Cancer Chemother Pharmacol (2009) 64:529–538
DOI 10.1007/s00280-008-0901-9

ORIGINAL ARTICLE

Evaluation of the antitumoral effect of dihydrocucurbitacin-B in both in vitro and in vivo models

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Received: 26 August 2008 / Accepted: 11 December 2008 / Published online: 30 January 2009

Abstract
Aims We evaluated both in vitro and in vivo antitumoral properties of an isolated compound from *Wilbrandia ebracteata*, dihydrocucurbitacin-B (DHCB), using B16F10 cells (murine melanoma).
Materials and methods We made use of MTT and ^{3}H-Thymidine assays to investigate the cell viability and cell proliferation, flow cytometry analysis to monitor cell cycle and apoptosis, western blot analysis to evaluate the expression of cell cycle proteins, imunofluorescence analysis and in vivo tumor growth and metastasis.
Results Dihydrocucurbitacin-B significantly reduced cell proliferation without important effects on cells viability. DHCB lead cells to accumulate in G2/M phases accompanied by the appearance of polyploid cells, confirmed by fluorescence assays that demonstrated a remarkable alteration in the cell cytoskeleton and formation of binuclear cells. Annexin-V-FITC incorporation demonstrated that DHCB did not induce apoptosis. About 10 μg/mL DHCB was found to decrease cyclin-A, and especially in cyclin-B1. The in vivo experiments showed that DHCB treatment (once a day up to 12 days; p.o.) was able to reduce the tumor growth and lung metastasis up to 83.5 and 50.3%, respectively.
Conclusions Dihydrocucurbitacin-B reduces cell proliferation due to a decrease in the expression of cyclins, mainly cyclin-B1 and disruption of the actin cytoskeleton, arresting B16F10 cells in G2/M phase. Taken together, the in vitro and in vivo experiments suggest that DHCB was effective against cancer, however, it remains to be proved if DHCB will be a good candidate for drug development.

Keywords Dihydrocucurbitacin-B · Cytoskeleton · Cyclins · *Wilbrandia ebracteata*

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Introduction

Cancer is increasingly viewed as a cell cycle disease, and it is commonly accepted that the understanding of the molecular mechanisms that produce inappropriate proliferation may lead to the identification of targets that could be therapeutically manipulated in the fight against tumors [1]. Unlike normal cells that only proliferate when triggered by developmental or other mitogenic signals in response to

tissue growth, the proliferation of cancer cells proceeds independently, suggesting that many cancers present defects in the cell cycle machinery. Such defects could occur in cell cycle regulators or elements of upstream signaling cascades that participate in the proliferative stimulus [2]. This does not mean that cancer cells cycles are necessarily different from those found in normal cells, but it implies that cancer cells proliferate because they are no longer subject to proliferation-inhibitory influences arising from the stroma or from changes in the gene expression pattern consequent to terminal differentiation, or because they do not necessarily require extrinsic growth factors to recruit them into or to maintain their proliferative state [3].

Drug discovery in medicinal plants has played an important role in the treatment of cancer, and most new clinical applications of plant secondary metabolites and their derivatives over the last half century have been in cancer treatment [4]. There are four main classes of natural anticancer compounds in clinical use: vinca alkaloids (such as vincristine and vinblastine) from *Catharanthus roseus* L. [5]; epipodophyllotoxins (etoposide) from *Podophyllum peltatum* L. [6]; taxanes (paclitaxel and derivatives) from *Taxus brevifolia* Nutt. and camptothecins (camptothecin) from *Camptotheca acuminata* Decne [7].

Cucurbitacins are polyhydroxylated triterpenes derived from plants with medicinal properties known since antiquity [8]. Some reports related cucurbitacins and cancer. Cucurbitacin B/E glucosides inhibited growth of MCF-7 and MDA-MB-231 human breast cancer cell lines [9]; Cucurbitacin I and Q specifically inhibited STAT3 phosphorylation in human and murine cancer cells [10, 11]; cucurbitacin E disrupted the actin and vimentin cytoskeleton in human prostate cancer cells [12]. Related to DHCB some papers reported a broad range of potent biological activities. Its ED50 to A549, SK-UV-3, SK-MEL-2, XF498 and HCT15 are 0.2–3.7 μg/mL [13]. Recently, we have demonstrated that DHCB exerts potent anti-inflammatory effects [14]; and others report the reduction in adjuvant-induced arthritis [15]; moreover, DHCB induces cell cycle arrest and apoptosis in human breast cancer (Bcap37 cells) [16].

Our present purpose was to investigate the antitumoral properties of a cucurbitacin-related compound, dihydrocucurbitacin-B (DHCB), isolated from *Wilbrandia ebracteata*, using in vitro and in vivo assays with murine melanoma B16F10 cells.

Materials and methods

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Dulbecco's Modified Eagle medium (DMEM), Propidium iodide, Hoechst 33342 and other salts used in immunoblot analysis were purchased from Sigma–Aldrich (St Louis, MO, USA). RNAse and Rhodamine-Phalloidin solutions were obtained from Molecular Probes/Invitrogen. Primary antibodies for the detection of cyclin-E (M-20), cyclin-A (H-432), cyclin-B1 (H-433), cdk1 (17), cdk2 (M2), $p27^{Kip1}$ (C-19) and actin (c-2) were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Secondary antibodies used for Western blot analysis, [methyl-^{3}H]-thymidine, Hybond™-ECL™ nitrocellulose membranes and ECL™ hyperfilms were obtained from Amersham-Pharmacia (Oakville, ON, Canada). ECL™ Western blot detection reagents were from PerkinElmer (USA). All other reagents were obtained from Sigma–Aldrich unless stated otherwise. Dihydrocucurbitacin-B was dissolved in dimethyl sulfoxide and the solvent was kept at $\leq$0.1% in the final solution.

Animals

In vivo experiments were conducted in male C57BL6 mice (18–25 g), housed at 22 ± 2°C under a 12/12 h light/dark cycle with free access to food and water until use. The animals were acclimatized to the laboratory for at least 24 h before testing and were used only once. All animal handling and experimental procedures were conducted with prior approval of the ethics committee on animal use of the Federal University of Santa Catarina (CEUA).

Plant material

Underground parts of *W. ebracteata* Cogn. were collected in Siderópolis, Santa Catarina, Brazil. The plant was identified by Dr. Mareni Farias (Department of Pharmaceutical Sciences of the Federal University of Santa Catarina). Voucher specimens (number FLOR34410) are deposited at the Herbarium of the Faculty of Pharmacy of the Federal University of Santa Catarina, Florianópolis, Brazil and at the Pharmaceutisches Institut der Universität Bonn, Germany.

