



Original Article

Flavonoids from the Brazilian plant *Croton betulaster* inhibit the growth of human glioblastoma cells and induce apoptosis



Paulo Lucas Cerqueira Coelho^a, Sandra Regina Villas-Boas de Freitas^a, Bruno Penas Seara Pitanga^a, Victor Diógenes Amaral da Silva^a, Mona Neves Oliveira^a, Maria Socorro Grangeiro^a, Cleide dos Santos Souza^a, Ramon dos Santos El-Bachá^a, Maria de Fátima Dias Costa^a, Pedro Rocha Barbosa^b, Ivana Lucia de Oliveira Nascimento^c, Silvia Lima Costa^{a,*}

^a Laboratório de Neuroquímica e Biologia Celular, Instituto de Ciências da Saúde, Universidade Federal da Bahia, Salvador, BA, Brazil

^b Instituto de Química, Universidade Federal da Bahia, Salvador, BA, Brazil

^c Laboratório de Imunologia e Biologia Molecular, Instituto de Ciências da Saúde, Universidade Federal da Bahia, Salvador, BA, Brazil

ARTICLE INFO

Article history:

Received 19 February 2015

Accepted 19 May 2015

Available online 1 October 2015

Keywords:

Flavonoids

Croton betulaster

Glioblastoma

Apoptosis

ABSTRACT

This study investigated the effects of the flavonoids 5-hydroxy-7,4'-dimethoxyflavone, casticin, and penduletin, isolated from *Croton betulaster* Müll Arg., Euphorbiaceae, a plant utilized in popular medicine in Brazil, on the growth and viability of the human glioblastoma cell line GL-15. We observed that 5-hydroxy-7,4'-dimethoxyflavone and casticin were not toxic to GL-15 cells after 24 h of exposure. However, casticin and penduletin inhibited the metabolic activity of glioblastoma cells significantly at a concentration of 10 μ M ($p \leq 0.05$). Flavonoids casticin and penduletin also induced a significant and dose-dependent growth inhibition beginning at 24 h of exposure, and the most potent flavonoid was penduletin. It was also observed that penduletin and casticin induced an enlargement of the cell body and a reduction of cellular processes, accompanied by changes in the pattern of expression of the cytoskeletal protein vimentin. Signs of apoptosis, such as the externalization of membrane phosphatidyl serine residues, nuclear condensation, and fragmentation, were also detected in cells treated with 50–100 μ M flavonoids. Our results indicate that flavonoids extracted from *C. betulaster* present antitumoral activity to glioblastoma cells, with penduletin proving to be the most potent of the tested flavonoids. Our results also suggest that these molecules may be promising supplementary drugs for glioblastoma treatment.

© 2015 Sociedade Brasileira de Farmacognosia. Published by Elsevier Editora Ltda. All rights reserved.

Introduction

Croton betulaster Müll. Arg., Euphorbiaceae, is a shrub found in the northern part of the Cadeia do Espinhaço, from Grão Mogol to the Serra do Sincorá in the Chapada Diamantina, Bahia, Brazil (Stannard, 1995). *C. betulaster* is a plant used in popular medicine to treat diabetes and diarrhea and is also known for its cicatrizing, anti-inflammatory, anti-malarial, anti-carcinogenic, anthelmintic and insecticidal properties. Barbosa et al. (2003) isolated flavonoids with different degrees of methylation from *C. betulaster*. Flavonoids are polyphenolic compounds of natural origin that are especially abundant in flowers and fruits and have raised interest in scientific community (Bruneton, 1991) due to their biological activities. These molecules have several functions

in plants: protecting leaves from ultra violet rays, inducing growth and attracting pollinators. Several studies have demonstrated that flavonoids have antioxidant, analgesic, antibacterial, antifungal, anti-inflammatory, antiviral, antitumor, antiallergic, and antiparasitic activities (Friedman, 2007; Ozcelik et al., 2006; Ye et al., 2007; Uyana, 2007; Bukhari et al., 2007; Cai et al., 2006; Pantev et al., 2006; Roy et al., 2007; Kawai et al., 2007; Mead and McNair, 2006). Recent studies demonstrated that flavonoids can cross the blood–brain barrier, especially when they are highly methylated (Youdim et al., 2003, 2004; Walle et al., 2007; Xu et al., 2007).

Glioblastoma multiforme (GBM) is the most aggressive and most frequent primary tumor of the central nervous system, comprising approximately 50% of cerebral gliomas. They are highly invasive, rapidly proliferating tumors that develop in cerebral hemispheres and present a poor prognosis. Surgical removal of the tumor constitutes the first line of therapy. Unfortunately, glioblastoma cells are highly mobile and also infiltrate the surrounding tissues. Thus, in most cases, surgery has to be followed by radiation therapy.

* Corresponding author.

E-mails: costasl@ufba.br, silvia.lima@pq.cnpq.br (S.L. Costa).

Standard chemotherapy consists in alkylating drugs, such as carboplatin, carmustine, fotemustine or temozolomide. Despite recent advances in these therapies, the median survival time for glioblastoma patients continues to be approximately 12 month. Agents such as tamoxifen, selenium, retinoids and cytokines have been proposed, but their effects remain a topic of discussion (Defer et al., 1997; Chambaut-Guérin et al., 2000; Costa et al., 2001; Roprai et al., 2007). New natural compounds with anti-tumor potential may offer an alternative to the traditional cytotoxic treatments. In this study, we used a model the human high proliferative glioblastoma cell line GL-15 to investigate the effects of the flavonoids 5-hydroxy-7,4'-dimethoxyflavone (dimethoxyflavone), 5',4'-dihydroxy-3,6,7-trimethoxyflavone (penduletin), and 5,3'-dihydroxy-3,6,7,4' tetramethoxyflavone (casticin) isolated from *C. betulaster* Müll. Arg. on the viability, proliferation and induction of morphological changes on malignant glioma cells.

Materials and methods

Extraction, isolation and characterization of flavonoids

The flavonoids 5-hydroxy-7,4'-dimethoxyflavone (dimethoxyflavone), 5',4'-dihydroxy-3,6,7-trimethoxyflavone (penduletin), and 5,3'-dihydroxy-3,6,7,4'-tetramethoxyflavone (casticin) were obtained from the leaves of *Croton betulaster* Müll. Arg., Euphorbiaceae (Barbosa et al., 2003). *C. betulaster* is a shrub found in the northern part of the Cadeia do Espinhaço, from Grão Mogol to the Serra do Sincorá in the Chapada Diamantina, Bahia, Brazil (Cordeiro, 1995). Aerial parts of *C. betulaster* were collected in June 1997, from Palmeiras, Chapada Diamantina, Bahia, Brazil, and were identified by Maria Lenise S. Guedes from the Instituto de Biologia, UFBA. A voucher specimen has been deposited in the herbarium of the Federal University of Bahia (ALCB number 031762), Brazil. The air-dried leaves (600 g) of *C. betulaster* were extracted with hexane, followed by dichloromethane and finally methanol. Successive silica gel columns (CC) of dichloromethane extracted from the leaves (45 g) using increasing amounts of EtOAc in hexane yielded casticin (450 mg) and penduletin (81 mg). The methanol extract was dissolved in MeOH–H₂O (9:1) and partitioned against hexane, dichloromethane, AcOEt and *n*-butanol. The AcOEt-soluble portion of the methanol extract chromatographed on a silica gel CC and eluted with a gradient of hexane–EtOAc yielded 5-hydroxy-7,4'-dimethoxyflavone (25 mg). The flavonoid structures were identified by an NMR data analysis and compared with the data reported in the literature.

Cell culture and treatments

The GL-15 cell line was derived from human glioblastoma multiforme and was cultured as previously described (Chambaut-Guérin et al., 2000; Costa et al., 2001). Briefly, cells were grown until confluence in the cell culture dishes (TPP) in Dulbecco's Modified Eagle's Medium (DMEM, Cultilab, SP, Brazil) and were supplemented with 100 UI/ml penicillin G, 100 µg/ml streptomycin, 7 mM glucose, 2 mM L-glutamine, 0.011 g/l pyruvate, and 10% fetal calf serum (Gibco, Grand Island, NY) in a humidified atmosphere with 5% CO₂ at 37 °C. The flavonoids from *C. betulaster* were dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO) at a concentration of 20 mM and stored in the dark at –20 °C. The flavonoids were then dissolved in medium at final concentrations of 10, 50 and 100 µM and incubated for 24–72 h. Control cells were treated with the same volume of DMSO, not exceeding 0.5%, and did not show any significant effect within the parameters analyzed compared to cultures not exposed to this solvent; therefore, these cells were adopted as the negative control.

