



O-naphthoquinone isolated from *Capraria biflora* L. induces selective cytotoxicity in tumor cell lines

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ABSTRACT. Biflorin is an o-naphthoquinone isolated from the roots of the plant *Capraria biflora* L. (Scrophulariaceae). In this study, the cytotoxic effects of biflorin were verified, and late apoptosis was detected in various cancer cell lines by *in situ* analysis. The cytotoxicity was further evaluated exclusively for 48 h of treatment in different tumor and non-tumor cell lines (Hep-2, HeLa, HT-29, A-375, and A-549, and HEK-293, respectively). The results indicated that biflorin induced selective cytotoxicity in tumor cells. HeLa cells were more susceptible to biflorin, followed by HT-29, A-549,

A-375, and Hep-2 at all concentrations (range 5-50 µg/mL), and the highest half-maximal inhibitory concentration IC_{50} (56.01 ± 1.17 µg/mL) was observed in HEK-293 cells. Late apoptotic/necrotic events, observed by *in situ* immunostaining with Annexin V, varied with each cell line; an increase in late apoptotic events was observed corresponding to the increase in biflorin dosage. Hep-2 cells showed a greater percentage of late apoptotic events among the tumor cell lines when treated with higher concentrations of biflorin ($69.63 \pm 2.28\%$). The non-tumor HEK-293 line showed greater resistance to late apoptotic events, as well as a lower level of cytotoxicity ($77.69 \pm 6.68\%$) than the tested tumor lines. The data presented indicate that biflorin showed an important, possibly selective, cytotoxicity against tumor cell lines, thereby revealing a promising novel substance with potential anticancer activity for tumor therapy.

Key words: Apoptosis; Biflorin; Cytotoxicity; O-naphthoquinone; Tumor

INTRODUCTION

Capraria biflora L. (Scrophulariaceae) is a perennial shrub distributed throughout North and South America (Wisintainer et al., 2014). Phytochemical investigations of this plant species have led to the isolation and characterization of biflorin [6,9-dimethyl-3-(4-methyl-3-pentenyl) naphtha [1,8-bc]pyran-7,8-dione] from its roots (Fonseca et al., 2003). Biflorin is an o-naphthoquinone with proven cytotoxic effects against tumor cells (anti-tumor), as well as anti-microbial and anti-mutagenic activities (Wisintainer et al., 2014). It is also strongly active against gram-positive and alcohol-acid-resistant bacteria (Gonçalves de Lima et al., 1958).

Biflorin has been shown to inhibit the proliferation of five tumor cell lines (CEM, HL-60, B16, HCT-8, and MCF-7) in a dose-dependent manner, at concentrations ranging from 0.39 to 25 µg/mL (Vasconcellos et al., 2005). Moreover, 1-20 µM biflorin exerted a cytotoxic effect on the SK-Br3 cell line (Montenegro et al., 2013b). A previous study also suggested that biflorin acts by inhibiting N-cadherin function in MDA-MB-435 melanoma cells; inhibitors of N-cadherin function have been reported to cause apoptosis and regulate the AKT-1 pathway (Montenegro et al., 2013b).

The antitumor activity of biflorin has also been evaluated *in vivo* in animals transplanted with Sarcoma 180 and Ehrlich carcinoma. A histopathological exam of the spleen, kidney, and liver of these transplanted animals displayed the promising antitumor effects and low toxicity of biflorin (Vasconcellos et al., 2007). Mouse *in vivo* studies have also revealed that biflorin coupling increased the effectiveness of 5-fluoracil chemotherapy (5-FU), and reduced the systemic toxicity (Vasconcellos et al., 2007). Moreover, the administration of 25 mg/day biflorin to animals transplanted with melanoma B16 for ten days induced significant tumor growth inhibition with no systemic toxicity (Vasconcellos et al., 2011).

However, there have been no reports on the selective cytotoxicity and effects of biflorin on the morphological changes and induction of apoptosis (in conjunction with annexin V) on tumor cells, apart from the cytotoxic screening of biflorin treatment in tumor cell lines. In this study, we evaluated the cytotoxic activity of biflorin in the tumor cell lines Hep-2 (human larynx carcinoma), HeLa (human cervical adenocarcinoma), HT-29 (human colon cancer), and A-375 (human melanoma), A-549 (human alveolar epithelial), and the non-tumor line HEK-293 (human embryonic kidney). We also attempted to determine the morphological aspects of the cells and induction of apoptosis after biflorin treatment via *in situ* analyses.

MATERIAL AND METHODS

Isolation of biflorin

Capraria biflora was collected from a plantation located in Fortaleza, Ceará, Brazil, in April 2005, and identified by Dr. Edson Nunes. A voucher specimen (No. 30848) was deposited in the Herbarium of the Federal University of Ceará. Air-dried powdered roots (6 kg) were extracted with light petroleum (4:1) for two days. The extract was partially evaporated at room temperature until the formation of a solid material was observed. The latter was filtered under vacuum and yielded purple solid compost (2 g). The purple solid material was subjected to Silica-gel chromatography, as well as isocratic elution using a binary mixture of light petroleum and EtOAc (9:1 v/v). The fractions were pooled together based on the results of thin-layer chromatography (TLC). Combined fractions containing the purified biflorin yielded 1.5 g compound.