Extraction and isolation procedures

The roots were first chopped; then, the cut, air-dried roots were successively extracted at room temperature with ethanol. The ethanolic extract was concentrated under reduced pressure and the residue suspended in water. This suspension was successively extracted with petroleum ether and dichloromethane. From the dichloromethane extract (WEDC), Dihydrocucurbitacin-B (DHCB, the major component) was isolated through a silica gel column (0.05–0.20 mm) with petroleum ether/ethyl acetate 1:2 as mobile phase. Finally, a second column with silica gel (0.04–0.063 mm) and the mobile phase petroleum ether/ethyl acetate/isopropanol 20:10:0.8 furnished DHCB. The identity of DHCB was

confirmed by thin layer chromatography, HPLC and ultra violet spectroscopy through comparison with an authentic sample. The structure of DHCB was determined and published by Farias et al. [17]. Using this method we obtained DHCB approximately 92% pure.

Cell culture

Murine melanoma B16F10 (number CRL-6475) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were maintained at subconfluence in a 5% CO_2 humidified atmosphere at 37°C. The medium used for routine subculture was Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM glutamine, 10 mM HEPES, 100 units/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum (FBS).

Cell viability assay (MTT)

The cell viability in the presence or absence of DHCB was measured by MTT assay as proposed by Mosmann [18]. B16F10 cells were plated into a 96-well plate at equal density (2.5×10^3 cells) in DMEM medium. After 24 h, the cells were stimulated with increasing concentrations of DHCB (0.001–100 μg/mL) for 48 h of incubation in a 5% CO_2 humidified atmosphere at 37°C. After this, cells were carefully washed with phosphate-buffered saline (PBS), and 200 μL of MTT solution (0.5 mg/mL) in fresh medium was added and incubated for 3 h. Then, the wells were washed with PBS and 200 μL of DMSO/well was added to dissolve the intracellular crystalline formazan product. The absorbance at 550 nm was read spectrophotometrically using a microplate reader. The results were expressed as a percentage of the absorbance of control cultures and all experiments were performed in triplicate.

[^{3}H] Thymidine incorporation

Asynchronous B16F10 cells were seeded into a 24-well plate at equal density (25×10^3 cells). After 24 h, the cells were stimulated with increasing concentrations of DHCB (0.001–100 μg/mL) and 2 μCi/mL of [^{3}H]-thymidine was added and incubated during the last 48 h in a 5% CO_2 humidified atmosphere at 37°C. After washing the cells two times with cold PBS and one time with 10% trichloroacetic acid, the cells were fixed with ethanol:ether (1:3) and then solubilized with sodium hydroxide (0.2 N, 0.6 mL/well), and counted in a scintillation counter. Each concentration point was repeated in triplicate wells, and at least three independent experiments were performed. Results are expressed as the standard error of the mean (mean ± SEM).

Flow cytometry analysis

Cell cycle

To estimate the proportion of cells in different phases of the cell cycle, in the presence or absence of DHCB, cellular DNA contents dyed with propidium iodide (PI) were measured by flow cytometry. Asynchronous B16F10 cells were plated in six-well plates. After incubation for 24 h, DHCB (10 μg/mL) or vehicle (DMSO 0.01%) was added and incubated for 0, 4, 8, 16, 24 and 48 h. After DHCB or vehicle treatment for various time periods, cells were carefully harvested. The cells were then permeabilized with Triton X-100 0.05% in slightly hypertonic buffer (buffer H; 20 mM HEPES pH 7.2; 0.16 M NaCl; 1 mM EGTA), fixed by addition of 2% cold paraformaldehyde and resuspended in buffer H (20 mM HEPES pH 7.2, 0.16 M NaCl and 1 mM EGTA) containing PI (10 μg/mL) and 200 μg/ml RNAse A. After 30 min at room temperature, the cells were analyzed by flow cytometry (Becton-Dickinson, San Jose, CA, USA) using an instrument equipped with a 488 nm argon laser. Data were analyzed using CellQuest software and the percentage of cells gated in each stage of the cell cycle was determined.

Annexin-V-FITC incorporation

Murine melanoma B16F10 cells were seeded into 6-well plates. After 24 h of growth, DHCB (10 μg/mL) or vehicle (DMSO 0.01%) was added. After 48 h of incubation, the culture supernatant (which contained some floating cells) was collected and the adherent cells harvested by a brief trypsinization. The two fractions were combined, centrifuged and washed with PBS. Cells were labeled with Annexin-V-FITC and propidium iodide (PI) and analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA). Data were analyzed using CellQuest software and the percentages of cells gated were indicated.

Western blot analysis for the cell cycle-regulatory proteins

B16F10 cells were seeded in 6-well plates. After 24 h of incubation, DHCB (10 μg/mL) or vehicle (DMSO 0.01%) was added and incubated for 0, 4, 8, 16, 24, 36 and 48 h. After each incubation time, cells were lysed in appropriate buffer (50 mM Tris–HCl pH 7.5, 1% Triton X-100, 100 mM NaCl, 5 mM EDTA pH 8.0, 40 mM β-glycerol-phosphate, 50 mM NaF, 200 μM orthovanadate, 5% glycerol and protease inhibitors). Proteins (35 μg) from whole cell lysates were separated by SDS-PAGE in 10 or 12% gels. Proteins were detected immunologically following electrotransfer onto nitrocellulose membranes (Amersham-Pharmacia Biotechnology). Protein and molecular weight

markers (BioRad, Mississauga, ON, Canada) were revealed by Ponceau Red staining. Membranes were blocked in PBS containing 5% powdered milk and 0.05% Tween-20 for 1 h at 25°C. Membranes were then incubated overnight at 4°C with primary antibodies in blocking solution and after with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit (1:1,000) IgG for 1 h. Blots were visualized using the PerkinElmer ECL system. Protein concentrations were measured using a modified Lowry procedure with bovine serum albumin as standard as described by [19].