Cell viability – MTT test

The flavonoids' effect on GL-15 cell viability was tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) test after 24 and 72 h exposure in 96-well plates (TPP Switzerland, 1 × 10⁴ cells/well). The cell viability was quantified by the conversion of yellow MTT by mitochondrial dehydrogenases of living cells to purple MTT formazan. Three hours before the end of the exposure duration, the medium was removed and replaced with DMEM without supplementation containing MTT (1 mg/ml). Afterwards, 100 µl/well of a buffer containing 20% SDS and 50% DMF, pH 4.7, was added, and the plate was kept for 12 h at 37 °C for complete dissolution of the formazan crystals. The optic absorbency of each sample was measured at a wavelength of 560 nm using a BIO-RAD 550PLUS spectrophotometer (Hercules, CA). Four replicate wells were used for each analysis. The results were determined as the average ± standard deviation (SD) and expressed as percentages of the control group viability.

Assay of proliferation – thymidine incorporation

The effect of flavonoids from *C. betulaster* on cellular proliferation was studied by measuring the incorporation of 2-(methyl-³H) thymidine (1 µCi/ml, Amersham). In brief, the cells were cultured into 96-well plates (TPP, Switzerland) (1 × 10⁴ cells/well) and incubated in four replicates with 1–100 µM flavonoids or 0.5% DMSO (control) for 24 and 72 h. The cells were pulsed-labeled for the final 5 h, and 1 µCi/200 µl of [³H]-thymidine was added to the culture medium. At the end of incubation, the cells were collected in a glass fiber filter (Packard) using a cell harvester (Filtermate 196, Packard, Meriden, CT, USA) and allowed to dry for 24 h. Incorporation of radioactive thymidine was determined by gas scintillation in a direct beta counter (Packard). Cell counts were based on four independent experiments, and the results (mean ± SD) were expressed as percentage of the incorporation of the control (considered as 100%).

Cell cycle analysis – flow cytometry

Flow cytometry analysis of DNA content was performed to assess the cell cycle phase distribution in control conditions (0.5% DMSO) or after flavonoids casticin or penduletin (50 µM) was added to logarithmically growing GL-15 cells. After 48 h exposure, GL-15 cells were harvested by trypsinization, fixed with cold ethanol and stained with propidium iodide (Biosource, Camarillo, CA). Flow cytometry was performed on a BD FACS Calibur. The software BD Cell Quest™ Pro was used to generate histograms and determine the cell cycle phase distribution. Experiments were performed at least three times.

Apoptosis detection – Annexin-V analysis and chromatin staining

Apoptosis was detected by monitoring the phosphatidylserine externalization using an Annexin-V Cy3/6-carboxyfluorescein diacetate (6-CFDA) staining kit (Sigma–Aldrich, St. Louis, MO). 6-CFDA is used to measure viability; upon entering living cells, this non-fluorescent compound is hydrolyzed by the esterases present, producing the fluorescent compound 6-carboxyfluorescein (6-CF). Treated and non-treated cells were cultured for 24 h in Plates 40 mm in diameter (1.5 × 10⁵ cells/plate). The cells were stained with 100 µl of binding buffer containing Annexin V-FITC (1 µg/ml), 6-CFDA (500 µM), dH₂O and binding buffer (100 mM HEPES/NaOH, pH 7.5; 1.4 M NaCl; 25 mM CaCl₂), as per the kit recommendations. After 15 min of incubation at room temperature in the dark, cells were analyzed using a fluorescence microscope (Olympus BX-2), and images were recorded using a Variocam digital camera (PCO,

Germany) connected to a personal computer. At least ten fields were analyzed and recorded, and the proportion of Annexin V/6-CF positive cells (referring to apoptosis) was determined. The apoptosis of GL-15 cells was also determined by the fluorescent dye Hoecht 33342 staining (Sigma–Aldrich, St. Louis, MO), which allows for the determination and quantification of the cells with fragmented and condensed nuclear chromatin. Control and treated cells seeded on 40 mm polystyrene culture plates (1.5×10^5 cells/plate) were rinsed three times with PBS and fixed with cold methanol at -20°C for 10 min. Chromatin was stained with Hoecht 33258 ($5 \mu\text{g}/\text{ml}$ in PBS) for 10 min at room temperature in a dark chamber. Thereafter, cells were analyzed using an epifluorescent microscope (Olympus BX-2), and images were recorded using a Variocam digital camera (PCO, Germany) connected to a personal computer. Ten randomized representative fields were analyzed each time. The proportion of fragmented nuclei stained with Hoecht 33258 was determined in 10 microscopic fields for each experimental point.

Fragmentation and nuclear condensation – chromatin staining by Hoecht-33258

The apoptosis of GL-15 cells was also determined by Hoecht-33258 staining (Sigma–Aldrich, St. Louis, USA), which allows for the determination and quantification of cells with fragmented and condensed chromatin. After washing with PBS, GL-15 cells cultured on 40-mm plates (1.5×10^5 cells/plate) were fixed for 10 min with methanol and stored at -20°C for 20 min. Subsequently, fixed cells were stained with the fluorescent dye Hoecht-33258 at a final concentration of $5 \mu\text{g}/\text{ml}$ in PBS for 10 min in a dark chamber at room temperature. The cells were then washed with PBS, and the plates were analyzed by fluorescent microscopy (Olympus AX70) and photographed. The apoptotic index represents the percentage of fragmented nuclei and was determined in a microscopic field of at least 100 cells/experimental point.

DNA fragmentation – comet assay

DNA integrity and single-strand breaks were monitored using single-cell gel electrophoresis (comet assay) performed under alkaline conditions based on a slightly modified version of the procedure described by Ribeiro et al. Briefly, all control and treated cells seeded on 40-mm polystyrene plates (1.5×10^5 cells/plate) were incubated for 24 or 72 h in the presence of flavonoids, with 0.5% DMSO as a negative control or 1 h direct exposure to UV light as a positive control. After treatment, the cells were scraped at 4°C and centrifuged at $1000 \times g$ for 10 min. The pellet was diluted in $150 \mu\text{l}$ of PBS. A total of $30 \mu\text{l}$ of the cell suspension was mixed with $300 \mu\text{l}$ 1% (w/v) low-melting-point agarose (Sigma–Aldrich), applied to the surface of a normal-melting-point agarose pre-coated slide to form a microgel, and allowed to set for 5 min at -20°C . Microgels were submerged in cell lysis buffer (14.61% NaCl, 3.72% EDTA, 0.12% Tris, pH 10.1%, Triton X-100, 10% DMSO) for one h at 4°C , protected from light. Following cell lysis, all slides were washed with PBS for 10 min to remove salt and detergent from the microgel. The slides were placed in a horizontal electrophoresis unit and were allowed to equilibrate for 20 min with an electrophoresis buffer (0.034% EDTA; 0.3 N NaOH, pH 13). Electrophoresis was performed for 25 min at 0.13 A and 25 V. Slides were rinsed with neutralization solution (12.12% Tris HCl, pH 7.5) three times for 5 min each time, fixed with 100% ethanol, air-dried, and stored in a light-protected location until analysis. For analysis and scoring, the DNA in the comet slides microgels was stained with 0.1% ethidium bromide ($25 \mu\text{l}$) for 5 min, and coverslips were applied before image analysis. The comet-assay samples were analyzed at $200\times$ magnification using an epifluorescent microscope (Olympus BX-2) and a rhodamine filter. Three replicate experiments were performed with

two slides per experiment and a UV irradiation point. The image of the electrophoresed DNA looks like a comet, with undamaged DNA as the head and fragmented, migrating DNA forming the tail. Comet images were recorded using a Variocam digital camera (PCO, Germany) connected to a personal computer. Two hundred randomly selected cells were scored from each slide (two slides per dose), and the percentage of comet cells (comet rate) was calculated. Tail length (comet length) of 100 randomly selected comet cells was also measured using a calibrated scale in the microscope's ocular to evaluate the distance of DNA migration using the software ImageJ 1.33u (Wayne Rasband, National Institutes of Health, USA).

