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Artigo

First-time Isolation of Flavonoids and Cytotoxic Potential of the Amazonian Shrub *Ptychopetalum olacoides* Benth

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Primeiro Isolamento de Flavonoides e Potencial Citotóxico do Arbusto Amazônico *Ptychopetalum olacoides* Benth

Resumo: No presente estudo, três flavonoides, 3-*O*-metilquercetina (**1**), 3,4'-*O*-dimetilquercetina (**2**) e 3,7-*O*-dimetilquercetina (**3**), foram isolados e caracterizados pela primeira vez a partir do extrato metanólico da espécie *Ptychopetalum olacoides* Benth. As estruturas das substâncias foram elucidadas por métodos espectroscópicos (1D-, 2D-RMN, EM e UV) e confirmadas por comparação com a literatura. A atividade citotóxica do extrato bruto foi avaliada *in vitro* contra três linhagens de células humanas cancerígenas. Foi observada atividade moderada (CI_{50} = 45.16 µg/mL) contra a linhagem de câncer de mama (MCF-7) e, além disso, o extrato bruto não foi citotóxico contra a linhagem de fibroblastos humanos não cancerígenos (MRC-5).

Palavras-chave: Antitumoral; câncer; flavonoides; Olacaceae; *P. olacoides*.

Abstract

In the present study, three flavonoids, 3-*O*-methylquercetin (**1**), 3,4'-*O*-dimethylquercetin (**2**) and 3,7-*O*-dimethylquercetin (**3**) were isolated and characterized for the first time from a methanol extract obtained from the species *Ptychopetalum olacoides*. The structures of compounds were identified by spectroscopic methods (1D-, 2D-NMR, MS and UV) and confirmed by comparison with the respective literature data. The cytotoxic effect of crude extract was evaluated *in vitro* against three human cancer cell lines. The results showed a mild cytotoxic activity (IC_{50} = 45.16 µg/mL) against breast cancer (MCF-7). However, crude extract did not exhibit any cytotoxic effect against normal cell human fibroblast (MRC-5).

Keywords: Antitumor; cancer; flavonoids; Olacaceae; *P. olacoides*.

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First-time Isolation of Flavonoids and Cytotoxic Potential of the Amazonian Shrub *Ptychopetalum olacoides* Benth

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1. Introduction

Ptychopetalum olacoides Benth. (Olacaceae) is a shrub or small tree widely known in Brazil as “muirapuama”, “marapuama”, “marapuana” and “muiratã”.[1] This is an endemic species to the Amazon rainforest and specially distributed in the north region of the country in Amazonas, Amapá and Pará states.[2] Preparations with the stems of *P. olacoides* have been used to treat "nervous weakness", fatigue, depression symptoms, tremor disorders, and sexual dysfunction.[3] The fluid root extract of this plant has been employed in phytotherapeutic formulations as Catuama®, a general tonic widely used in some regions

of Brazil.[4] However, there are few data related to the phytochemical profile of this species. Montrucchio and co-workers previously reported the isolation of saturated fatty acids (stearic and palmitic acids), methylxanthine caffeine, triterpenoid lupeol and steroid β-sitosterol.[5] Other studies described the isolation of clerodane diterpenoids,[6] benzoic acid derivatives such as vanillic and protocatechuic acids, and methylxanthine theobromine.[7] Despite validation of the total flavonoids content from *P. olacoides*,[8] there are no reports concerning isolation and characterization of flavonoids in this genus.

2. Materials and methods

2.1. General procedures

TLC was performed on plates pre-coated with silica gel 60 F_{254} (Merck, Germany). Preparative HPLC was performed on a Phenomenex C18 (30 cm x 10 mm x 5 µm, Torrance, Canada) equipped with a Shimadzu LC-10AS pump and a SPD10A UV/Vis detector (Shimadzu, Kyoto, Japan). The UV spectra were recorded on a JASCO V-370 Bio spectrophotometer (Tokyo, Japan). The NMR spectra were measured on Varian VNMRS 500 MHz spectrometer for ^{1}H and 125 MHz for ^{13}C (Palo Alto, USA), and chemical shifts were reported in ppm downfield from TMS. The MS data were recorded on a Flexar SQ 300 LC/MS system (PerkinElmer, Shelton, CT, USA) using an analytical C18 column (PerkinElmer, 150 mm × 4.6 mm, 3 µm). A micro-splitter valve was used to send 45% of the flow to the mass spectrometer. The quadrupole mass spectrometer equipped with electrospray ionization(ESI-MS) was operated under positive ion mode. The MS parameters were set at 12 L/min for drying gas flow, 80 psi for nebulizer pressure and 300 °C for drying gas temperature. Column chromatography was carried out on Sephadex LH-20.