Fluorescence microscopy

B16F10 cells were seeded on sterile, circular glass coverslips in 6-well plates. After 24 h of incubation, DHCB (10 μg/mL) or vehicle was added and incubated for 48 h. Then, cells were washed twice with ice-cold PBS. Cultures were fixed in formaldehyde 3.7% in PBS for 30 min at 4°C, permeabilized with 0.1% Triton X-100 in PBS for 30 min at 4°C, and Rhodamine-phalloidin solution (to stain actin filaments), together with Hoechst 33342 (to stain the nucleus) was then added for 30 min in the dark. The coverslips were mounted on glass slides and examined with a Nikon ECLIPSE TE2000-U microscope (Ontario, Canada) using a Hamamatsu CCD Meteor II Digital camera and images were acquired using SimplePCI software by Compix Inc (OR, USA).

In vivo protocols

Tumor-growth

C57BL6 mice (seven to nine mice in each group) were lightly anesthetized with an intraperitoneal injection of a combination of ketamine:xylazine (1:1; final volume 50 μL). B16F10 cells (1×10^6) were injected s.c. to the right flank in 100 μL DMEM (FBS 10%) using a 26-gauge needle attached to a syringe. Around 7 days after tumor cells injection, when a solid mass appears (3–5 mm diameter), the oral treatments was started with DHCB (1, 3 or 10 mg/kg) or vehicle (control, 0.1 mL/10 g body weight), once a day for 12 days. In the 12th experimental day, the animals were sacrificed in CO_2 chamber and tumors were carefully removed. The excised tumors were weighed in analytical balance. Data were expressed as the mean ± SEM for each group of animals. Dunnett's test was used to compare data of DHCB treated and vehicle-treated mice. P values < 0.05 were considered statistically significant.

Lung metastasis

B16F10 cells were supplied with fresh DMEM 1 day before their harvest for tail vein injection. Subconfluent cells (70–80%) were washed with phosphate-buffered saline and detached by brief exposure to trypsin (0.25% trypsin and 0.2% EDTA) and washed with complete DMEM (FBS 10%). Cells were resuspended and viability was determined by trypan blue exclusion and was always more than 95%. Twenty microliters of a tumor cell (1×10^6 cells) suspension was injected to the lateral tail vein of mice by using a 27-gauge needle. In the same day of tumor cell injection, DHCB (1 or 10 mg/kg) or vehicle treatments were started, once a day for 12 days. Mice containing lung tumors were sacrificed on day 12 after tumor cell injection. Lungs were kept in Bouin's fixative for 24 h before counting. Individual lobes were separated, and the number of surface-visible metastases was determined by using a stereomicroscope (2× magnification). Statistical analysis was performed by using the Dunnett's test and P values < 0.05 were considered statistically significant.

Results

Cell viability and proliferative rates

To investigate the effects of DHCB treatment on the cell viability and proliferation status, we used the MTT and incorporation of [^{3}H]-thymidine assays. In this experiment, increasing concentrations of DHCB (0.001–100 μg/mL), reduced the cell viability, after 48 h incubation, up to 48.43% (24.02–48.43% for 0.1–100 μg/mL, respectively) as compared to control (vehicle) (Fig. 1a).

In the proliferation assay, the uptake of [^{3}H]-thymidine into DNA is a widely used technique in cell biology to measure levels of DNA synthesis in normal or tumor cells [20]. In this assay, at the same concentrations and time of incubation mentioned above, DHCB was able to reduce the incorporation of [^{3}H]-thymidine by up to 95.22% (47.64–95.22% for 1–100 μg/mL, respectively) (Fig. 1a). These results suggest that DHCB, mainly at 10 μg/mL, was able to reduce the proliferation rates without important effects on the cell viability. Moreover, morphologic analysis by videomicroscopy (time lapse), showed that DHCB (10 μg/mL) promotes important alterations in cell spreading, with cells becoming rounded and reducing proliferation up to 48 h of incubation (Fig. 1b).

Flow cytometry analysis

The effect of DHCB addition on the cell cycle was evaluated by flow cytometry, after nucleus staining with propidium iodide. In this assay, the percentage of cells gated in M1 (cells in G1), M3 (cells in G2/M) and M4 (indicative of polyploidy) were registered (Fig. 2b). The results demonstrated that DHCB (10 μg/mL) induced an accumulation of

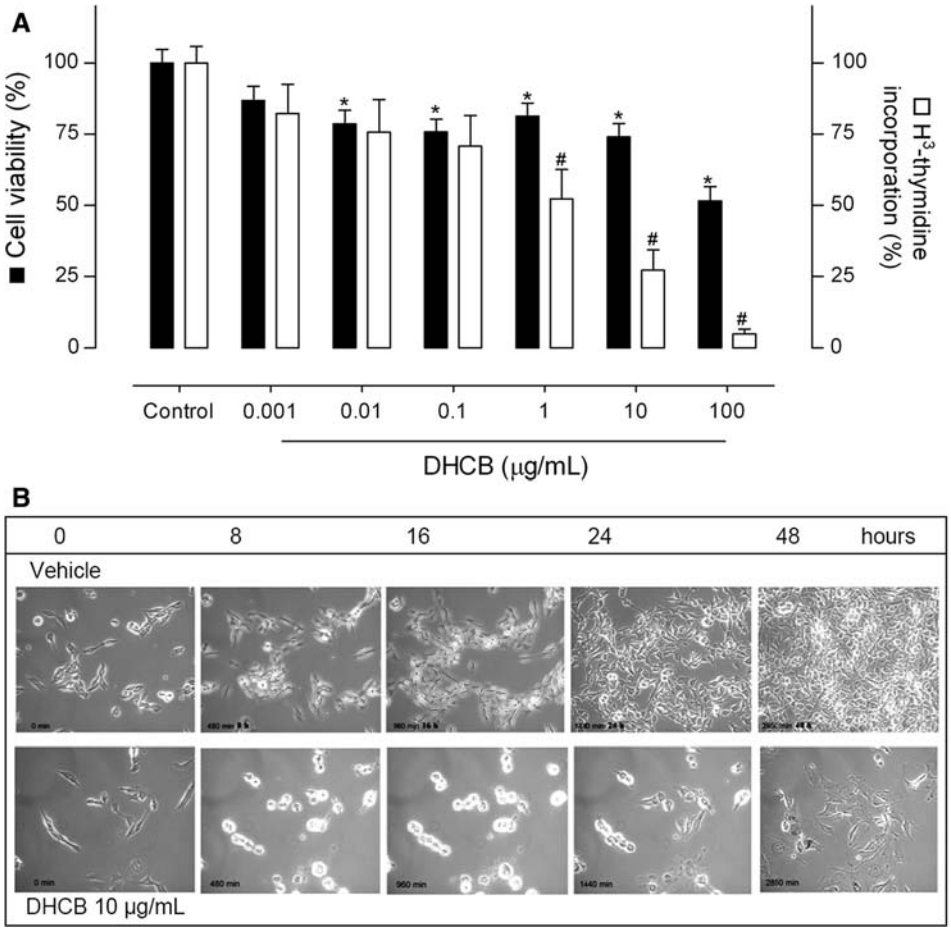

Fig. 1 **a** Effect of a single addition of DHCB (0.001–100 μg/mL; 48 h of incubation) on the viability (MTT, *closed bars*) and proliferation ([H^3]-thymidine incorporation, *open bars*) of B16F10 asynchronous cells. *Each bar* represents the mean ± SEM of at least three different experiments. $^*P < 0.01$ (MTT) or $^\#P < 0.01$ ([H^3]-thymidine incorporation) indicate significant differences from control group. **b** Time lapse of B16F10 in the presence or absence of DHCB 10 μg/mL (0–48 h) for morphology observations (×20 objective)

