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Antiproliferative activity of *Eremanthus crotonoides* extracts and centratherin demonstrated in brain tumor cell lines

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Abstract: The genus *Eremanthus* is recognized by the predominance of sesquiterpene lactones from the furanoheliangolide type, a class of substances extensively tested against cancer cell lines. Thus, the species *E. crotonoides* (DC.) Sch. Bip., Asteraceae, obtained on "restinga" vegetation was evaluated against U251 and U87-MG glioma cell lines using the MTT colorimetric assay. Dichloromethane fraction was cytotoxic to both glioblastoma multiforme cell lines. We then conducted UPLC-PDA-ESI-MS/MS analysis of the dichloromethane fraction, which allowed the identification of the sesquiterpene lactones centratherin and goyazensolide. The isolation of centratherin was performed using chromatographic techniques and the identification of this substance was confirmed according to NMR data. Cytotoxic activity of centratherin alone was also evaluated against both U251 and U87-MG cells, which showed IC50 values comparable with those obtained for the commercial anticancer drug doxorubicin. All the tested samples showed cytotoxic activity against glioblastoma multiforme cells which suggests that *E. crotonoides* extracts may be important sources of antiproliferative substances and that the centratherin may serve as prototype for developing new antiglioblastoma drugs.

Article

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Introduction

Plants from the Asteraceae family are commonly used in Brazilian folk medicine, health care and as foodstuffs. The subtribe Lychnophorinae (Vernonieae, Asteraceae) is endemic to Brazil and many of their species are used in anti-inflammatory preparations. However, other biological activities have been described such as antimicrobial and cytotoxic properties, especially for the *Lychnophora* and *Eremanthus* species (Santos et al., 2009; Keles et al., 2010). The phytochemical profile of this subtribe indicates a predominance of sesquiterpene lactones and flavonoids. In current medical research, extracts obtained from species rich in sesquiterpene lactones have been extensively tested against cancer cell lines, allowing for the discovery of promising anticancer drugs. These include thapsigargin, parthenolide and derivatives of these compounds that are currently in clinical trials for the treatment of cancer (Ghantous et al., 2010).

The genus *Eremanthus* Less comprises 27 species of trees and shrubs that are normally found in "cerrado" (Brazilian savanna), but *E. crotonoides* is an exception that can also be found in "restinga" (sandy

coastal plains) (Santos et al., 2009). Studies carried out for species from this genus indicated the predominance of sesquiterpene lactones from the furanoheliangolide class (goyazensolide and eremantholide types) as their characteristic constituents (Vichnewski & Gilbert, 1972; Raffauf et al., 1975; Bohlmann et al., 1980, 1981, 1982; Vichnewski et al., 1989; Rüngeler et al., 1998; Sacilotto et al., 2002; Sakamoto et al., 2005).

Therefore, the aim of the present work was to evaluate antiproliferative effects of extracts and sesquiterpene lactone from *Eremanthus crotonoides* against two brain tumor cell lines.

Materials and Methods

Plant material

The leaves of *Eremanthus crotonoides* (DC.) Sch. Bip., Asteraceae, used in this work were collected in Restinga de Jurubatiba National Park, Rio de Janeiro, Brazil, and identified by the botanist Dr. Marcelo Guerra Santos, Universidade do Estado do Rio de Janeiro, Brazil. A voucher specimen (M. Guerra Santos 2150) has been deposited at the Herbarium of the Faculdade de Formação de Professores, Universidade Estadual do Rio de Janeiro, Brazil.

Preparation of extracts

The air-dried and powdered leaves (1.8 kg) of *E. crotonoides* were extracted with ethanol (98%) at room temperature for seven days with daily agitation. After evaporation of the ethanol under reduced pressure, the ethanolic extract (ECE, 145.3 g) was suspended in water and then sequentially extracted with *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol to give *n*-hexane (ECH, 58.3 g), dichloromethane (ECD, 36.5 g), ethyl acetate (ECEA, 12.9 g), and *n*-butanol (ECB, 27.6 g) fractions, respectively. All solvents were purchased from Tedia (USA).

