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Article

Chemical Constituents, Antiproliferative and Antioxidant Activities of *Vernonanthura nudiflora* (Less.) H. Rob. Aerial Parts

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Sesquiterpene lactones are an important class of secondary metabolites frequently isolated from *Vernonanthura* genus that present a variety of biological properties, including antiproliferative activity. Due to the limitation of pharmacological studies on *Vernonanthura nudiflora*, the aim of this work was to investigate their antioxidant potential and antiproliferative activity against human tumor cells, as well as to isolate and identify the chemical constituents present in their aerial parts. The phytochemical investigation resulted in the isolation of the sesquiterpene lactones piptocarphins A, B, D, and a new hirsutinolide derivative, 8α-tigloyloxy-10α-hydroxy-hirsutinolide, besides triterpenes, glycosylated steroids, flavonoids, and one chlorogenic acid derivative. Also, other sesquiterpene lactones were identified by ultra-high performance liquid chromatography-high resolution tandem mass spectrometry (UHPLC-HRMS/MS) from dichloromethane fraction. This fraction showed activity against the tumor cells tested, mainly against leukemia, glioma, ovarian and kidney, with growth inhibitory activity (GI_{50}) less than 0.80 μg mL^{-1}. Piptocarphins A and B, in mixture, showed strong activity against all human cancer cell lines tested, with GI_{50} values ≤ 0.15 μg mL^{-1}. Piptocarphin D was selective for glioma and resistant ovarian cell lines. The new hirsutinolide derivative showed potent activity against breast (GI_{50} = 0.96 μg mL^{-1}) and resistant ovarian (GI_{50} = 3.60 μg mL^{-1}) cell lines.

Keywords: Asteraceae, *Vernonanthura nudiflora*, sesquiterpene lactones, new hirsutinolide, pharmacological potential

Introduction

Vernonanthura H. Rob. (Asteraceae family) comprises approximately 70 species widely distributed from southern Mexico to central Argentina,[1-4] of which 41 occur in Brazil, concentrated mainly in South and Southeast regions.[5]

Some species now assigned to *Vernonanthura* were originally placed in *Vernonia* genus. Robinson[4] segregated most of the South America species in 22 new genera, including *Vernonanthura*. Several *Vernonanthura* species are known in Brazil as "assa-peixe", and are used in traditional medicine for the treatment of flu and colds.[6,7] Species of this genus have also been reported to possess pharmacological activities,[8] such as antiplasmodial,[9] antileishmanial,[10-12] antimicrobial,[13-15] antioxidant,[11] antinociceptive and anti-inflammatory.[16,17]

Vernonanthura nudiflora (Less.) H. Rob., popularly known as "alecrim do campo", is a sub-shrub, 50-80 cm

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high, flowering showy, native to South America, with distribution in Argentina, Brazil and Uruguay.[5,18,19] A provoked experimental intoxication in sheep showed that *V. nudiflora* has moderate toxic action on the digestive system of this animal, nevertheless no significant effects on the respiratory and circulatory system were observed.[20] Previous experiment undertaken by Dobereiner and Tokarnia[21] also showed that *V. nudiflora* provoke irritation on the digestive tract mucosa of cattle and sheep.

V. nudiflora ethanolic extract presented potent antiproliferative activity against leukemia tumor cell lines, evidencing promising therapeutic potential.[22] Previous phytochemical investigation of this species revealed the presence of triterpenes, steroids, flavonoids, and mainly sesquiterpene lactones of glaucolide, hirsutinolide and cadinanolide classes.[23,24] Sesquiterpene lactones are still an important class of secondary metabolites, providing new therapeutic leads, especially for development of anti-inflammatory and anticancer agents.[8,25,26]

Hirsutinolide-type sesquiterpene lactones isolated from natural products, and semi-synthetic analogues, showed *in vitro* and *in vivo* activity against human glioma cell lines.[27] Additionally, Youn *et al.*[28] reported that hirsutinolides isolated from *Vernonia cinerea* were able to inhibit glioblastoma (U251MG), and breast tumor cells (MDA-MB-231), which demonstrate the potential of this class of compounds.