cells in G2/M phases until 24 h and the appearance of polyploid cells beginning at 16 h. After 48 h of incubation, we observed that the cell cycle profile was completely altered and that polyploidy was predominant (Fig. 2a, b). In another assay we investigated the influence of DHCB addition on the induction of apoptosis, using the incorporation of Annexin-V analyzed by flow cytometry. The results demonstrated that DHCB did not induce important increase the number of apoptotic cells when compared to control, since the percentages of viable cells (lower left), apoptotic cells (lower right) and necrotic cells (upper right) were 81.78, 6.84 and 10.37%, respectively (Fig. 2c).

Effect of DHCB on the expression of cell cycle proteins

The interference of DHCB in the expression of cell cycle proteins was examined in B16F10 cells in culture after 0, 4, 8, 16, 24, 36 and 48 h of incubation. The results showed that DHCB addition (10 μg/mL) was able to reduce the expression of cyclin-A (Fig. 3a) and, mainly cyclin-B1 (Fig. 3b). Other proteins were evaluated including cyclin-E, and p27 (Fig. 3c), but no important differences were found when compared to control. Gel-pro analyzer 4.0 software was used to determine the density of bands, which is shown as arbitrary units.

Fluorescence analysis

To confirm the appearance of polyploid cells observed in flow cytometry analysis, we used Rhodamine-phalloidin/Hoechst 33342 to stain actin filaments and nuclei, respectively. Our qualitative results demonstrated that after 48 h of incubation with DHCB (10 μg/mL) there was a predominance of binuclear cells and, interestingly, an important alteration in the actin cytoskeleton as demonstrated in Fig. 4a and b.

DHCB effects on tumor growth and metastasis in vivo

In these set of experiments we observed the in vivo effect of DHCB on the primary tumor and metastasis. Given by oral route, once a day for 12 days, DHCB suppressed both tumor growth and the number of tumor colonies in the lung. The tumor volume of primary melanoma tumors in DHCB (1, 3 and 10 mg/kg) treated mice was significantly reduced by 49.3; 57.8 and 83.5%, respectively, compared with

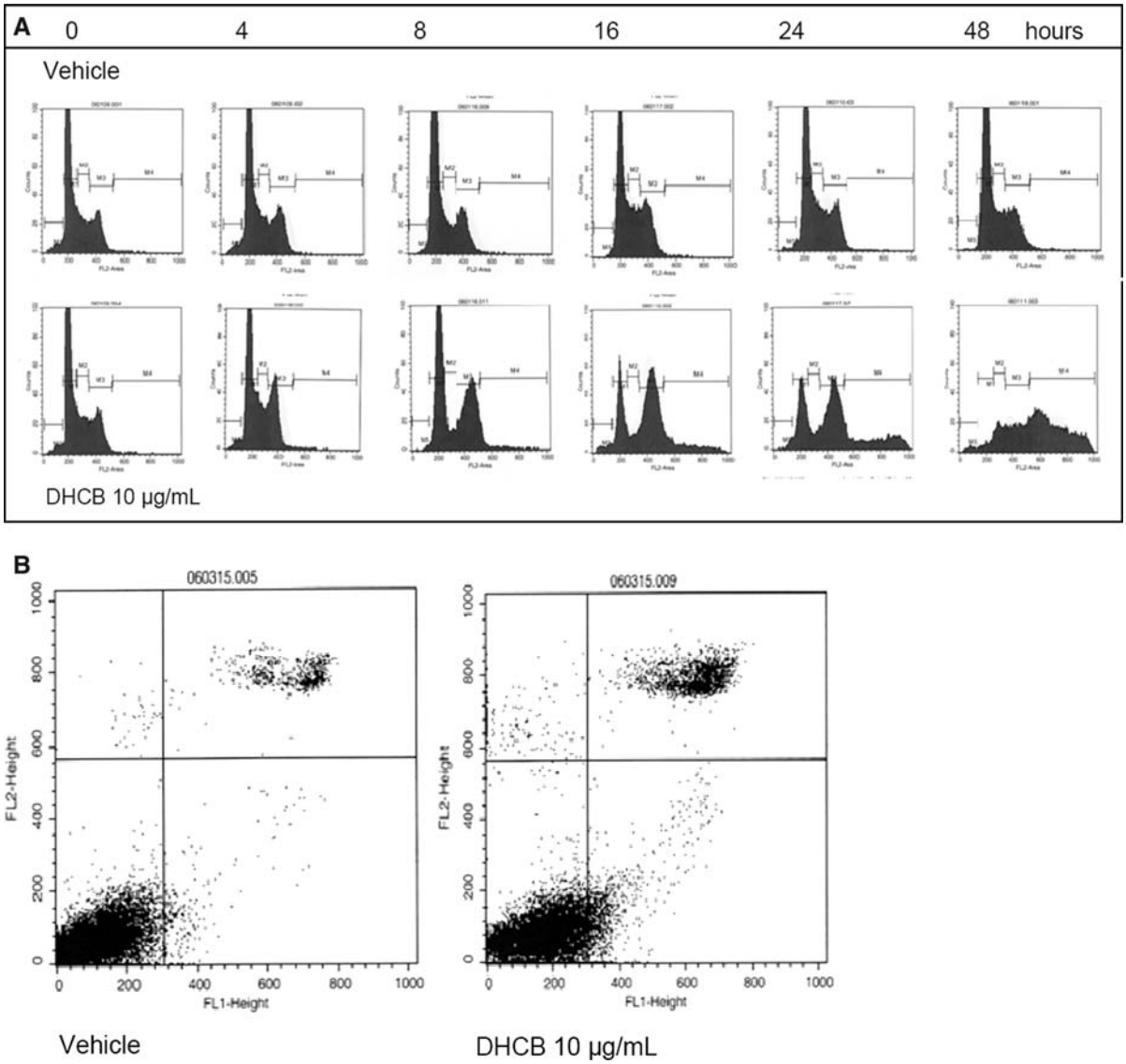

Fig. 2 Flow cytometry investigations. **a** Effect of a single addition of DHCB (10 µg/mL; 0, 4, 8, 16, 24 and 48 h of incubation) on cell cycle distribution of B16F10 asynchronous cells. *M1* cells gated in G1, *M3* cells gated in G2/M and *M4* polyploidy. **b** Annexin-V FITC incorporation in the presence or absence of DHCB (10 µg/mL; 48 h of incubation). In each figure, the lower left *square* represents viable cells, lower right apoptotic cells and upper right necrotic cells