Phenotypic analysis – Rosenfeld's staining

Morphological changes were primarily assessed by analysis of the Rosenfeld's staining. All control and treated cells seeded on 40-mm polystyrene plates (1.5×10^5 cells/plate) were rinsed three times with PBS (without Ca^{2+} and Mg^{2+}) and fixed for 10 min with methanol at -20°C . Fixed cells were stained according to the protocol established by Rosenfeld (Rosenfeld, 1947). Rosenfeld's reagent (1 ml) was added to the plates, which were then incubated for 20 min at room temperature. Thereafter, the plates were rinsed with water, air-dried, analyzed in an optic phase microscope (Nikon TS-100) and photographed using a digital camera (Nikon E-4300).

Morphological changes – immunocytochemistry

Morphological changes were studied by immunocytochemistry for the cytoskeletal protein vimentin. Control and treated cells were rinsed three times with PBS and fixed with cold methanol at -20°C for 10 min. The cells were incubated with mouse monoclonal anti-vimentin (1/500, clone V9, Boehringer, Mannheim) for 2 h and tetramethylrhodamine isothiocyanate conjugated sheep anti-mouse (1/250, Biomarker, Israel) antibodies for 60 min at room temperature under slow agitation. Chromatin integrity or nuclear fragmentation/condensation was assessed by co-staining the nuclear chromatin of fixed cells for 10 min with the fluorescent dye Hoecht-33258 (Sigma, St. Louis, MO) at a final concentration of $5 \mu\text{g}/\text{ml}$ in PBS in a dark chamber at room temperature. Thereafter, the cells were analyzed by fluorescent microscopy and photographed (Olympus AX70).

Statistical analysis

Results are expressed as the mean \pm standard deviation. One-way ANOVA test followed by a Student–Newman–Keuls test was used to determine the significant differences among groups that differ by only one parameter. Values of $p < 0.05$ were considered significant.

Results

Effects on cell viability and growth

To determine sensitivity of the GL-15 cells of the flavonoids casticin, penduletin, and 5-hydroxy-7,4'-dimethoxyflavone, we conducted the MTT test that establishes cell viability by measuring the functionality of mitochondrial dehydrogenases. Compared to the control (0.5% DMSO), neither 5-hydroxy-7,4'-dimethoxyflavone nor casticin showed toxicity to GL-15 cells after 24 h of exposure at any of the concentrations tested (1–100 μM) (Fig. 1A and B), only observed after 72 h treatment with 100 μM casticin. However, penduletin demonstrated toxicity beginning at a concentration of 10 μM . These effects were also evident after 72 h of exposure and demonstrated the greater sensitivity of the glioblastoma cell line GL-15 to penduletin.

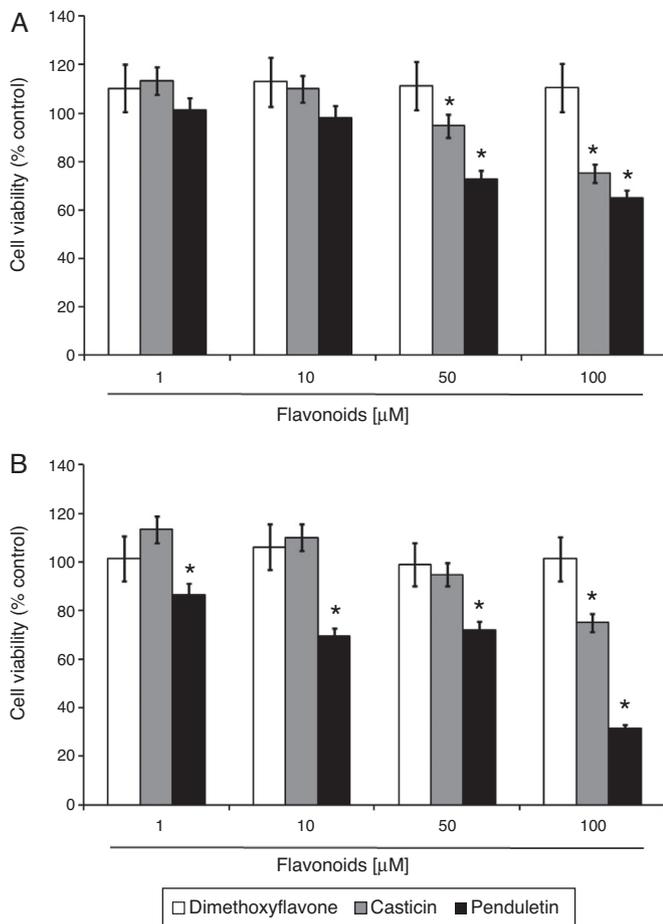


Fig. 1. Analysis of metabolic activity by MTT test in GL-15 cells exposed to 5-hydroxy-7,4'-dimethoxyflavone, casticin, and penduletin at concentrations of 10, 50 and 100 µM. (A) GL-15 cells after 24 h exposure to flavonoids. (B) GL-15 cells after 72 h exposure to flavonoids. Cells under control conditions were treated with 0.5% DMSO, a vehicle for dilution of drugs. Results expressed in percentage in relation to the control, taken as 100% (* $p < 0.05$).

Because flavonoids casticin and penduletin displayed both reductions on viability and growth inhibition of GL-15 cells since 24 h after treatment, we analyzed the cell cycle distribution by flow cytometry. In control conditions, the proportion of GL-15 cells in the G1 phase increased with time with a concomitant decrease in S phase (Fig. 2). However, after 48 h exposure to 50 µM flavonoids changes in the cell cycle distribution were observed, and cells accumulated in the G2 phase.

Effects on proliferation GL-15 cells

The effect on cell proliferation was studied by the incorporation of ^3H -thymidine. The treatment of exponentially dividing GL-15 cells with casticin, penduletin, and 5-hydroxy-7,4'-dimethoxyflavone induced a strong, dose-dependent growth inhibition, with penduletin proving to be the most potent flavonoid. Growth inhibition occurred with values ranging from 78 to 95% after 24 h of exposure and was also evident after 72 h of exposure (Fig. 2A and B).

Because flavonoids casticin and penduletin displayed an inhibitory effect on GL-15 cell growth, we analyzed the cell cycle distribution after treatment by flow cytometry. In control conditions, the proportion of GL-15 cells in the G0/G1 phase increased with time with a concomitant decrease in S phase (Fig. 3A). Flavonoids induced changes in the cell cycle distribution after 48 h exposure. In the cultures exposed to 50 µM castin the proportion

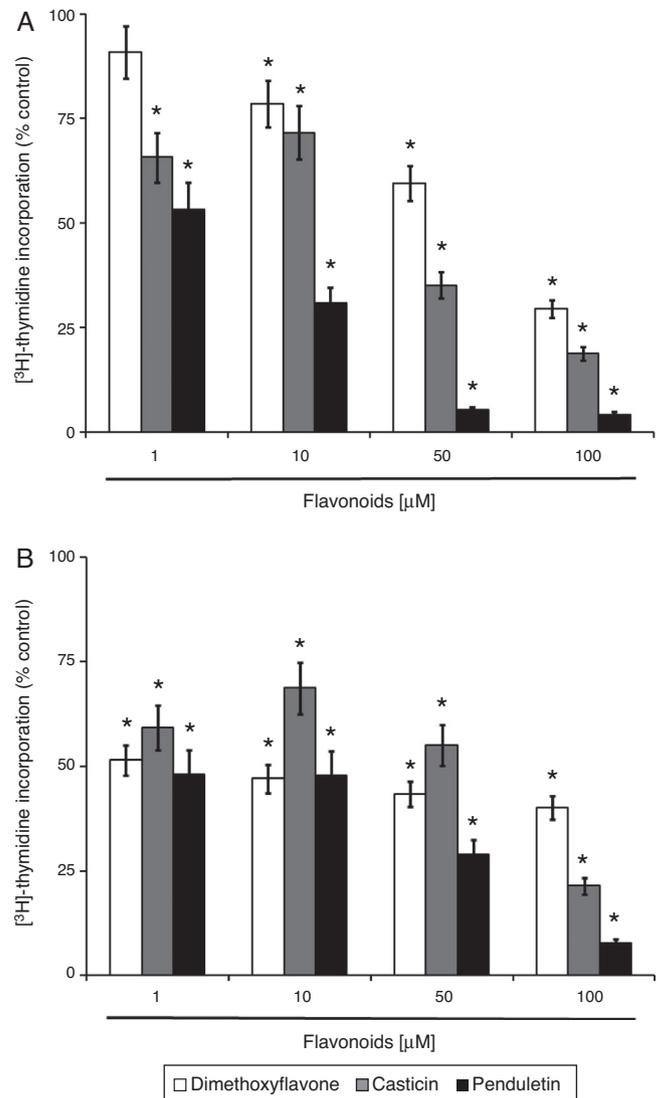


Fig. 2. Analysis of cell proliferation by ^3H -thymidine incorporation into the DNA of GL-15 cells exposed to different concentrations of 5-hydroxy-7,4'-dimethoxyflavone, casticin, and penduletin at concentrations of 10, 50 and 100 µM. (A) Results after 24 h of exposure to flavonoids. (B) Results after 72 h of exposure to flavonoids. Cells under control conditions were treated with 0.5% DMSO, a vehicle for dilution of the flavonoids. Results expressed were in percentage in relation to the control, taken as 100% (* $p < 0.05$).

of cells in G0/G1 phase was reduced with and concomitant increase in proportion of cells in S phase. Moreover, a drastic reduction in proportion of cells in G0/G1 phase was evident after penduletin treatment, and cells accumulated in the S and G2 phase.

