stress-induced or microbial-induced and the produced methoxylated flavonoids seems to be responsible for plant adaptation and for antimicrobial response.[20,24,39] According to Liu *et al.*[21] the occurrence of methoxyl at position 8 of flavonoids, as found here in compounds **8**, **9** and **14**, is uncommon.[21] Therefore, our findings may contribute to characterize *P. glazioviana* as a prolific source of methoxylated flavonoids with antimicrobial potential.

Total phenolic compounds, flavonoids content and DPPH· radical scavenging assay

A spectrophotometric method was used to quantify the phenolic compounds and flavonoids in *P. glazioviana* CEE. A calibration curve was built with gallic acid to quantify the phenolic compounds. The linearity coefficient found was $R^2 = 0.99303$ and the obtained equation was used to calculate the total phenolics. The total phenol content assay showed the presence of 44.28 ± 1.79 mg of EAG g^{-1} of CEE, pointing the intense production of phenolics by the studied species. When compared to other crude extracts from Malvaceae, the CEE of *P. glazioviana* showed to possess greater content of phenolics. For example, extracts of *Sidastrum micranthum* and *Sida rhombifolia*, previously evaluated by the same method, showed 38.22 ± 0.43 and 39.37 ± 2.54 mg EAG g^{-1}, respectively.[40] Researchers in pharmaceutical field are very interested in phenolic compounds because of their biological properties, which include antioxidant and anti-inflammatory activities. Studies evaluating the phenolic compounds in grapes have shown a strong relationship between these compounds and antioxidant activity of wines.[41]

To determine the total flavonoid content, a calibration curve was constructed with quercetin. Many flavonoids have been related to antioxidant activity and prevention of cardiovascular disease, inflammation, among others heath conditions.[16] Flavonoids are the most studied group of phenolics, characterized by the 2-phenyl-benzyl-γ-pyrone nucleus. Thus, the flavonoid content is often investigated in plant extracts. The total flavonoid content in

P. glazioviana was determined as 33.68 ± 0.76 mg EQ g^{-1} of extract. The result shows that *P. glazioviana* is a great producer of flavonoids, as demonstrated by the isolated compounds. Flavonoids are related to the attenuation of oxidative stress, acting as electron donors, and reducing the occurrence of inflammatory and chronic-degenerative diseases.[42]

To evaluate the antioxidant potential by radical scavenging of *P. glazioviana*, appropriate concentrations of its crude extract were used to calculate the absorbance corresponding to the 50% reduction in the DPPH absorbance (EC_{50}). The determined DPPH EC_{50} calculated for *P. glazioviana* CEE was 6.36 ± 0.029 mg mL^{-1}, showing a greater potential than those previously reported using the same method for other Malvaceae species[40] for example: *Sidastrum micranthum* (EC_{50} = 125.733 ± 0.291 mg mL^{-1}), *Wissadula periplocifolia* (EC_{50} = 125.733 ± 0.291 mg mL^{-1}), *Sida rhombifolia* (EC_{50} = 125.733 ± 0.291 mg mL^{-1}) and *Herissantia crispa* (EC_{50} = 120.06 ± 3.10 mg mL^{-1}). Despite that, other species from *Pavonia* genus, such as *Pavonia xanthogloea* and *Pavonia speinoide* showed greater antioxidant activity than *P. glazioviana*.[3,43] Our findings corroborate with the literature data by presenting the great antioxidant potential of species from *Pavonia* genus.

Evaluation of antimicrobial activity

Tables 1 and 2 show the results of the antimicrobial evaluation of the major substances: the compounds **8**, **9** and **10**.