In this paper, we describe the isolation and structural elucidation of one new hirsutinolide derivative, along with sixteen known compounds. In addition, other nine sesquiterpene lactones were identified by ultra-high pressure liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS/MS) from dichloromethane fraction. Moreover, due to the limitation of pharmacological studies on *V. nudiflora*, the evaluation of their antioxidant potential, and antiproliferative activity against human tumor cells, were carried out.

Experimental

General experimental procedures

Chromatography separations were performed on silica gel 60 (70-230 mesh, Merck), silica gel flash (230-400 mesh, Acros Organics), Sephadex LH-20® (Sigma) or polyvinylpolypyrrolidone (PVPP, Sigma-Aldrich) chromatography columns (CC). Thin layer chromatography (TLC) was performed on normal phase pre-coated silica gel 60G or $60GF_{254}$ (Merck) plates. Visualization of the compounds on TLC was accomplished by UV irradiation at 254 and 366 nm, and/or by spraying with H_2SO_4/anisaldehyde/acetic acid (1:0.5:50 mL) solution followed by heating at 100 °C. HPLC separations were performed on a Shimadzu instrument (Mod. Prominence) with two LC-20AR pumps, degasser DGU-20ASR, detector UV-Vis SPD-M20A model and injection system automatic SIL-10AF, and equipped with a Shim-pack PREP-ODS (250 × 20 mm; 15 μm) column. The mobile phase consisted of water (Milli-Q, Millipore) and methanol (Sigma Chemicals Co). Nuclear magnetic resonance (NMR) spectra were recorded on a VARIAN Mercury Plus spectrometer operating at 300 and 75.5 MHz, and Bruker avance III HD spectrometer operating at 500 and 125 MHz, using $CDCl_3$ and dimethyl sulfoxide (DMSO-d_6) as solvents. The UHPLC analysis was performed in a Shimadzu Nexera X2 instrument, equipped with a CBM-20A a system controller, two LC-30AD pumps, a CTO-30A column oven and SIL-30AC autosampler. The mass spectra were recorded on Bruker IMPACT II mass spectrometer, with electrospray ionization source (ESI) in the positive ion mode, quadrupole-time of flight (Q-TOF) analyzer and multichannel plate (MCP) detector. The optical rotation was measured on a PerkinElmer polarimeter at 24 °C and λ = 589 nm (sodium D-line), using a 1 cm microcell (*c* 0.15, $CHCl_3$). UV-Vis spectra were recorded using a PHARO 300 spectrophotometer (Merck). The solvent was removed using Rocket Synergy sample concentrator (Genevac).

Plant material

The aerial parts of *V. nudiflora* were collected at Ponta Grossa city, Paraná State, Brazil (25°05'16" S, 50°05'43" W) on March 2016, and indentified by Dr Marta Regina Barrotto do Carmo. A voucher specimen was deposited at the herbarium at Universidade Estadual de Ponta Grossa (HUPG 21694).

Extraction and isolation

Air-dried aerial parts of *V. nudiflora* (765.0 g) were ground, exhaustively extracted with ethanol at room temperature and concentrated under reduced pressure at 37 ºC, to yield the crude extract (CE, 42.0 g). A part of the crude extract (38.0 g) was suspended in $MeOH/H_2O$ (1:1, v/v, 400 mL), and successively partitioned with *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol. The solvents were removed under reduced pressure to give *n*-hexane (HE; 16.0 g), dichloromethane (DC; 3.7 g), ethyl acetate (EA; 4.2 g), butanolic (BU; 6.6 g) and residual hydromethanolic (HM; 6.8 g) fractions.