controls ($P < 0.05$) (Fig. 5a). The formation of B16F10 cell tumor colonies in lungs of DHCB (10 mg/kg) treated mice was also significantly reduced by 50.3% ($P < 0.05$) when compared to control (Fig. 5b).

Discussion

We have previously characterized *W. ebracteata* as a source of cucurbitacins and cucurbitacin-related compounds [17, 21–24]. In this study our purpose was to evaluate the in vitro and in vivo antitumoral properties of dihydrocucurbitacin-B (DHCB), a compound isolated from *W. ebracteata* roots, on murine melanoma B16F10 cells.

Our research group has characterized the structures of several cucurbitacins from *W. ebracteata* and demonstrated few structural differences. For example, the structural differences between cucurbitacin B (CUCB) and dihydrocucurbitacin-B occur between carbons 23–24 (a double-bond for CUCB and a single-bond for DHCB) [17, 25]. In the MTT assay, CUCB was found to be around 10 times more potent and also more cytotoxic than DHCB (data not shown).

The present study was initiated with the MTT and [H^3]-thymidine incorporation assays to evaluate cell viability and cell proliferation, respectively. To perform these assays, we attempted to synchronize B16F10 cells by serum deprivation, but this was not possible. Rodriguez-Ayerbe

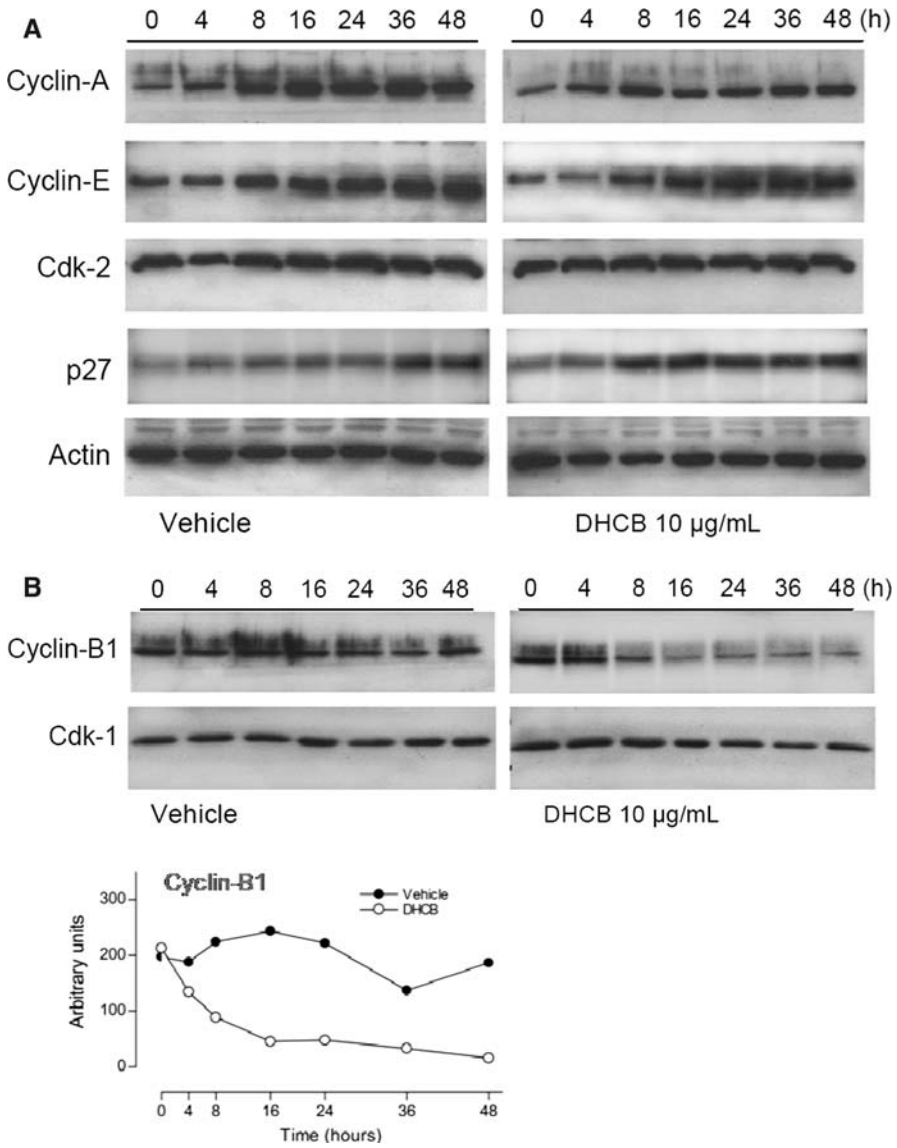

Fig. 3 Western blot analysis of cell cycle proteins in the presence or absence of DHCB 10 µg/mL (0, 4, 8, 16, 24, 36 and 48 h of incubation). **a** Blots for cyclin-A, cyclin-E, cdk-2 and p27 proteins. **b** Blots for cyclin-B1 and cdk-1; the *graph* represent the optical density expressed as arbitrary units of cyclin-B1. Actin was used as protein control

and Smith-Zubiaga [26], using analysis of cell viability, DNA synthesis and proliferation assays, demonstrated that B16F10 cells show a classical cell cycle "freeze", and cannot be synchronized by the serum deprivation followed by refeeding protocol which is successful in normal cell cultures. Our initial results demonstrated that DHCB addition was able to reduce the proliferative status without important effects on cell viability, up to 48 h, as evaluated in the protocol. In addition, analysis of cell dynamics by time lapse showed that DHCB promotes important alterations in the cell morphology, as well as a reduction in the cell growth (as shown in Fig. 1b). Taken together, the results suggest that DHCB could interfere in the mechanisms that control the cell proliferation machinery.