2.2. Plant material

The powdered wood/bark of *P. olacoides* was acquired from Santosflora Herbs Ltda. (CNPJ: 51569309/0001-38, IBAMA registration No. 35867 and ANVISA's authorization No. 6.02.671-1) in June 2013. The species was collected in February 2013, lot code MARPP01/0213 and validity period from 02.04.2013 to 02.04.2016.

2.3. Extraction and isolation

The bark and wood powder of *P. olacoides* (500 g) was extracted with a solvent gradient of increasing polarity under sonication using *n*-hexane, ethyl acetate and methanol. After extraction and removal of the solvent under vacuum in a rotatory evaporator, a dark residue was obtained (4.10 g) from the methanol extract (ME). The ME fraction (2.6 g) was restructured in methanol and filtered using Fisherbrand nylon 0.2 µm filter to obtain a particle-free extract. The extract was then chromatographed on lipophilic Sephadex LH-20 (25-100 µm) and eluted with MeOH,[9] resulting mainly in 12 fractions (MEF1-12). MEF8 (20 mg) was subsequently purified by preparative RP-HPLC [mobile phase: H_2O/AcOH (99:1) (solvent A) and MeOH (solvent B) at a constant flow rate of 5 mL/min, using 65% solvent B and 35% solvent A, detection at 340 nm] resulting in substances **1** (1.0 mg), **2** (1.0 mg), and **3** (1.0 mg).

2.4. Identification

The NMR spectra of compounds **1-3** were acquired with ^{1}H, COSY, HSQC and HMBC techniques. The MS data were obtained by LC/MS analysis of MEF8 using as mobile phase a gradient of H_2O/AcOH (99:1) (solvent A) and MeOH (solvent B) starting with 35-80% of B (20 min), 80-92% of B (20-25 min), maintaining at 92% for 8 min, with a flow rate of 1 mL/min. Data acquisition was

accomplished with the Chromera® software version 3.4.1. Compounds were further analyzed by UV spectroscopy with the shift reagents $AlCl_3$ and $AlCl_3$/HCl.[10]

3-*O*-methylquercetin (**1**): yellow oil; UV (MeOH): λ_{max} (log ε): 245, 300, 355; (MeOH + $AlCl_3$): λ_{max} (log ε): 235, 265, 440; (MeOH + $AlCl_3$/HCl): λ_{max} (log ε): 230, 265, 405; ^{1}H NMR (500.00 MHz, CD_3OD): δ (ppm) = 6.21 (s, 1H, H-6), 6.40 (s, 1H, H-8), 7.65 (s, 1H, H-2'), 6.93 (d, *J* = 8.3 Hz, 1H, H-8'), 7.56 (d, *J* = 8.3 Hz, 1H, H-6'), 3.81 (s, 3H, OCH_3-3); MS: *m/z* 317 $[M+H]^+$.

3,4'-*O*-dimethylquercetin (**2**): yellow oil; UV (MeOH): λ_{max} (log ε): 255, 295, 355; (MeOH + $AlCl_3$): λ_{max} (log ε): 235, 355, 415; (MeOH + $AlCl_3$/HCl): λ_{max} (log ε): 225, 270, 405; ^{1}H NMR (500.00 MHz, CD_3OD): δ (ppm) = 6.23 (d, *J* = 2.1 Hz, 1H, H-6), 6.44 (m, 1H, H-8), 7.75 (d, *J* = 2.1 Hz, 1H, H-2'), 6.98 (d, *J* = 8.5 Hz, 1H, H-5') e 7.67 (dd, *J* = 2.1, 8.5 Hz, 1H, H-6'), 3.83 (s, 3H, OCH_3-3), 3.97 (s, 3H, OCH3-4'); ^{13}C NMR (125.0 MHz, CD_3OD): δ (ppm) = 56.4 (CH_3, OMe-4'), 60.4 (CH_3, OCH_3-3), 94.7 (CH, C-8), 99.7 (CH, C-6), 112.8 (CH, C-2'), 116.3 (CH, C-5'), 138.2 (OCH_3-3), 147.6 (OCH_3-4'); MS: *m/z* 331 $[M+H]^+$.

3,7-*O*-dimethylquercetin (**3**): yellow oil; UV (MeOH): λ_{max} (log ε): 240, 300, 370; (MeOH + $AlCl_3$): λ_{max} (log ε): 235, 440; (MeOH + $AlCl_3$/HCl): λ_{max} (log ε): 235, 355, 410; ^{1}H NMR (500.00 MHz, CD_3OD): δ (ppm) = 8.6 (brs, OH-5), 6.35 (d, *J* = 2.2, 1H, H-6), 6.61 (d, *J* = 2.2, 1H, H-8), 7.65 (d, *J* = 2.2, 1H, H-2'), 4.83 (brs, OH- 3'-4'), 6.91 (d, *J* = 8.5 H, H-5'), 7.56 (dd, *J* = 2.2, 8.5 H, H-6'), 3.80 (s, 3H, OCH_3-3), 3.89 (s, 3H, OCH_3-7); ^{13}C NMR (125.0 MHz, CD_3OD): δ (ppm) = 56.2 (CH_3, OCH_3-7), 60.2 (CH_3, OCH_3-3), 92.7 (CH, C-8), 98.6 (CH, C-6), 105.3 (C, C-10), 116.1 (CH, C-5'), 116.2 (CH, C-2'), 121.0 (C, C-1'), 122.1 (CH, C-6'), 138.3 (OCH_3-3), 145.1 (C, OH-3'), 148.7 (C, OH-4'), 156.9 (C, C-2), 161.4 (C, OH-5), 165.9 (OCH_3-7); MS: *m/z* 331 $[M+H]^+$.