Sample preparation for UPLC analysis

ECD fraction was analyzed by chromatography using UPLC-PDA-ESI-MS/MS to identify the presence of sesquiterpene lactones. An aliquot of 2.5 mg of ECD was weighed in a glass vial and then was added to 2.5 ml of a solution of MeOH-H_2O (4:6). The final solution (1 mg/mL) was filtered on a 13 mm GHP, 0.22 µm microporous membrane and then submitted to UPLC analysis (5.00 µL) by autosampler.

UPLC method condition

The analysis was performed using a Waters Acquity UPLC system equipped with a waters Acquity UPLC eλ PDA detector and managed by MassLynx software version 4.1 (SCN 714). The analytical chromatography column used to separate the components of the sample was an Acquity 1.7 µm (2.1 x 50 mm) BEH (*Ethylene Bridged Hybrid*) C18, equipped with an equivalent material pre-column. The gradient program employing a flow rate of 0.3 mL/min was as follows: solvent A: water; solvent B: methanol; elution profile: 0-10 min, 10-100% B (linear gradient), 10-12 min, 100% B (isocratic), 12-12.5 min, 100-10% B (linear gradient), 12.5-15 min, 10% B (isocratic). The PDA detector was monitored to record between 200 nm and 400 nm and the column temperature was established at 30º C.

Electrospray ionization (ESI) MS/MS conditions

The MS/MS system utilized was a Waters Acquity TQD tandem quadrupole mass spectrometer integrated with a UPLC system via an ESI ion source. The analyses were performed in the positive mode (ES+). The conditions used were as follows: capillary voltage, 3.49 kV; cone voltage, 28.08 V; source temperature, 148 ºC; desolvation temperature, 250 ºC; cone gas flow, 2.0 l/h; desolvation gas flow (N2), 497 L/h; collision gas flow, 0.10 mL/min; collision gas, argon; nebulizer gas, high-purity nitrogen. The value of 10 eV was the best energy collision, because it permits the display of the maximum intensity of product ions.

Fractionation and isolation

ECD fraction (6.0 g) was submitted to a rapid silica-gel (70-230 mesh) column clean-up under vacuum conditions by using the solvents *n*-hexane (1,0 L), *n*-hexane (0,5 L), dichloromethane (1,0 L), dichloromethane-ethyl acetate (6:4) (0,5 L), dichloromethane-ethyl acetate (4:6) (0,5 L), ethyl acetate (1,0 L) and ethanol (1,0 L) (Tedia, USA), to give fractions FR1, FR2, FR3, FR4, FR5, FR6 and FR7, respectively. These six fractions were analyzed by TLC GF254 (Macherey-Nagel) using *n*-hexane-ethyl acetate-methanol (5:5:1) as eluent system. Subsequently, a visual inspection was performed under short-wave UV (254 nm). FR4 (250.0 mg) was submitted to reversed–phase (C18) vacuum-liquid chromatography eluted with ethanol-water (4:6), ethanol-water (6:4), ethanol and ethyl acetate to obtain the sub fractions SUB-1, SUB-2, SUB-3, and SUB-4 respectively. The SUB-1 (138.5 mg) was purified by preparative TLC eluted with *n*-hexane-ethyl acetate-methanol (5:5:1) yielding the majority compound EC1 (118.7 mg). ^{1}H and ^{13}C NMR analysis of this substance were carried out in Bruker DPX-200 MHz spectrometer. Deuterated chloroform (Cambridge Isotope Laboratories, USA) was used for solubilization and TMS peak was used as internal standard.