Induction of morphological changes

The induction of morphological changes of 5-hydroxy-7,4'-dimethoxyflavone, casticin, and penduletin on GL-15 cells was analyzed by microscopy after staining cells with Rosenfeld's dye (Fig. 4) and after performing immunocytochemistry for the intermediate filament protein vimentin. Under control conditions, GL-15 cells form a monolayer and the cells present a bipolar phenotype. However, exposure of the cells to 100 µM of 5-hydroxy-7,4'-dimethoxyflavone, casticin and penduletin induced significant morphological changes beginning 24 h after treatment. A reduction in the number of adhered cells was observed in cultures exposed to 100 µM flavonoids. The GL-15 cells exposed to 5-hydroxy-7,4'-dimethoxyflavone showed a bipolar phenotype and a retracted

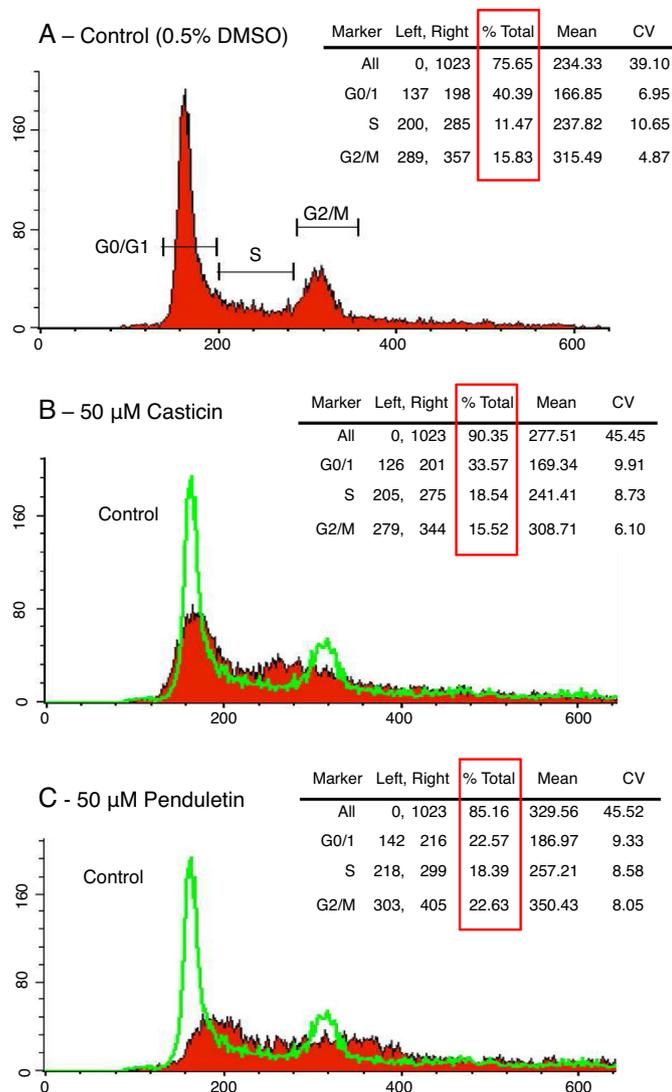


Fig. 3. Analysis by flow cytometry of cell cycle distributions of GL-15 cells in control conditions and exposed to different concentrations of casticin and penduletin at concentration of 50 µM, after a 48 h experiment. The DNA content is shown by relative fluorescence and the number of cells in the G0/G1 phase is represented in the first peak, whereas those in the G2/M phase are in the second peak. Cells in S phase are present in the area between the G0/G1 and G2/M peaks.

cytoplasm with tight, thin extensions. Furthermore, in cultured cells treated with casticin or penduletin, there was a dramatic reduction in adherent cells and the remaining cells showed an irregular phenotype and a distended cell body that was occasionally accompanied by thick filaments and branching projections. This effect was more intense in cultures treated with 100 µM flavonoids and after 72 h of treatment. Morphological changes were also detected in GL-15 cells by immunolabeling the cytoskeletal protein vimentin (Fig. 5). We observed that GL-15 cells express the vimentin protein homogeneously and that it is distributed in the cytoplasm, where it forms intermediate filaments. However, when the cells were exposed to flavonoids from *C. betulaster*, they altered the pattern of protein distribution, which was more diffuse in the cytoplasm following phenotypic changes.

Apoptosis detection

The ability to induce programmed cell death of the flavonoids extracted from *C. betulaster* was initially determined *in situ* with Annexin-V, which detects phosphatidylserine residues in the

outlet of membranes, and with CFDA dye, an indicator of viable cells. Twenty-four hours after treatment, cells treated with 100 µM flavonoids demonstrated a significant increase in the proportion of Annexin-V positive cells, thus indicating that apoptosis occurred (Fig. 6A–E).

Induction of apoptosis in GL-15 cells was also investigated by nuclear chromatin staining with the DNA intercalating dye Hoechst-33258 (Fig. 7A–E). Microscopy with ultraviolet radiation revealed that after 72 h of exposure to 100 µM flavonoids, 30–40% of the remaining adherent cells demonstrated nuclear condensation and chromatin fragmentation, which are features of apoptosis. These characteristics were not observed in cells under control conditions. The comet test, which identifies fragmentation of genetic material individually in cells, was then performed to investigate DNA fragmentation level in GL-15 cells. We observed that GL-15 cells exposed to 10–100 µM 5-hydroxy-7,4'-dimethoxyflavone, casticin, and penduletin demonstrated DNA fragmentation after 72 h of treatment (Fig. 8). The highest comet rates were observed after exposure to 100 µM flavonoids and were approximately 10.91% in cells exposed to 5-hydroxy-7,4'-dimethoxyflavone, 8.65% in cells exposed to casticin, and 21.21% in cells exposed to penduletin. Among the flavonoids studied, penduletin induced the most severe fragmentation of chromatin with the highest comet rate. The ultraviolet radiation, adopted as a positive control, caused lesions in GL-15 cells with ratios of approximately 65% due to the wavelength's capability to generate free radicals that act directly on DNA to induce damage (data not shown). A small proportion of GL-15 cells with the comet form (~1.97%) was observed in control cultures treated with the solvent DMSO (0.5%). However, according to Tice et al., the comet test has demonstrated sensitivity capable of detecting DNA damage at very low levels, and these findings could inform our experimental model or the experimental conditions adopted.

Discussion

The use of medicinal plants to treat diseases is a constant practice among the Brazilian population and is most prevalent in the Northeast region due to cultural influences. The use of *in vitro* cell lines to investigate the biological activity of natural products is one of the first steps in the search for novel drugs (Yunes and Calixto, 2001). In our study, we used the GL-15 cell line derived from human multiforme glioblastoma as an experimental model (characterized and established by Bocchini et al., 1993) to investigate the antitumor activity of the flavonoids casticin, 5-hydroxy-7,4'-dimethoxyflavone and penduletin, which can be extracted from the leaves of *C. betulaster* (Barbosa et al., 2003).