2.5. Cytotoxic activity assay

Crude methanol extract (ME) was evaluated for *in vitro* cytotoxicity against three human tumor cell lines, melanoma (SK-Mel 28), gastric ascites (AGP-01) and breast carcinoma (MCF-7), and one non-tumor cell line, fetal lung fibroblast (MRC5), using the Alamar Blue (AB) assay.[11] Doxorubicin was used as positive control. The concentration of samples resulting in 50% growth inhibition (IC_{50}) was calculated for each cell line in GraphPad Prism® 5.0.

3. Results and discussion

Three flavonoids, 3-*O*-methylquercetin (**1**), 3,4'-*O*-dimethylquercetin (**2**) and 3,7-*O*-dimethylquercetin (**3**) were isolated from the methanolic extract of the bark and wood of *P. olacoides* (Fig. 1) for the first time. The structures of compounds are supported by 1D (^{1}H and ^{13}C) and 2D (COSY, HSQC and HMBC) NMR experiments, UV and LC-ESI-MS analysis, and are in agreement with those reported in the literature.[12] UV shifts data confirmed the position of the free hydroxyl groups. These confirmations were possible once the use of the shift reagents $AlCl_3$ and $AlCl_3$/HCl permits differentiation of the formation of acid-stable complexes between hydroxyls and neighboring ketones, and acid-labile complexes with *orto*-dihydroxyl groups. Thus, the bathochromic shift caused by $AlCl_3$/HCl on band 1 of the spectra of **1**-**3** is characteristic of a free hydroxyl group in carbon atom C-5, and the absence of oxygen atom at carbon C-6 along with the bathochromic shift caused by $AlCl_3$ on band 1 of the spectra of **1** and **3**, is characteristic of *orto*-dihydroxyl groups on B-ring.[10]

Crude methanol extract was evaluated *in vitro* for its cytotoxic activity against gastric ascites (AGP-01), breast (MCF-7) and melanoma (SK-Mel-28) cancer cells. The crude extract presented a moderate cytotoxic effect against MCF-7, with IC_{50} of 45.16 µg/mL, when compared with doxorubicin (positive control). In addition, the extract did

not display cytotoxicity against MRC-5 (non-tumor human fibroblast cells).

Although we have not studied the cytotoxic activity of the isolated flavonoids, literature reports that these quercetin derivatives exhibit a variety of biological activities, including antiproliferative and antioxidant properties.[13] For instance, Talib and co-workers[13b] described the antiproliferative activity of **1** against MCF-7 cells with an IC_{50} value of 11.23 µg/mL. Therefore, the compounds described in this paper may be important for the cytotoxic activity against MCF-7 cancer cells.

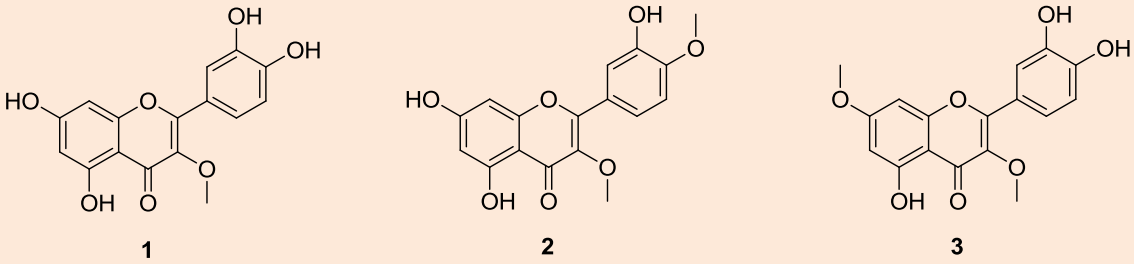

Figure 1. Structures of flavonoids **1-3** isolated from the methanolic extract of wood and bark of *Ptychopetalum olacoides* Benth

4. Conclusion

This is the first report on isolation of flavonoids and cytotoxic activity for the genus *Ptychopetalum*. Bioguided assays should be further performed in order to confirm that these flavonoids are the main active compounds involved in the cytotoxic activity.

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