Cytotoxicity assay

The human glioma cancer cell lines U87-MG and U251 were acquired from ATCC (Washington, DC, USA). These cells were maintained in DMEM (GIBCO, SP, Brazil) supplemented with 10% fetal bovine serum inactivated at 56 °C, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, SP, Brazil) at 37 °C in a humid atmosphere containing 5% CO_2. Briefly, Cells (5 x 10^4/well) were plated in 96-well plates and on the next day incubated with EEC, ECD, or centratherin at different concentrations (0.005-160 µg/mL) over a period of 24 h. Doxorubicin (Bergamo Ltda, SP, Brazil) was used as a positive control, while DMSO (Merck, RJ, Brazil) as a negative control. After incubation, cytotoxicity was assessed by MTT (Sigma Aldrich, St. Louis, USA) reduction (Mosman, 1983) and quantified with a spectrophotometer (mQuant, Biotek Instruments) at 545 nm. The results of 3 experiments in triplicate were used to calculate IC50 by nonlinear regression using GraphPad prism 5.0 Software.

Morphological analysis

The morphology of U87-MG and U251 cell lines treated with 10 µg/mL of ECE, ECD and centratherin or DMSO 0.01% for 24 h was directly observed and photographed with an inverted light microscope (Bel Photonic INV100-FL). All photos were taken at 200x magnification.

Results and Discussion

Regarding literature data, it can be observed that sequential partition of ethanolic extract from *Eremanthus* species with solvents of increasing polarity allow the achievement of a sesquiterpene enriched dichloromethane fraction (Sakamoto et al., 2005, 2010). Thus, chromatographic fingerprint analysis of the dichloromethane fraction (ECD) from leaves of *Eremanthus crotonoides* (DC.) Sch. Bip., Asteraceae, was carried out by UPLC-PDA-MS and MS/MS. In the total ion chromatogram obtained by UPLC-PDA-

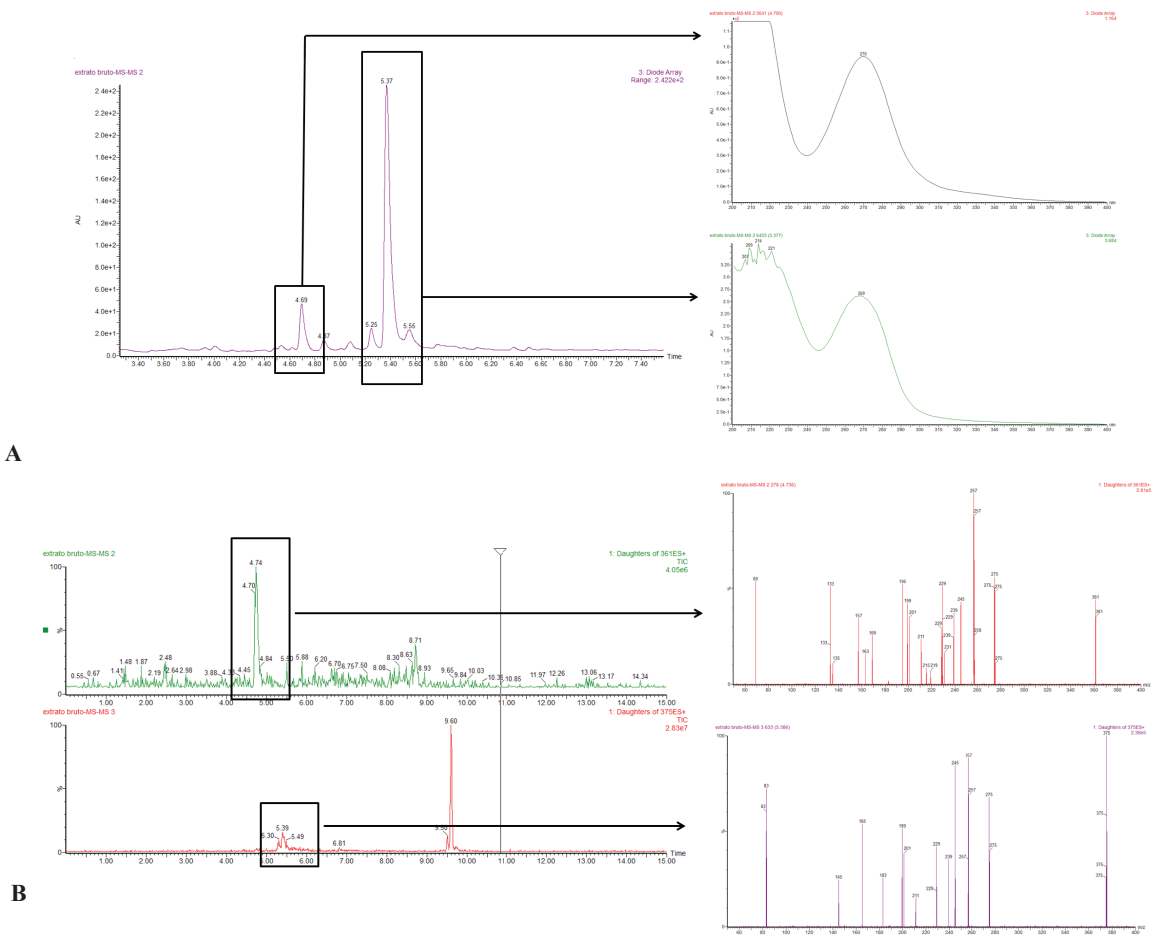
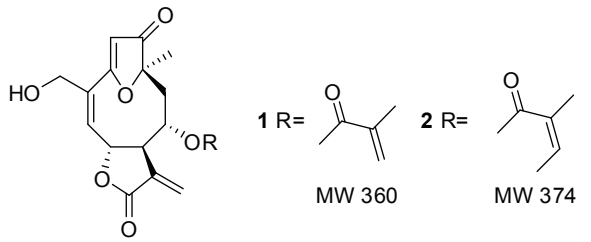