Initially, we used the MTT test to determine the effects of flavonoids on growth and cell viability by measuring the incorporation of ³H-thymidine into the DNA of GL-15 cells and the metabolic activity after exposure to different concentrations of the different flavonoids. We observed that all tested molecules inhibited cell proliferation in a dose-dependent manner and interfered with the mitochondrial function of GL-15 cells, thus reducing its viability. Penduletin was the most potent flavonoid, causing an inhibition of proliferation and cell viability of approximately 50–60% after 24 h of treatment at a concentration of 10 µM.

Our studies also demonstrated that flavonoids from *C. betulaster* induced programmed cell death in GL-15 cells, shown by labeling externalized phosphatidylserine residues in the cell membrane by Annexin V, a primary event of the apoptotic process (Jellinger, 2006; Greene et al., 2007). Flavonoid-induced apoptosis was also determined by identification of characteristics of cells undergoing apoptosis, such as nuclear condensation, as determined by the DNA intercalant dye Hoechst 33258, and DNA fragmentation, as determined by the comet test (Benkovic et al., 2008). These findings

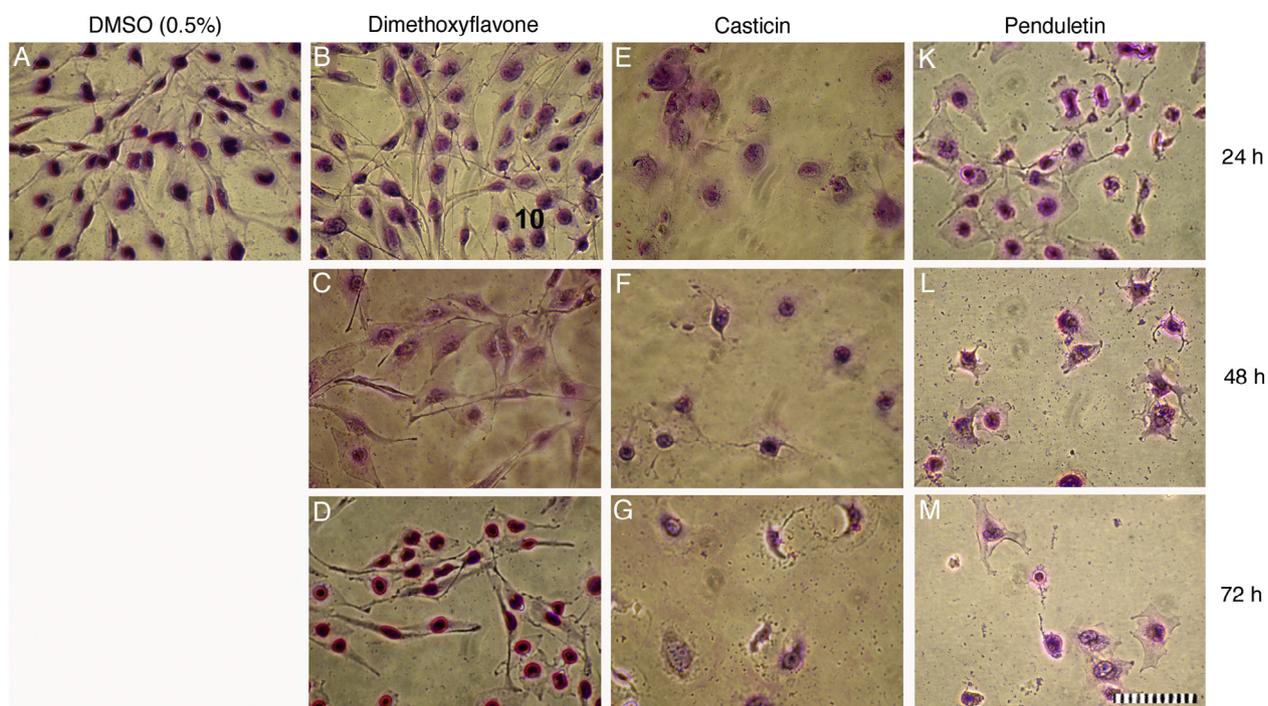


Fig. 4. Analysis of morphological changes after Rosenfeld's staining of GL-15 cells under control conditions (0.5% DMSO, A) or after 24 h, 48 h and 72 h exposure to 100 μ M flavonoids from *C. betulaster*: 5-hydroxy-7,4'-dimethoxyflavone (B, C, D), casticin (E, F, G), or penduletin (K, L, M). Objective 20 \times . Scale = 100 μ m.

demonstrate that the flavonoids tested herein can reduce proliferation and alter the metabolism of the glioblastoma cell line GL-15 and can also induce apoptosis, all of which are essential activities in the screening of drugs for tumor treatment.

Several studies have shown flavonoids as active principles responsible for various biological effects, including antitumor activity (Middleton et al., 2000). As demonstrated by Scheck et al.

(2006), the extract from *Scutellaria baicalensis* containing flavonoids inhibits dose-dependent effects on the viability of human glioblastoma cells and induces growth inhibition and apoptosis. Ferguson et al. (2006) also showed that flavonoids present in *Vaccinia macrocarpa* inhibit cell proliferation, cause cell cycle arrest, and induce apoptosis in the human glioblastoma cell line U87. Moreover, Braganhol et al. (2006) showed that the flavonoid quercetin

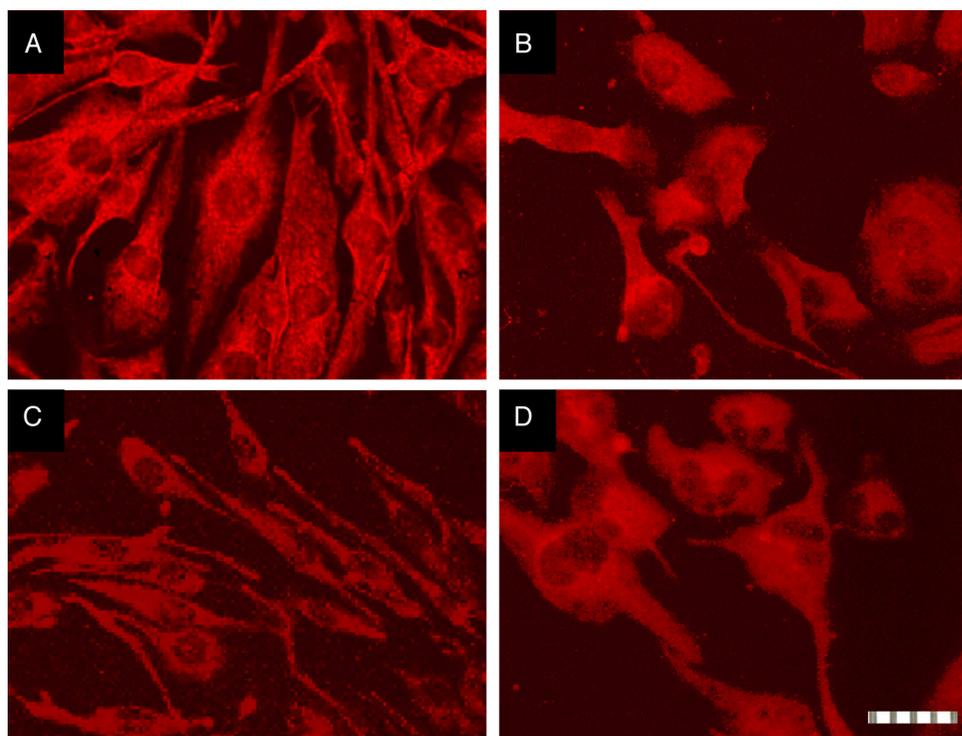


Fig. 5. Morphological analysis by immunocytochemistry for the cytoskeletal protein vimentin in GL-15 cells (A) under control conditions (0.5% DMSO) or after 72 h treatment with 100 μ M flavonoids from *C. betulaster*, (B) 5-hydroxy-7,4'-dimethoxyflavone, (C) casticin, or (D) penduletin. Object 20 \times . Scale = 100 μ m.