It is well recognized that the understanding of molecular mechanisms leading to cell proliferation allows the identification of targets that could be manipulated to treat tumors. To investigate the effect of DHCB on cell proliferation, we used cell cycle analysis by flow cytometry and western blot assays. In the cell cycle analysis of cells labeled with propidium iodide, we observed that the DHCB incubation lead to a switch of cells to G2/M starting at 4 h and continuing until 24 h. In the investigation of the expression of cell cycle proteins, our results showed that the DHCB addition promotes a tenuous decrease in the expression of cyclin-A. On the other hand, we observed a remarkable decrease in the cyclin-B1 expression beginning at 16 h and sustained up to 48 h of incubation, as determined in the protocol.

The cell cycle is directed by a complex of protein kinases formed by a catalytic subunit, the cdk and a regulatory subunit, the cyclin which confers specificity, each one with its partner (e.g. Cyclin-E/cdk-2). In the G2/M phases, the complex cyclin-B/Cdk-1 is generally known to be the key mediator of phase transition in all eukaryotic cells.

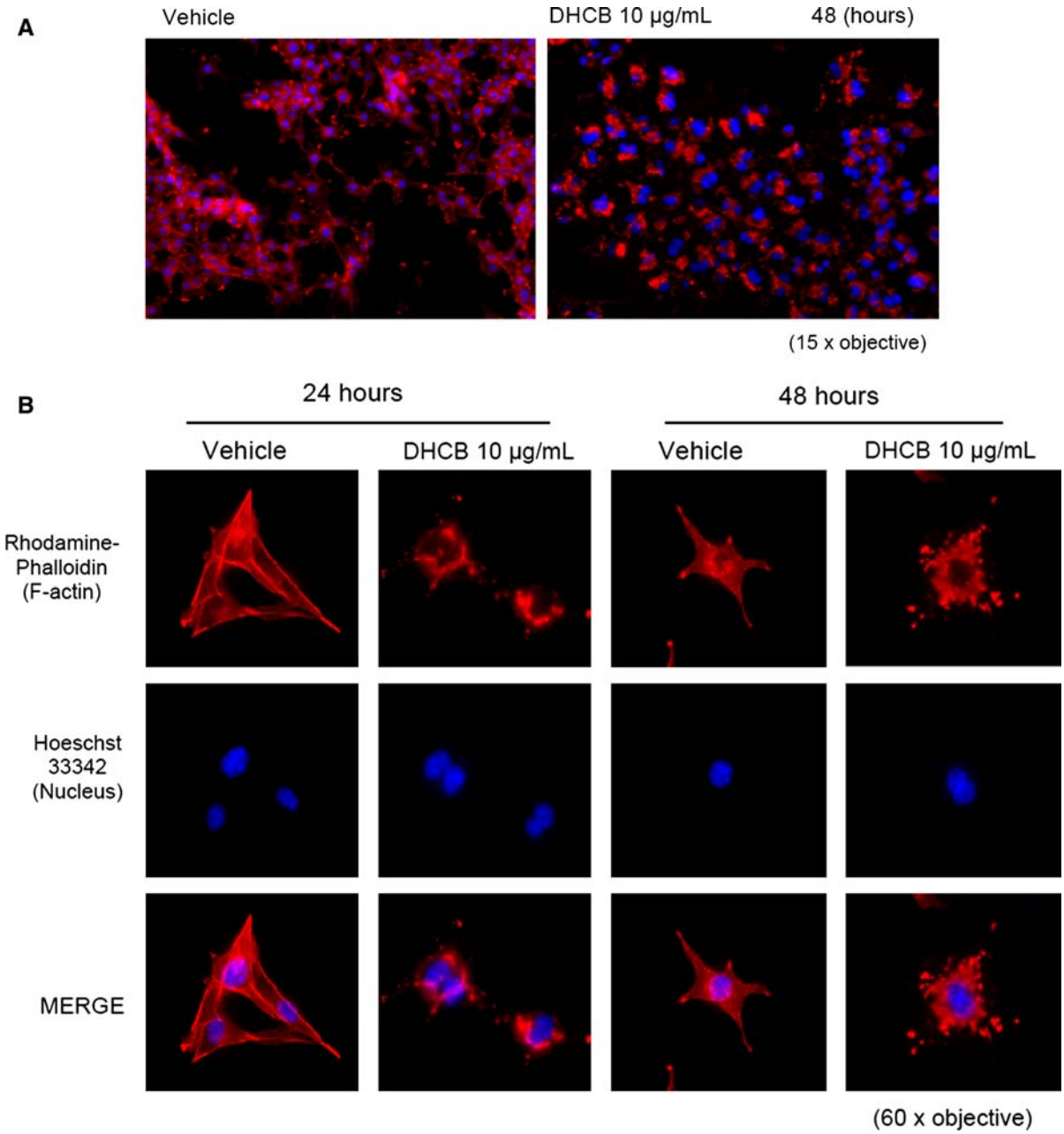

Fig. 4 Fluorescence analysis of B16F10 cells in the presence or absence of DHCB (10 µg/mL; 24 or 48 h of incubation). F-actin and nucleus staining with Rhodamine-Phalloidin and Hoechst 33342, respectively. **a** After 48 h of incubation with DHCB or vehicle, ×15 objective. **b** After 24 and 48 h of incubation with DHCB or vehicle, ×60 objective

This active mitotic kinase (also called MPF or mitosis-promoting factor) is regulated at several levels: the formation of cyclin-B/Cdk-1 complex depends on the availability of cyclin-B, which starts to be produced in late S phase, accumulates until mitosis and is rapidly degraded as cells enter the next G1 phase; it also depends on the subcellular localization of cyclin-B and the regulation of MPF activity by the phosphorylation state. In this process, phosphatases such as cdc25B and cdc25C are responsible for activating while wee1 and myt-1 protein kinases are responsible for inactivating the MPF complex [27]. We found a decrease in the expression of cyclin-B1 that could characterize a reduction in availability. Thus, this fact lead us to believe that it is reasonable to hypothesize that the cell accumulation in G2/M observed after DHCB treatment, at least in part, is due to reduction, but not inhibition, of the activity of the cyclin-B/cdk-1 complex (necessary in the transition in G2 and M phases).

Reports in the literature have described the relationship between cyclin B/Cdk-1 and multinuclear cell formation.