Figure 1. Chromatograms obtained from UPLC-PDA-ESI-MS/MS analysis in the dichloromethane fraction (ECD) obtained from leaves of *Eremanthus crotonoides*. A. PDA chromatogram and the UV spectra at 4.7 and 5.4 min peaks, and B. MS/MS chromatogram and the product ion spectrum in the positive ion mode, obtained by MS/MS analysis, from the ions at *m/z* 361 and 375, respectively.

MS/MS analysis (Figure 1A,B), peaks at 4.7 and 5.4 min, exhibited precursor ions at *m/z* 361 and 375, respectively, as well as typical sesquiterpene lactone UV spectra (λ_{max}: 270 nm) (Moraes et al., 2009). The product ion spectrum in the positive mode, obtained by MS/MS analysis, from precursor ion at *m/z* 361 $[M+H]^+$ showed initially a loss of 86 units leading to the formation of an ion at *m/z* 275, which have been associated with the corresponding esters in C-8, being eliminated as carboxylic acid [$C_3H_5CO_2H$]. After this, 18 and 28 units were lost from the fragment ion at *m/z* 275 [M+H - $C_3H_5CO_2H$] to generate ions at *m/z* 257 [M+H - $C_3H_5CO_2H$ - H_2O] + and *m/z* 247 [M+H – $C_3H_5CO_2H$-CO], which are formed by the loss of H_2O and CO respectively. The ion at *m/z* 257 (presented as the base peak) lost CO (28 units) to produce a signal at *m/z* 229, followed by the loss of H_2O (18 units) to generate an ion at *m/z* 211. The ion at m/z 201 was generated by the loss of CO (28 units) from the ion at *m/z* 257. The MS spectra obtained from precursor ion at *m/z* 361 was compared with literature data (Crotti et al., 2005) and therefore allowed the identification of goyazensolide (**1**). Subsequently, the MS/MS product ion spectrum from the precursor ion at *m/z* 375 [M+H] showed the loss of 100 units leading to the formation of a product ion at *m/z* 275. This process is characterized by the loss of esters in C-8 (eliminated as carboxylic acid). Afterward, the ion at *m/z* 275 [M+H - $C_4H_7CO_2H$] lost H_2O (18 units) and CO (28 units) leading the formation of ions at *m/z* 257 and at *m/z* 247. The fragment ion at *m/z* 257 (base peak) lost 28 units (CO) to produce a signal at *m/z* 229, followed by the loss of 18 units (H_2O) leading to the formation of an ion at *m/z* 211. There is a signal at *m/z* 239, which was generated by the loss of 2 molecules of water (36 units) from the ion at *m/z* 275. The MS spectra obtain from precursor ion at *m/z* 375 was compared with literature data (Crotti et al., 2005) allowing the identification of centratherin (**2**).