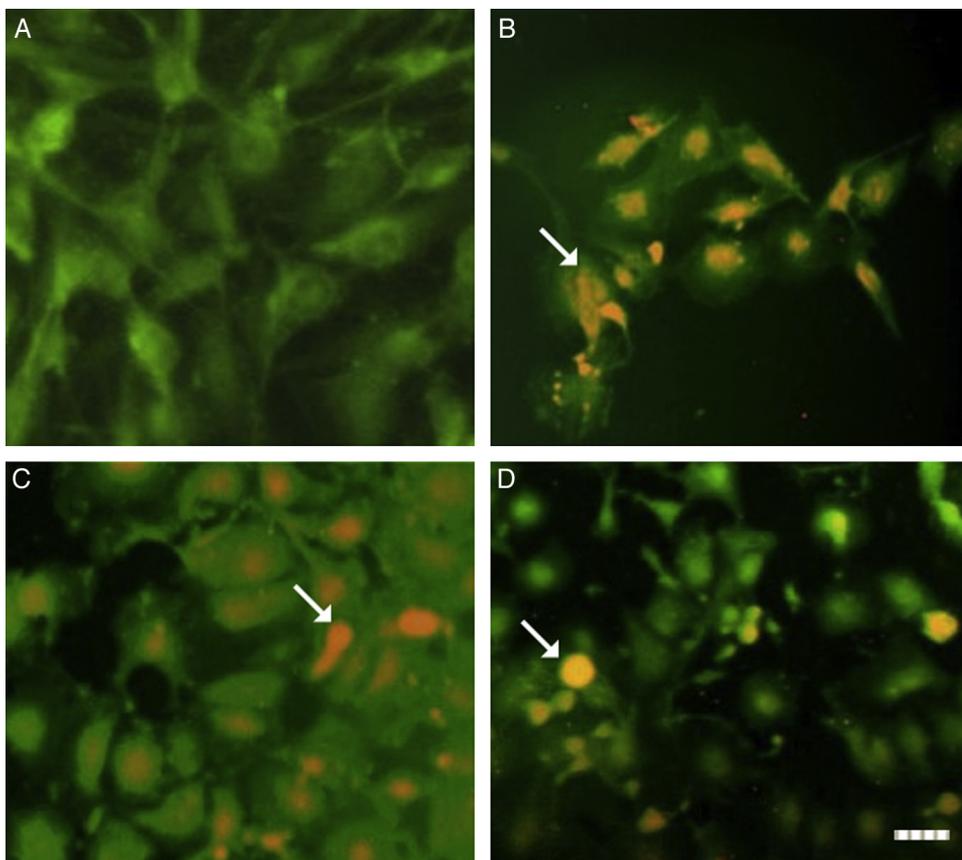


Fig. 6. Detection of the induction of programmed cell death in GL-15 cells under control conditions (0.5% DMSO) or after 24 h treatment with 100 μ M flavonoids from *C. betulaster* by marking with Annexin V/CFDA. (A) 0.5% DMSO, (B) 5-hydroxy-7,4'-dimethoxyflavone, (C) casticin, or (D) penduletin. The photomicrograph represents the juxtaposition of the images of CFDA (green) positive cells and images of Annexin-V (orange red) positive cells. Object 20 \times . Scale = 100 μ m. (E) Quantification of Annexin-V/CFDA positive cells (apoptotic cells) expressed in percentage of total cells counted (* $p < 0.05$).

inhibits cell proliferation in the glioblastoma multiforme cell line U138MG, thus inhibiting cell viability and the induction of apoptosis. Together with our results, these findings suggest that flavonoids are promising supplementary molecules for developing new treatments for malignant gliomas.

The inhibitory effect of flavonoids has also been found in other types of tumor cells. Roy et al. (2007) demonstrated *in vitro* that the flavonoid baicalein inhibits the proliferation of tumor cells of colon (HT-29) and prostate cancer (DU-145). Ye et al. (2007) demonstrated that the flavonoid extracted from *Cleistocalyx operculatus* induces the inhibition of metabolic activity and apoptosis in lung cancer (SMMC-7721), pancreatic cancer (8898), chronic leukemia (K562), uterine cervical cancer (HeLa) and lung cancer (95-D) cell lineages.

Rosenfeld's staining was used to demonstrate the ability of flavonoids from *C. betulaster* to induce morphological changes in GL-15 cells. We observed that all the flavonoids tested induced morphological changes. The GL-15 cells exposed to 5-hydroxy-7,4'-dimethoxyflavone demonstrated cell body retraction and filamentous extension formation. However, in the presence of casticin and penduletin, GL-15 cells had enlarged cells bodies and a polygonal phenotype with short, fine extensions. These morphological changes in GL-15 cells were also detected by immunolabeling vimentin. Vimentin is a cytoskeletal protein that is part of the intermediate filament of immature cells. Immunocytochemical analyses with GL-15 cells conducted by Bocchini et al. combined with the observations of this and other studies have revealed that these cells constitutively express the protein vimentin and exhibit a homogeneous distribution in all cell bodies,

contributing to the cell's bipolar phenotype. Our results showed that flavonoids from *C. betulaster* are able to induce phenotypical changes in GL-15 glioblastoma cells, suggesting that a morphogenic potential that may be investigated using markers of different cell populations in the CNS. The morphogenic potential of casticin was already evidenced in the primary cultures of rat cortical neurons (Sampaio-Spohr et al., 2010). Research carried out by Xie et al. (2005) has confirmed that polyphenolic compounds are able to induce morphological changes and programmed cell death in lung cancer cells. In fact, in a previous study, we observed that the flavonoid rutin induced astrocytic differentiation in GL-15 cells (Santos et al., 2011).

A study by Haidara et al. (2006), suggested that the flavonoid casticin may be important to anti-cancer therapy, due to its inhibition of the *in vitro* proliferation of the epidermal carcinoma cell line. Kobayakawa et al. (2004) administered casticin to arrest the cell cycle in the G2-M phase in lung cancer and colon carcinoma cell lineages, thus confirming this flavonoid's important antitumor activity. A study conducted by Li et al. (2005) disclosed that penduletin demonstrates antitumoral activity with breast cancer cells. However, the biological activity of 5-hydroxy-7,4'-dimethoxyflavone has been not yet been explored. Research conducted by El-Ghorab et al. (2003) demonstrated that this flavonoid has antioxidant activity. Studies conducted by Wang et al. (1992) and Martini et al. (2004) have shown that 5-hydroxy-7,4'-dimethoxyflavone has significant antibacterial activity.

Even inhibitory and morphogenic effects were observed in our experimental procedures for all flavonoids tested; the GL-15 cells of the flavonoid tested presented distinct patterns of