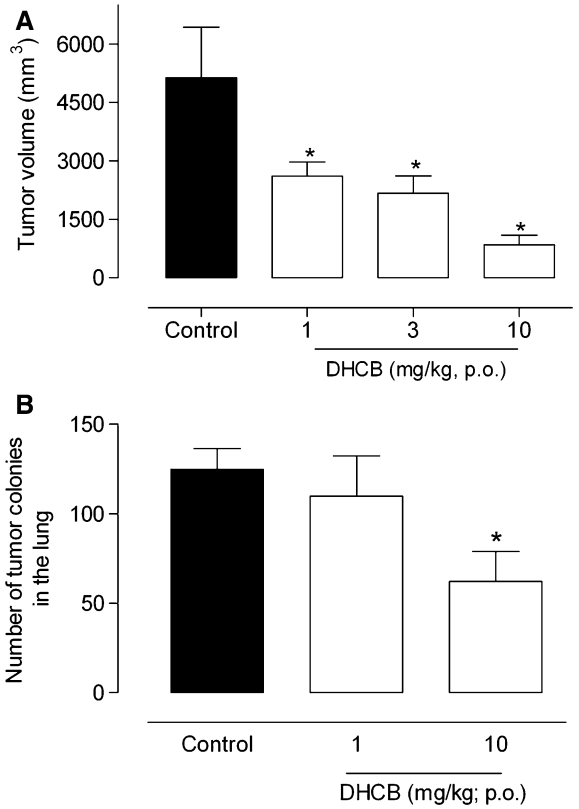

Fig. 5 DHCB suppressed tumor growth and inhibited lung metastasis in the lung in C57BL/6 mouse model. **a** DHCB administered once a day (p.o.) significantly suppressed primary tumor growth when compared to control mice (n = 7–9 animals). **b** DHCB (once a day, 12 days, p.o.) significantly reduces the number of tumor colonies in the lung (n = 7–9 animals). In each graph, bars represent the mean ± SEM; *$P < 0.05$ indicates significant differences from control group

Zhang et al. [28] reported that the cytokinesis, but not karyokinesis, in megakaryocytes appears to be bypassed completely with subnormal levels of cyclin B/Cdk-1 activity due to the increased degradation of cyclin-B. This observation was accompanied by the generation of multinuclear cells. In another set of experiments, was demonstrated that SKF-104976 (an inhibitor of cholesterol biosynthesis) induced a delay in G2-M transition followed by the formation of polyploid cells in human leukemia lineage HL-60. This finding was also accompanied by a decrease in the cyclin-B1/Cdk-1 activity [29].

The cyclin-B/Cdk-1 complex also participates in the reorganization of the cell cytoskeleton architecture required for cell cycle progression. This complex is related to the phosphorylation of proteins implicated in the correct assembly of the mitotic spindle [30] as well as the actin-binding protein caldesmon and several regulators of the small GTPases Rho and CDC42 that mediate the rearrangements of the actin cytoskeleton during mitosis [31].

Our results about the cell cycle analysis by flow cytometry, also indicated the appearance of polyploid cells beginning at 16 and continuing up to 48 h after DHCB incubation. To confirm these data concerning polyploidy, nuclei and actin filaments were stained, respectively, with Hoechst 33342 and Rhodamine-phalloidin and were observed by fluorescence. We observed that mainly after 48 h of DHCB incubation the cells were binucleated and, we also observed an important effect on the actin cytoskeleton.

Some reports have revealed that cucurbitacins can directly modulate the actin cytoskeleton as proposed by Duncan et al. [12], who demonstrated that cucurbitacin E acts as a potent disruptor of cytoskeletal integrity by increasing the filamentous or polymerized actin fraction in prostate carcinoma cells. Moreover, the structural similarity of cucurbitacins to cholesterol also suggests the possibility of a partially membrane-mediated effect, such as disruption of regulatory proteins in the membrane which may affect cytoskeletal architecture as suggested by Fenton et al. [32].

The final step in cell division in which the cytoplasm is divided to form two daughter cells is known as cytokinesis. During late stages of the cell cycle, a cortical ring containing actin and myosin is constructed at the site of cell–cell separation. Following segregation of genetic material and organelles into the two halves of the cell, contraction of the actomyosin ring causes invagination of the plasma membrane and the consequent separation of the two cells. The formation and contraction of the actomyosin ring are tightly regulated processes, ensuring that cell–cell separation occurs at the proper time [33]. Since cytokinesis involves the assembly and disassembly of actin filaments, compounds that interfere with actin polymerization or its spatial organization will block this process and generate multinucleated cells by uncoupling nuclear and cytoplasmic division [34]. These reports complement our findings regarding the effects of DHCB on the actin cytoskeleton and could have direct implications for the formation of polyploid cells and concomitant reduction in cell proliferation. The in vivo assays we have demonstrated the antitumoral effect of DHCB given by oral route. In contrast of other members of cucurbitacins family, DHCB did not show signals of toxicity such as diarrhea, bleedings, behavioral changes or lethality (data not shown), and the most important, was effective in reduce both tumor growth and metastasis. Possibly the effects observed in vitro could be responsible, at least in part, to the in vivo effects, but there are other aspects related to DHCB that we cannot discard, such as the potent COX-2 inhibition recently described [14]. Additional experiments to investigate the in vivo distribution of DHCB and/or its metabolites produced after oral administration are in course to assist the elucidation of in vivo results demonstrated.

In summary, we demonstrated that DHCB promotes a decrease in the cyclin-B expression that could reduce cyclin-B1/Cdk-1 activity and bring about the disorganization

of the actin cytoskeleton. These events could be responsible for the emergence of binuclear cells. Moreover, DHCB was also effective in reduce in vivo tumor growth and lung metastasis. Our findings could have implications in pathological events such as the migration and invasion of tumor cells, however, it remains to be proved if DHCB will be a good candidate for drug development.

Acknowledgments This study was supported by grants from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Apoio à Pesquisa Científica e Tecnológica de Santa Catarina (FAPESC) and Université de Sherbrooke, QC, Canada.