Aditional column fractionation and preparative thin-layer chromatography allowed the obtainment of an amorphous white powder (118.7 mg). The isolated compound was confirmed as centratherin (2-butenoic acid, 2-methyl-, (3a*R*,4*S*,6*R*,10*Z*,11a*R*)-2,3,3a,4,5,6,7,11a-octahydro-10-(hydroxymethyl)-6-methyl-3-methylene-2,7-dioxo-6,9 epoxycyclodeca [*b*]furan-4-yl ester, (2*Z*)-) by comparison of its ^1H and ^{13}C NMR spectra with those published in the literature (Ohno et al., 1979; Vichnewski et al., 1990). In addition, PDA chromatogram indicated that centratherin (Rt 5.4 min) (Figure 1A) presented the major relative percentual on ECD.



The first phytochemical investigation of leaves from *E. crotonoides*, which were collected in "Cerrado" (Brazilian savanna), described three new furanoheliangolides, in addition to six known furanoheliangolides (Bohlmann et al., 1982). However, to our knowledge, the sesquiterpene lactones from the furanoheliangolide class centratherin (**2**) and goyazensolide (**1**) had not been previously identified in this species. It is interesting for an ecological overview that *E. crotonoides* was collected in "restinga" habitat. Since secondary metabolites represent a chemical interface between plants and surrounding environment (Gobbo-Neto et al., 2007), this aspect must be considered regarding to the difference in chemical composition of the present study, characterized by the high content of centratherin, when compared to its previous phytochemical characterization.

ECE and ECD fractions obtained from leaves of *E. crotonoides* were cytotoxic to both tested glioblastoma multiforme cell lines (U87-MG and U251) (Table 1). Glioblastoma multiforme is the most aggressive form of glioma and, despite recent research advances into glioma genesis and treatment, it continues to be a lethal disease with a dismal prognosis (Adamson et al., 2009). Previous studies described antiproliferative effects of centratherin against small cell lung cancer (NCI-H187) (Vongvanich et al., 2006) and goyasenzolide against seven different tumor cell lines, especially leukemic cell lines (Santos et al., 2004). As part of ongoing studies for discovering substances with potential antiproliferative action against glioblastoma cells, the isolated sesquiterpene lactone centratherin was also tested. It showed an IC50 of 3.57 and 8.06 µg/mL (Table 1) against U87-MG and U251 cell lines, respectively.

Table 1. IC50 values for antitumor effects obtained by treatment with ECE, ECD, and centratherin against glioma cell lines (U251 and U87-MG). Doxorubicin was used as positive control.

Cell line	IC50 (µg/mL)			
	ECE	ECD	Centratherin	Doxorubicin
U251	61.06±(44.48-83.82)	19.56±(15.67-24.42)	8.06±(7.00-9.22)	10.43±(6.35-17.13)
U87	46.14±(32.72-65.07)	16.25±(6.78-30.97)	3.57±(2.39-5.33)	3.85±(0.89-16.63)

Furanoheliangolides often exhibit anti-inflammatory, trypanocidal, cytotoxic, and genotoxic activities (Grael et al., 2000; Sakamoto et al., 2003; Santos et al., 2004; Vasconcelos et al., 2007;). The furanoheliangolides possess two functionalities in the form of an α-methylene-γ-lactone and an α,β,γ,δ unsaturated carbonyl group (Rüngeler et al., 1999) that can react through a stereospecific Michael addition with biological nucleophiles (mainly with the thiol groups of cysteine residues present in proteins) forming stable adducts (Ghantous et al., 2010).