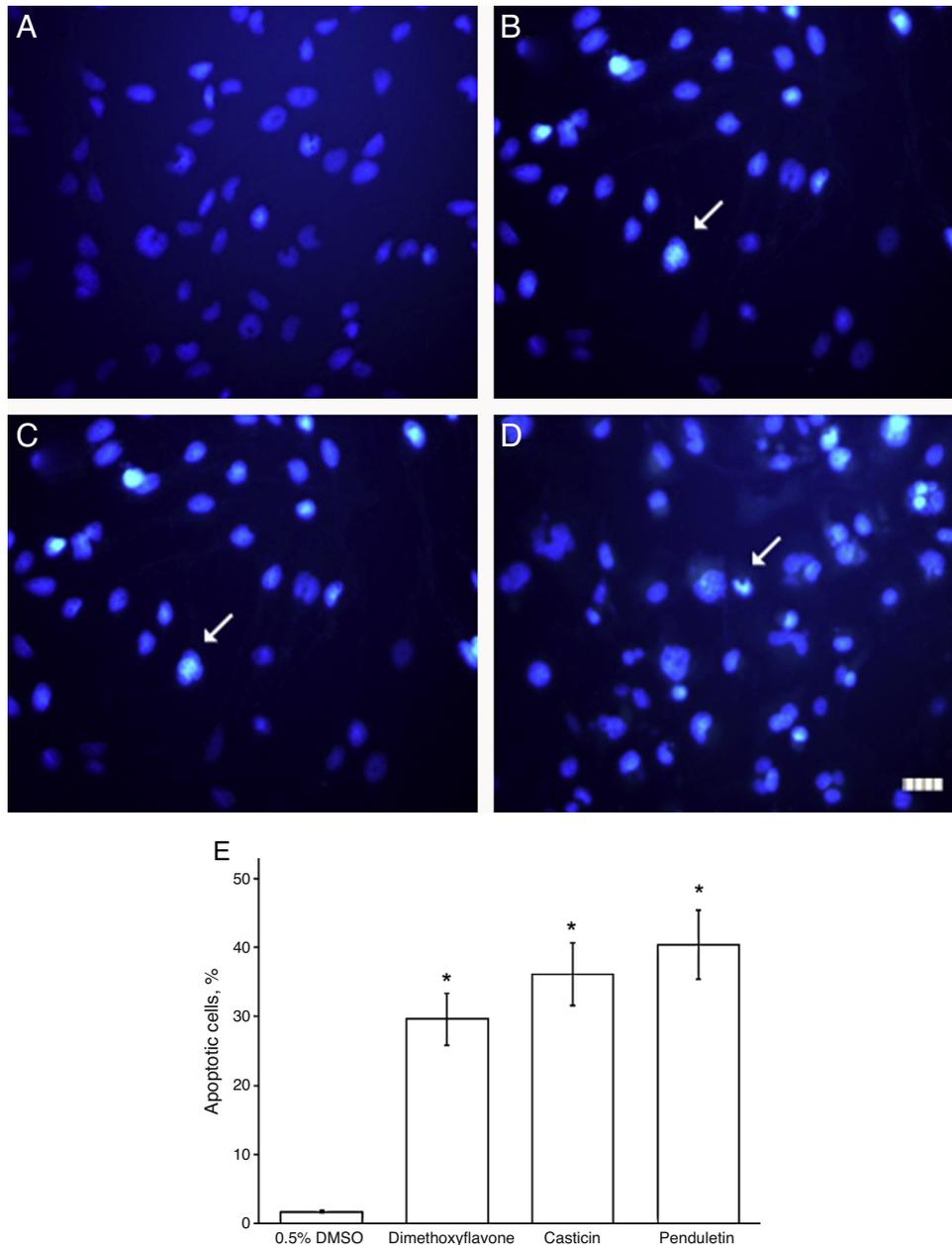


Fig. 7. Induction of programmed cell death in the GL-15 cells after treatment of *C. betulaster* with flavonoids and under control conditions (0.5% DMSO) after 72 h treatment. (A) Analysis by the intercalating dye Hoechst 33258 in cells under control conditions (without treatment) and exposed to the vehicle DMSO (0.5%) or treated with 100 μ M of flavonoids, (B) 5-hydroxy-7,4'-dimethoxyflavone, (C) casticin, or (D) penduletin; object 20 \times , (E) quantification of cells presenting nuclear chromatin condensation and/or fragmentation under control conditions or after 72 h treatment with 100 μ M flavonoids.

response intensities and of phenotypic changes in function. These flavonoids have different amounts of hydroxyl and methyl groups in their structures. Several studies have shown this to be an important feature, as the degree of hydroxylation and methylation favored an increase or decrease in the potential actions, respectively. Of the flavonoids used in our experiments, 5-hydroxy-7,4'-dimethoxyflavone was clearly the least effective, and casticin (5,3'-dihydroxy-3,6,7,4'-tetramethoxyflavone) and penduletin (5,4'-dihydroxy-3,6,7-trimethoxyflavone) were the most effective. These findings suggest that the number of hydroxyl and methyl groups is essential to activity of flavonoids in GL-15 glioblastoma cells.

According to Sathornsumetee and Rich (2007), gliomas have a high recurrence and are the most aggressive tumor in the

CNS. However, the conventional treatment associated with anti-angiogenesis therapy can reduce the rate of tumor recurrence, improving patient survival. In a literature review, Gerstner et al. examined the same issue, reporting the results of clinical trials conducted with patients using conventional therapy and inhibitory molecules of growth factors to improve patient survival. Therefore, compounds that show antitumor activity may be a key factor for the improvement of the conventional treatment of CNS tumors. The *C. betulaster* flavonoids tested in our study demonstrated biological activity against the human malignant glioblastoma cell line GL-15, presenting basic properties such as proliferation inhibition and apoptosis induction. Penduletin proved to be the most potent flavonoid, and this study suggests that flavonoids may be promising supplementary drugs for glioblastoma treatment.

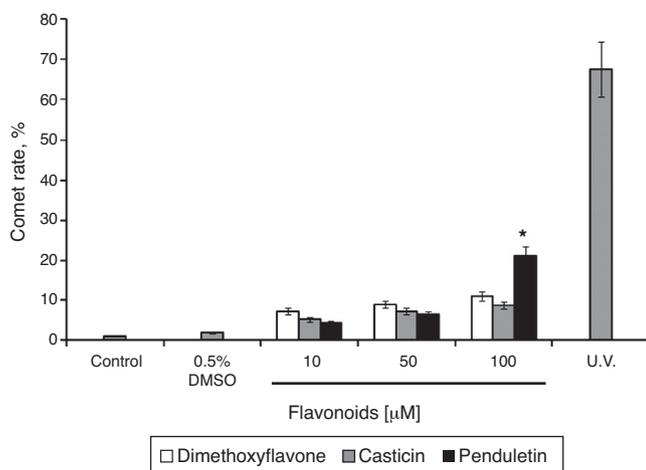


Fig. 8. Analysis of DNA fragmentation by the comet test in GL-15 cells exposed to different concentrations of 5-hydroxy-7,4'-dimethoxyflavone, casticin, or penduletin. The frequency of inferred and estimated damage was calculated based on the number of cells with a tail versus those without a tail.

Authors' contributions

SLC, RSE-B, PRB, and MFDC, planned the research and designed the experiments; PRB for drugs purification and characterization; PLCC, SRV-BF, BPSP, MNO, CSS, MSG, VDAS, and SLC performed culture-related experiments and contributed to data analysis; CSS, VDAS, RSE-B, ILON, MFDC, and SLC contributed with reagents/materials/analysis tools; PLCC, SRV-BF, BPSP, MNO, CSS, MSG, VDAS, RSE-B, and SLC for paper writing. All authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq – 470807/2011-3), Fundação de Apoio à Pesquisa do Estado da Bahia (FAPESB – PNE 004/2011), Coordenação de Apoio de Pessoal de Nível Superior (CAPES) and the Graduate Program in Immunology at the Federal University of Bahia.

References

Barbosa, P.B., Fascio, M., Martins, D., Roque, N.F., 2003. Triterpenes of *Croton betulaster* (Euphorbiaceae). *Biochem. Syst. Ecol.* 31, 307–308.

Benkovic, V., Horvat-Knezevica, A., Dikica, D., Lisicica, D., Orsolica, N., Basica, I., Kosalec, I., Kopjar, N., 2008. Radioprotective effects of propolis and quercetin in c-irradiated mice evaluated by the alkaline comet assay *Phytomedicine* 15, 851–858.

Bocchini, V., Beccari, T., Arcuri, C., Bruyere, L., Fages, C., Tardy, M., 1993. Glial fibrillary acidic protein and its encoding mRNA exhibit mosaic expression in a glioblastoma multiform cell line of clonal origin. *Int. J. Dev. Neurosci.* 11 (4), 485–492.

Braganhol, E., Zamin, L.L., Canedo, A.D., Horn, F., Tanajusuku, A.S., Wink, M.R., Salbego, C., Battastini, 2006. Antiproliferative effect of quercetin in the human U38MG glioma cell line. *Anticancer Drugs* 17, 663–671.

Bruneton, J., 1991. *Fitoquímica y de farmacognosia*. Editorial Acriba, S.A.

Bukhari, I.A., Khan, R.A., Gilani, A.U., Shah, A.J., Ahmad, V.U., 2007. The analgesic, anti-inflammatory and calcium antagonist potential of *Tanacetum artemisiodes*. *Arch. Pharm. Res.* 30, 303–312.

Cai, S.Q., Wang, R., Yang, X., Shang, M., Ma, C., Shoyama, Y., 2006. Antiviral flavonoid-type C-glycosides from the flower of *Trollius chinensi*. *Chem. Biodivers.* 3, 343–348.

Chambaut-Guérin, A.M., Costa, S.L., Lefrançois, T., Fages, C., Gauthereau, X., Tardy, M., 2000. Effects of retinoic acid and tumour necrosis factor alfa on GL-15 glioblastoma cells. *Neuroreport* 2, 389–393.

Cordeiro, I., 1995. Euphorbiaceae. In: Stannard, B.L., Harvey, Y.B., Harley, R.M. (Eds.), *Flora of the Pico das Almas*. Chapada Diamantina, Bahia, Brazil, p. 303.

Costa, S.L., Paillaud, E., Fages, C., Rochette-Egly, C., Jouault, H., Perzelova, A., Tardy, M., 2001. Effects of a novel synthetic retinoid on malignant glioma in vitro: Inhibition of cell proliferation, induction of apoptosis and differentiation. *Eu. J. Cancer* 37, 520–530.