References

1. Malumbres M, Carnero A (2003) Cell cycle deregulation: a common motif in cancer. Prog Cell Cycle Res 5:5–18
2. Pines J (1995) Cyclins and cyclin-dependent kinases: a biochemical view. Biochem J 308(Pt 3):697–711
3. Deshpande A, Sicinski P, Hinds PW (2005) Cyclins and cdks in development and cancer: a perspective. Oncogene 24(17):2909–2915
4. Balunas MJ, Kinghorn AD (2005) Drug discovery from medicinal plants. Life Sci 78(5):431–441
5. van Der Heijden R, Jacobs DI, Snoeijer W, Hallard D, Verpoorte R (2004) The Catharanthus alkaloids: pharmacognosy and biotechnology. Curr Med Chem 11(5):607–628
6. Gordaliza M, Garcia PA, del Corral JM, Castro MA, Gomez-Zurita MA (2004) Podophyllotoxin: distribution, sources, applications and new cytotoxic derivatives. Toxicon 44(4):441–459
7. Oberlies NH, Kroll DJ (2004) Camptothecin and taxol: historic achievements in natural products research. J Nat Prod 67(2):129–135
8. Lavie D, Glotter E (1971) The cucurbitanes, a group of tetracyclic triterpenes. Fortschr Chem Org Naturst 29:307–362
9. Tehila TS, Shlomo G, Sara D, Hugo EG, Margalit B (2007) Growth inhibitory activity of cucurbitacin glucosides isolated from Citrullus colocynthis on human breast cancer cells. Biochem Pharmacol 73:56–67
10. Blaskovich MA, Sun J, Cantor A, Turkson J, Jove R, Sebti SM (2003) Discovery of JSI-124 (cucurbitacin I), a selective Janus kinase/signal transducer and activator of transcription 3 signaling pathway inhibitor with potent antitumor activity against human and murine cancer cells in mice. Cancer Res 63:1270–1279
11. Sun J, Blaskovich MA, Jove R, Livingston SK, Coppola D, Sebti SM et al (2005) Cucurbitacin Q: a selective STAT3 activation inhibitor with potent antitumor activity. Oncogene 24:3236–3245
12. Duncan KL, Duncan MD, Alley MC, Sausville EA (1996) Cucurbitacin E-induced disruption of the actin and vimentin cytoskeleton in prostate carcinoma cells. Biochem Pharmacol 52:1553–1560
13. Shi YR, Seung HL, Sang UC, Chong OL, Zaesung N, Jong WA (1994) Antitumor activity of *Trichosanthes kirilowii*. Arch Pharmacol Res 17:348–353
14. Siqueira JM, Peters RR, Gazola AC, Krepsky PB, Farias MR, Rae GA, Brum-Fernandes AJ, Ribeiro-do-Valle RM (2007) Anti-inflammatory effects of a triterpenoid isolated from *Wilbrandia ebracteata* Cogn. Life Sci 80:1382–1387
15. Escandell JM, Recio MC, Máñez S, Giner RM, Cerdás-Nicolás M, Ríos JL (2006) Dihydrocucurbitacin B, isolated from *Cayaponia tayuya*, reduces damage in adjuvant-induced arthritis. Eur J Pharmacol 532:145–154
16. Yang L, Wu S, Zhang Q, Liu F, Wu P (2007) 23, 24-Dihydrocucurbitacin B induces G2/M cell-cycle arrest and mitochondria-dependent apoptosis in human breast cancer cells (Bcap37). Cancer Lett 256:267–278
17. Farias MR, Schenkel EP, Mayer R, Rücker G (1993) Cucurbitacins as constituents of *Wilbrandia ebracteata*. Planta Med 59:272–275
18. Mosmann T (1982) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth 65:55–63
19. Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83:346–356
20. Naito K, Skog S, Tribukait B, Andersson L, Hisazumi H (1987) Cell cycle related [^{3}H]thymidine uptake and its significance for the incorporation into DNA. Cell Tissue Kinet 20(4):447–457
21. Schenkel EP, Farias MR, Mayer R, Breitmaier E, Rücker G (1992) Cucurbitacins from *Wilbrandia ebracteata*. Phytochemistry 31:1329–1333
22. Peters RR, Farias MR, Ribeiro-do-Vale RM (1997) Anti-inflammatory and analgesic effects of cucurbitacins from *W. ebracteata*. Planta Med 63:525–528
23. Peters RR, Saleh TF, Lora M, Patry C, de Brum-Fernandes AJ, Farias MR, Ribeiro-do-Vale RM (1999) Anti-inflammatory effects of the products from *W. ebracteata* on carrageenan-induced pleurisy in mice. Life Sci 64(26):2429–2437
24. Peters RR, Krepsky PB, Siqueira-Junior JM, Rocha JCS, Bezerra MM, Ribeiro RA, de Brum-Fernandes AJ, Farias MR, Rocha FAC, Ribeiro-do-Valle RM (2003) Nitric oxide and cyclooxygenase may participate in the analgesic and anti-inflammatory effect of the cucurbitacins fraction from *Wilbrandia ebracteata*. Life Sci 73(17):2185–2197
25. Rodriguez-Ayerbe C, Smith-Zubiaga I (2000) Effect of serum withdrawal on the proliferation of B16F10 melanoma cells. Cell Biol Int 24(5):279–283
26. Valente LMM (2004) Cucurbitacins and their main structural characteristics. Quim Nova 27(6):944–948
27. Molinari M (2000) Cell cycle checkpoints and their inactivation in human cancer. Cell Prolif 33:261–274
28. Zhang Y, Wang Z, Liu DX, Pagano M, Ravid K (1998) Ubiquitin dependent degradation of cyclin B is accelerated in polyploid megakaryocytes. J Biol Chem 273:1387–1392
29. Fernandez C, Lobo MdMdelV, Gomez-Coronado D, Lasuncion MA (2004) Cholesterol is essential for mitosis progression and its deficiency induces polyploid cell formation. Exp Cell Res 300:109–120
30. Ubersax JA, Woodbury EL, Quang PN, Paraz M, Blethrow JD, Shah K, Shokat KM, Morgan DO (2003) Targets of the cyclin-dependent kinase Cdk1. Nature 425:859–864
31. Yamashiro S, Yamakita Y, Hosoya H, Matsumura F (1991) Phosphorylation of non-muscle caldesmon by p34cdc2 kinase during mitosis. Nature 349:169–172
32. Fenton RG, Kung HF, Longo DL, Smith MR (1992) Regulation of intracellular actin polymerization by prenylated cellular proteins. J Cell Biol 117:347–356
33. Huckaba TM, Pon LA (2002) Cytokinesis: rho and formins are the ringleaders. Curr Biol 12(23):R813–R814
34. Giganti A, Friederich E (2003) The actin cytoskeleton as a therapeutic target: state of the art and future directions. Prog Cell Cycle Res 5:511–525