Morphological analysis indicated that cells treated with ECE, ECD and centratherin showed cytotoxicity effects, like rounded shape and decreased number caused by cell death. Centratherin was more effective against U87 cells than to U251 cells (Figure 2), corroborating results obtained through IC50 values. This difference would be due to different genetic backgrounds of cell lines such as *TP53* status. *TP53* codes the p53 protein, an important apoptosis regulator. Mutations in *TP53* were related to chemoresistence (Bossi & Sacchi, 2007). Considering *TP53* status, U87 cell line is *TP53* wild type and U251 is mutant (Chen et al., 1995). The IC50 values obtained for the centratherin was comparable to those obtained for the commercial anticancer drug doxorubicin (Table 1).

Conclusion

The present study demonstrated the antiproliferative activity of phytochemicals derived from *E. crotonoi*des. The ethanolic extract obtained from leaves, and the dichloromethane fraction that originated from this extract, as well as the furanoheliangolide centratherin were shown to be active against glioblastoma multiforme cells (U87-MG and U251). Therefore, this substance may be useful as prototype of anticancer substances, suggesting the great potential of this species as source of bioactive substances.

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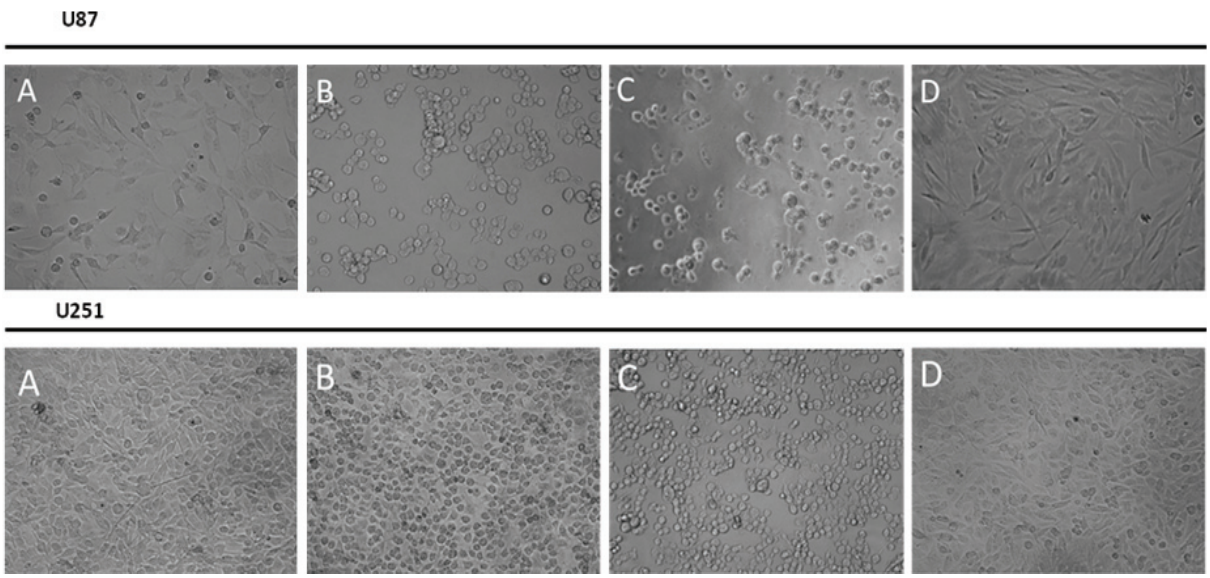

Figure 2. Morphological analysis of U87-MG and U251 cell lines after 24 h treatment. Cells treated with A. ECE (10 µg/mL); B. ECD (10 µg/mL); and C. centratherin (10 µg/mL) showed cytotoxicity effects, like rounded shape and decreased number, when compared with negative control treated with 0.01% DMSO (D). At 10 µg/mL concentration ECE and ECD had lower cytotoxic effect than centratherin.

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