An aliquot of HE fraction (7.0 g) was submitted to vacuum liquid chromatography (VLC) (ϕ (internal diameter) = 4.9 cm × h (heigth) = 14.8 cm on silica gel

column, using a gradient of hexane/EtOAc, to afford the subfractions HE-1 to HE-13. The subfraction HE-2 (1.65 g) was subjected to silica gel CC (ϕ = 3.0 cm × h = 26.1 cm) using *n*-hexane/EtOAc gradient as eluents, in order to increasing gradient of polarity, and afforded compound **1** (4.2 mg). Subfraction HE-3 (0.5 g) afforded a mixture of compounds **1-5** (25.2 mg) after CC (ϕ = 2.3 cm × h = 27.0 cm) in silica gel flash using *n*-hexane/EtOAc gradient as eluent. Subfraction HE-5 (150.0 mg) was also submitted to silica gel CC (ϕ = 1.8 cm × h = 22.8 cm) using *n*-hexane/EtOAc gradient as eluent, resulting in twelve fractions (HE-5-1 to HE-5-12). Subfraction HE-5-8 (50.0 mg) was fractionated by CC on silica flash (ϕ = 1.1 cm × h = 27.1 cm) with *n*-hexane/EtOAc gradient, affording compounds **6** and **7** (1.0 mg) in mixture.

An aliquot of DC fraction (1.20 g) was submitted to silica gel CC (ϕ = 2.6 cm × h = 29.6 cm) eluted with *n*-hexane/$CHCl_3$/MeOH gradient giving subfractions DC-1 to DC-15. Subfraction DC-5 afforded compound **10** (3.5 mg). Subfraction DC-6 (370.4 mg) was subjected to silica flash CC (ϕ = 1.9 cm × h = 23.5 cm) using *n*-hexane/acetone gradient as eluents to afford the subfractions DC-6-1 to DC-6-11. Subfractions DC-6-3 (41.4 mg) and DC-6-4 (75.9 mg) were submitted to purification on Sephadex LH-20 CC (ϕ = 0.8 cm × h = 18.6 cm) using $CHCl_3$/MeOH (2:8, v/v) isocratic as mobile phase. Compounds **6** and **7** (10.5 mg, in mixture) and compounds **11** and **12** (2.5 mg, in mixture) were identified in subfractions DC-6-4-3 and DC-6-4-6, respectively. Subfractions DC-6-3-2 (51.8 mg) were submitted to semi-preparative HPLC purification (conditions: Shim-pack PREP-ODS (250 × 20 mm; 15 µm) column, mobile phase MeOH:H_2O (1:1, v/v), 15 mL min^{-1} flow, analysis time: 30 min and λ = 254 nm) affording compounds **8** (2.7 mg, t_R = 10 min) and compound **9** (2.4 mg, t_R = 11 min), and the reisolation of compounds **6** (2.7 mg, t_R = 12 min) and **7** (1.4 mg, t_R = 15 min). Subfraction DC-9 (56.3 mg) was purified by recrystallization in acetone to yield compounds **13** and **14** (9.0 mg) in mixture.

An aliquot of EA fraction (2.0 g) was submitted to Sephadex LH-20® CC (ϕ = 2.1 cm × h = 23.2 cm) using MeOH/H_2O, in decreasing polarity gradient, to obtain subfractions EA-1 to EA-16. Further purification of EA-3 (180.6 mg) using Sephadex LH-20® CC (ϕ = 1.1 cm × h = 24.1 cm) and MeOH/H_2O (1:1, v/v) isocratic as mobile phase, resulted in the isolation of compound **15** (14.6 mg). Compound **16** (35.6 mg) was isolated from subfraction EA-12 (215.6 mg) after CC in Sephadex LH-20® (ϕ = 2.3 cm × h = 23.0 cm) using MeOH/H_2O in decreasing polarity gradient. Compounds **11** and **12** (6.5 mg, in mixture) and **16** (22.4 mg) were reisolated from EA-14 fraction (48.6 mg) after TLC preparative purification using $CHCl_3$:MeOH (6:4, v/v) as mobile phase. Subfraction EA-15 (52.2 mg) was submitted to a PVPP CC (ϕ = 1.2 cm × h = 7.5 cm), using MeOH (isocratic elution), to yield compound **17** (3.0 mg). Compound **15** (6.8 mg) and compound **16** (21.6 mg) were reisolated from HM fraction (1.50 g) after CC in Sephadex LH-20® CC (ϕ = 2.1 cm × h = 23.2 cm) using MeOH/H_2O in decreasing polarity gradient.