Defer, G.L., Adle-Biassette, H., Ricolfi, F., Martin, L., Authier, F.J., Chomienien, C., Degos, L., Degos, J.D., 1997. All-trans retinoic acid in relapsing malignant gliomas: clinical and radiological stabilization associated with the appearance of intratumoral calcifications. *J. Neur. Oncol.* 34, 169–177.

El-Ghorab, A.H., El-Massry, K.F., Marx, F., Fadel, H.M., 2003. Antioxidant activity of Egyptian *Eucalyptus camaldulensis* var. *brevirostris* leaf extracts. *Nahrung* 47, 41–45.

Friedman, M., 2007. Overview of antibacterial, antioxidant, antiviral, and antifungal activities of tea flavonoids and teas. *Mol. Nutr. Food. Res.* 51, 116–134.

Ferguson, P.J., Kurowska, E.M., Freeman, D.J., Chambers, A.F., Kororpatnick, 2006. In vivo inhibition of growth of human tumor lines by flavonoid fractions from cranberry extract. *Nutr. Cancer* 56, 86–94.

Greene, L.A., Liu, D.X., Troy, C.M., Biswas, S.C., 2007. Cell cycle molecules define a pathway required for neuron death in development and disease. *Bioch. Bioph. Acta.* 1772, 392–401.

Haidara, K., Zanir, L., Shi, Q.W., Batist, G., 2006. The flavonoid casticin has multiple mechanisms of tumor cytotoxicity action. *Cancer Lett.* 242, 180–190.

Jellinger, K.A., 2006. Challenges in neuronal apoptosis. *Review. Curr. Alzheimer Res.* 3, 377–391.

Kawai, M., Hirano, T., Higa, S., Arimitsu, J., Maruta, M., Kuwahara, Y., Ohkawara, T., Hagiwara, K., Yamadori, T., Shima, Y., Ogata, A., Kawase, I., Tanaka, T., 2007. Flavonoids and related compounds as anti-allergic substances. *Allergol. Int.* 5, 113–123.

Kobayakawa, J., Sato-Nishimori, F., Mariyasu, M., Matsukawa, Y., 2004. G2-M arrest and antimetabolic activity mediated by casticin, a flavonoid isolated from *Vitex Fructus* (*Vitex rotundifolia* Linne fil.). *Cancer Lett.* 10, 59–64.

Li, W.X., Cui, C.B., Cai, B., Wang, H.Y., Yao, X.S., 2005. Flavonoids from *Vitex trifolia* L. inhibit cell cycle progression at G2/M phase and induce apoptosis in mammalian cancer cells. *J. Asian Nat. Prod. Res.* 7, 615–626.

Martini, N.D., Katerere, D.R., Eloff, J.N., 2004. Biological activity of five antibacterial flavonoids from *Combretum erythrophyllum* (Combretaceae). *J. Ethnopharmacol.* 93, 207–212.

Mead, J., McNair, N., 2006. Antiparitic activity of flavonoids and isoflavones against *Cryptosporidium parvum* and *Encephalitozoon intestinalis*. *Microbiol. Lett.* 259, 153–157.

Middleton Jr., E., Kandaswami, C., Theoharides, T.C., 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Res.* 4, 673–751.

Ozcelik, B., Orhan, I., Toker, G., 2006. Antiviral and antimicrobial assessment of some selected flavonoids. *Z. Naturforsch* 61, 632–638.

Pantev, A., Ivancheva, S., Staneva, L., Serkedjieva, J., 2006. Biologically active constituents of a polyphenol extract from *Geranium sanguineum* L. with anti-influenza activity. *Z. Naturforsch* 61, 508–516.

Rooprai, H.K., Kyriazis, I., Nuttall, R.K., Edwards, D.R., Zicha, D., Aubyn, D., Davies, D., Gullan, R., Pilkington, G.J., 2007. Inhibition of invasion and induction of apoptosis by selenium in human malignant brain tumour cells *in vitro*. *Int. J. Oncol.* 5, 1263–1271.

Rosenfeld, G., 1947. Corante pancreático para hematologia e citologia clínica: Nova combinação dos componentes do May Grunwald e do Giemsa num só corante de emprego prático. *Mem. Inst. Butantã* 20, 329–335.

Roy, M.K., Nakahara, K., Trakoontivakorn, G., Takenaka, M., Isobe, S., Tsushida, T., 2007. Baicalin, a flavonoid extracted from a methanolic extract of *Oroxylum indicum* inhibits proliferation of a cancer cell line *in vitro* via induction of apoptosis. *Pharmazie* 62, 149–153.

Sampaio-Spohr, T.C.L., Stipursky, J., Sasaki, A.C., Barbosa, P.R., Martins, V., Benjamim, C.F., Roque, N.D., Costa, S.L., Gomes, F.C.A., 2010. Effects of the flavonoid casticin from Brazilian *Croton betulaster* in cerebellar cortical progenitors *in vitro*: Direct and indirect action through astrocytes. *J. Neurosci. Res.* 88, 530–541.

Santos, B.L., Silva, A.R., Pitanga, B.P.S., Souza, C.S., Grangeiro, M.S., Fragomeni, B.O., Coelho, P.L.C., Oliveira, M.N., Menezes-Filho, N.J., Costa, M.F.D., El-Bachá, R.S., Vellozo, E.S., Sampaio, G.P., Freire, S.M., Tardy, M., Costa, S.L., 2011. Antiproliferative, proapoptotic and morphogenic effects of the flavonoid rutin on human glioblastoma cells. *Food Chem.* 127, 404–411.

Sathornsumetee, S., Rich, J.N., 2007. Antiangiogenic therapy in malignant glioma: promise and challenge. *Curr. Pharm.* 13, 3545–3558.

Scheck, A.C., Perry, K., Hank, N.C., Clark, W.D., 2006. Anticancer activity of extracts derived from the mature roots of *Scutellaria baicalensis* on human malignant brain tumor cells. *BMC Complement. Altern. Med.* 6 (27), 1–9.

Uyana, H., 2007. Artificial polymeric flavonoids synthesis and applications. *Macromol. Biosci.* 7, 410–422.

Xu, Y.C., Leung, S.W., Yeung, D.K., Hu, L.H., Chen, G.H., Che, C.M., Man, R.Y., 2007. Structure–activity relationships of flavonoids for vascular relaxation in porcine coronary artery. *Phytochemistry* 68, 1179–1188.

Walle, T., Ta, N., Kawamori, T., Wen, X., Tsuji, P.A., Walle, U.K., 2007. Cancer chemopreventive properties of orally bioavailable flavonoids–methylated versus unmethylated flavones. *Biochem. Pharmacol.* 73, 1288–1296.

Wang, S.X., Zhang, F.J., Feng, Q.P., Li, Y.L., 1992. Synthesis, characterization, and antibacterial activity of transition metal complexes with 5-hydroxy-7,4'-dimethoxyflavone. *J. Inorg. Biochem.* 46, 251–257.

- Xie, J., Chen, Q.Y., Zhou, J.Y., 2005. Study on mechanism of tea polyphenols in inducing human lung cancer cell apoptosis in vitro. *Zhongguo Zhong* 25, 244–247.
- Ye, C.L., Liu, Y., Wei, D.Z., 2007. Antioxidant and anticancer activity of 3'-formyl-4',6'-dihydroxy-2'-methoxy-5'-methylchalcone and (2S)-8-formyl-5-hydroxy-6-methylflavanone. *J. Pharm. Pharmacol.* 59, 553–559.
- Youdim, K.A., Qaiser, M.Z., Begly, D.J., Rice-evans, C.A., Abbott, N.J., 2004. Flavonoid permeability across an in situ model of the blood–brain barrier. *Free Radic. Biol. Med.* 1, 592–604.
- Youdim, K.A., Dobbie, M.S., Kuhnle, G., Prottogente, A.R., Abbott, N.J., Rice-Evans, C.A., 2003. Interaction between flavonoids and the blood–brain barrier. *In Vitro Stud.* 35, 180–192.
- Yunes, R.A., Calixto, J.B., 2001. Flavonóides Antioxidantes de Plantas Medicinais e Alimentos: Importância e Perspectivas Terapêuticas. In: *Plantas Medicinais Sob a Ótica da Química Medicinal Moderna*. Argos 1., pp. 321–331.