The compounds 5-hydroxy-3,7,8,4'-tetramethoxyflavone (**8**) and tiliroside (**10**) did not inhibited the bacterial growth of any strains used in the biological assay. Regarding the antifungal activity for compounds **8** and **10**, the compound tiliroside (**10**) showed moderate activity against the strains of *Candida tropicalis* ATCC-13803 and *Aspergillus fumigatus* ATCC-40640 (Table 1). Antifungal activity of tiliroside has been previously reported.[44]

Among the three compounds evaluated, the flavonoid 5,7-dihydroxy-3,8,4'-trimethoxyflavone (**9**) showed strong antibacterial activity (MIC = 512 μg mL^{-1}) against *Escherichia coli* and *Pseudomonas aeruginosa* (Table 2). Besides that, the compound **9** also showed strong antifungal activity (MIC = 512 μg mL^{-1}) inhibiting the tested fungi strains of *Candida albicans*, *C. tropicalis*, *Candida parapsilosis*, *Aspergillus flavus* and *A. fumigantus* (Table 1). The MFC for this compound was established at the concentration of 1024 μg mL^{-1}. *Candida* and *Aspergillus* species are pathogens commonly reported in immunocompromised and hospitalized patients. These infections currently tend to increase due to higher rates of hospitalization due COVID-19, population aging and the occurrence of chronic diseases.[45] Invasive aspergillosis, for example, is found in 50% of patients with hematological malignancies. It is the most common fungus in humans and is considered the most invasive one, affecting the brain and kidneys[46] therefore, the search for new active compounds against *Aspergillus* species is a relevant task.

CONCLUSIONS

The first assessment on *P. glazioviana* species showed that this plant possesses a diverse specialized metabolism, producing chlorophyll derivatives, steroids, terpenoids and phenolic compounds. The species can produce several polymethoxylated flavonoids, including uncommon structures with methoxyl bearing 8 positions of flavonoid scaffold. Polymethoxylated flavonoids are reported as phytoalexins with antimicrobial activity. In our study, the compound 5,7-dihydroxy-3,8,4'-trimethoxyflavone showed strong activity against *E. coli*, *P. aeruginosa*, *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *A. flavus* and *A. fumigantus*. Our findings contributed to characterize *P. glazioviana* as a great producer of methoxylated flavonoids with antimicrobial potential.

Table 1. Results of the antifungal minimum inhibitory concentration (MIC) determination (μg mL^{-1}) of substances isolated from *Pavonia glazioviana*

Tested products	MIC / (μg mL^{-1})				
	C. albicans ATCC-60193	*C. tropicalis* ATCC-13803	*C. parapsilosis* ATCC-60193	*A. flavus* LM-248	*A. fumigatus* ATCC-40640
Compound **8**	+	+	+	+	+
Compound **9**	512	512	512	512	512
Compound **10**	+	1024	+	+	1024
Culture medium	–	–	–	–	–
Culture medium + microorganism	+	+	+	+	+
Amphotericin B (32 μg mL^{-1})	–	–	–	–	–

(+): Growth of microorganism; (–): no growth of microorganism.

Table 2. Results of the antibacterial minimum inhibitory concentration (MIC) determination (μg mL^{-1}) of substances isolated from *Pavonia glazioviana*

Tested products	MIC / (μg mL^{-1})		
	S. aureus ATCC-25923	*E. coli* ATCC-18739	*P. aeruginosa* ATCC-25853
Compound **8**	+	+	+
Compound **9**	+	512	512
Compound **10**	+	+	+
Culture medium	–	–	–
Culture medium + microorganism	+	+	+
Gentamicin (64 μg mL^{-1})	–	–	–

(+): Growth of microorganism; (–): no growth of microorganism.

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AUTHOR CONTRIBUTION

M. S. O.; O. S. C.; D. A. F. and M. F. V. S. carried out the phytochemical work and spectroscopic identification of the compounds. M. R. R. M. M. assisted the extraction and isolation of the compounds. A. S. C. and J. B. L. carried out the plant collection and identification. Y. C. F. T.; C. M. S. and W. A. M. Q. performed the spectrophotometric analysis and contributed to the discussion. E. O. L. and G. L. F. performed the biological assay.

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