Analysis of the dichloromethane fraction by UHPLC-HRMS/MS

The sample was prepared in MeOH (1.0 mg mL^{-1}) and chromatographic separations were performed using UHPLC on a Symmetry C18 column (75 × 2.0 mm i.d.; 1.6 µm Shim-pack XR-ODS III), maintained at a temperature of 40 ºC. The mobile phase consisted of H_2O (solvent A) and 0.1% formic acid in CH_3CN (solvent B). The gradient program was as follows: initial 0-1 min, using elution A-B (95:5, v/v), 1-3 min (30:70 v/v), 3-12 min (5:95 v/v) and kept at 95% B for 16 min at a flow rate of 0.2 mL min^{-1}. Injection volume was 3 µL. High resolution mass spectrometry analysis were carried out in a Q-TOF mass spectrometer via an electrospray ionization interface. The capillary voltage was operated in positive ionization mode, set at 4500 V, using sodium formate (10 µM) as calibrant. The dry gas parameters were set to 8 L min^{-1} at 200 ºC with a nebulization gas presure of 4 bar. Colision-induced dissociation (CID) fragmentation was performed using argon (Ar) collision gas and collision energy from 0-30 eV. Spectra data of the investigated compounds were collected from *m/z* 50-1300 with a resolution of 50.000, and with an acquisition rate of 5 spectrums *per* second. The ions of interest were selected by auto MS/MS scan fragmentation. The data processing software was Data analysis 4.3 (Bruker). Moreover, the mass error value was calculated. Only molecular formulas ≤ 5 ppm of error were considered in this study.[29]

In vitro antiproliferative assay

In vitro antiproliferative activity experiments on human cell lines were performed according to Monks *et al.*[30] The crude extract and fractions of *V. nudiflora* were evaluated *in vitro* against ten human tumor cell lines [U251 (glioma, CNS), UACC-22 (melanoma), MCF-7 (breast), NCI-ADR/RES (ovarian expressing the multiple drug resistance phenotype), 786-0 (renal), NCI-H460 (lung, non-small cells), PC-3 (prostate), OVCAR-3 (ovarian), HT-29 (colon), and K-562 (leukemia)], kindly provided by the National Cancer Institute (Frederick, MA, USA). More, some of the isolated compounds (**6** + **7**, **8**, **9**, **10**) were tested

against six human tumor cell lines [U251 (glioma, CNS), MCF-7 (breast), NCI-ADR/RES (ovarian expressing the multiple drug resistance phenotype), NCI-H460 (lung, non-small cells), PC-3 (prostate), HT-29 (colon)]. All samples were also evaluated against the immortalized human keratinocytes (HaCat) cell line (provided by Prof Dr Ricardo Della Coletta, UNICAMP). Stock solution (0.1g mL^{-1}) of each samples was prepared in DMSO and successively diluted in RPMI 1640, supplemented with 5% fetal bovine serum and 1% penicillin:streptomycin mixture (1000 UI mL^{-1}:1000 μg mL^{-1}) to final concentrations (0.25, 2.5, 25 and 250 μg mL^{-1} for the extract and fractions, and 0.15, 1.50, 15.0 and 150.0 μg mL^{-1} for the isolated compounds). The chemotherapeutic doxorubicin chloridrate (0.025, 0.25, 2.5 and 25 μg mL^{-1}) was used as a positive reference standard to determine the sensitivity of cell lines. Cells in 96-well plates (100 μL cells *per* well) were exposed to sample concentrations, in triplicate, for 48 h at 37 °C and 5% of CO_2. The final DMSO concentration (≤ 0.25%) did not affect cell viability. Before (plate control) and after sample addition, cells were fixed with 50% trichloroacetic acid, and cell proliferation was determined by spectrophotometric quantification of cellular protein content at 540 nm (Molecular Devices, model VersaMax) using sulforhodamine B. Two effective concentrations, named growth inhibition 50% (GI_{50}) and total growth inhibition (TGI), were calculated by non-linear regression analysis (sigmoidal fit) using Origin 7.5® (OriginLab Corporation).[31] The selectivity index (SI) was calculated as SI = GI_{50} HaCat / GI_{50} tumor cell line.[32]

In vitro antioxidant assays

DPPH free-radical scavenging activity

The free radical scavenging effect of the crude extract and fractions of *V. nudiflora* was investigated using the DPPH (1,1-diphenyl-2-picrylhydrazyl) (Sigma-Aldrich) assay, based on the method proposed by Boroski *et al.*[33] with slight modifications. Serial concentrations (5, 10, 25, 50, 75, 100, 150 and 250 μg mL^{-1}) of extract and fractions were prepared by addition of 20 mg of samples and 10 mL of methanol and 2 mL of DPPH methanolic solution (3.20 mg DPPH in 100 mL) were mixed with these solutions. The mixture was thoroughly vortex-mixed and kept in the dark for 30 min. Absorbance was measured using a UV-Vis spectrophotometer at 517 nm. Rutin and ascorbic acid were used as standards antioxidant compounds. All samples were tested in triplicate. The results were expressed as percent inhibition of the DPPH radical, which was calculated using the equation 1:

$$\% \text{ inhibition} = [(A_{DPPH} - A_{sample}) / A_{DPPH}] \times 100] \quad (1)$$

where A_{DPPH} is the absorbance of the DPPH solution and A_{sample} is the absorbance of the DPPH solution with the sample tested. The sample concentration that afforded 50% inhibition (IC_{50}) was obtained by plotting the concentrations of the sample solutions *versus* percent inhibition.

$ABTS^{\bullet+}$ radical scavenging activity

The ABTS method was performed according to method described by Re *et al.*[34] with some modifications. Briefly, 7.0 mM of ABTS (2,2-azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid) (Sigma-Aldrich) and potassium persulfate (140 mM) were mixed, and keeping in the dark for 16 h at room temperature. For the analysis, the $ABTS^{\bullet+}$ solution was diluted in ethanol (PA) to afford the $ABTS^{\bullet+}$ work solution (absorbance of 0.700 ± 0.050 at 734 nm). Aliquots (30 μL) of each extract and fractions, at different concentrations in triplicate, were homogenized with $ABTS^{\bullet+}$ work solution (3 mL) and, after 6 min, the absorbance was measured at 734 nm using a UV-Vis spectrophotometer. The results were expressed as percent inhibition of the ABTS radical, using the equation described in the DPPH assay. Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid; Sigma Aldrich) was used as antioxidant standard.

Total phenolic compounds (TPC)

The total phenolic content (TPC) was determined using the Folin-Ciocalteu method as described by Singleton and Rossi,[35] and adapted by Boroski *et al.*[36] One aliquot (0.25 mL) of each sample in MeOH (2.5 mg mL^{-1}) was diluted in distilled water (4 mL), and mixed with Folin-Ciocalteu reagent (0.25 mL) and saturated sodium carbonate solution (0.50 mL). This mixture was kept in the dark at room temperature (23-25 ºC) for 1 h before the absorption measurement at 765 nm. Gallic acid was used as standard in the calibration curve. Results were expressed as milligrams of gallic acid equivalents *per* gram of sample (mg GAE g^{-1} extract).

Total flavonoids (TF)

The total flavonoid (TF) content of crude extract and fractions was determined by aluminium chloride colorimetric method,[37] adapted by Boroski *et al.*[36] Aliquot (0.5 mL, in triplicate) of each sample diluted in MeOH (2.5 mg mL^{-1}) was mixed with 5% $AlCl_3$ solution (0.25 mL) and methanol (4.25 mL) in graduated centrifuge tubes (15 mL). The resulting mixture was allowed to rest for 30 min at room temperature, protected from light. After that, the absorbance at 415 nm was measured. Quercetin was used as standard.