Mass spectrometric (MS) analysis - chemical identification

The dry fraction was dissolved in a mixture containing 50% (v/v) chromatographic grade acetonitrile (Tedia, Fairfield, OH, USA), 50% (v/v) deionized water, and 0.1% formic acid. The solution was infused directly into the electrospray ionisation (ESI) source at a flow rate of 100 $\mu\text{L}\cdot\text{min}^{-1}$, with the assistance of a high performance liquid chromatograph (HPLC; Shimadzu, Tokyo, Japan). ESI (+)-MS and ESI (-)-MS were acquired using a hybrid high-resolution and high accuracy (5 $\mu\text{L/L}$) micro-time of flight (TOF) (Q-TOF) mass spectrometer (Bruker Scientific®, Billerica, MA, USA) under the following conditions: capillary and cone voltage, + 3500 V and + 40 V, respectively; and de-solvation temperature, 180°C. A TOF control software (Bruker Scientific®) was used for data acquisition and processing. The data was collected in an m/z range of 70-800 and at a speed of 2 scans/second; a resolution of 50,000 (FWHM) was obtained at m/z 200. No important ions were observed below m/z 100 or above m/z 600; therefore, the ESI (+)-MS data at the m/z range 295-350 is shown.

Cell culture and cytotoxic assay

The Hep-2 (human larynx carcinoma), HeLa (human cervical adenocarcinoma), HT-29 (human colon cancer), A-375 (human melanoma), A-549 (human alveolar epithelial), and HEK-293 (human embryonic kidney) cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with antibiotics and 10% fetal bovine serum (Gibco; Life Technologies, Carlsbad, CA, USA) at 5% CO_2 and 37°C. In order to assess the cytotoxic activity, cells were seeded in 96-well flat-bottom microplates at a density of approximately 5×10^4 cells/well. Following attachment, the cells were treated with serial dilutions of biflorin in culture media: in order to identify a suitable incubation time, HeLa and HEK-293 cells were incubated for 24, 48, and 72 h; the cytotoxic curve profiles for the different times of cultivation with biflorin were similar in these cells. Therefore, the 48 h incubation period was utilized to determine the cytotoxic activity of biflorin in all tumor cell lines.

The cell viability was determined using the tetrazolium salt assay (MTT) (Denizot and Lang, 1986). Briefly, 5×10^4 cells/well were cultured in 96-well plates, and treated with biflorin, dissolved in increasing concentrations (5-50 $\mu\text{g/mL}$) of dimethyl sulfoxide (DMSO), for 48 h. DMSO was added after incubating the cells with MTT solution at room temperature for 2 h; the absorption was determined at 540 nm (SpectraMAX M2/M2e; Molecular Devices, Sunnyvale, CA, USA). At

least three independent experiments were performed for each experimental cell line, and the half maximal inhibitory concentration (IC_{50} ; $\mu\text{g/mL}$) was determined (Monks et al., 1991). Biflorin was prepared immediately prior to each treatment. The negative control was exposed to an equivalent concentration of DMSO solvent.

Detection of late apoptosis by *in situ* analysis

Apoptotic cell death was detected by *in situ* analysis using Annexin V staining and visualized by fluorescence microscopy. Hep-2, HeLa, HT-29, A-375, A-549, and HEK-293 cells were seeded (1.0×10^5 cells/mL) on to coverslips in 24-well plates with 500 μL , supplemented culture media. After 24 h, the cells were treated with 5, 10, and 40 $\mu\text{g/mL}$ biflorin (negative controls were exposed to an equivalent concentration of DMSO) and incubated at 37°C under 5% CO_2 conditions for 48 h. The media was subsequently removed, and the cells incubated with 2.5 $\mu\text{g/mL}$ Annexin V (ab114196; Abcam, Cambridge, UK) primary antibody in binding buffer (HEPES 10 mM, NaCl 150 mM, KCl 5 mM, MgCl_2 1mM, CaCl_2 1.8 mM, pH 7.4) for 30 minutes. The cells were then washed once with binding buffer, and incubated with 2.5 $\mu\text{g/mL}$ Dylight® 488 (ab115637; Abcam, Cambridge, UK) secondary antibody. The cells were also stained with propidium iodide (PI) to verify possible necrotic activation (P4170; Sigma-Aldrich, St. Louis, MO, USA). Results were expressed as the percentage of cells undergoing early stage (Annexin-V-positive) or late stage (Annexin-V-positive/ PI-positive) apoptosis: at least 500 cells were counted in 10 different fields in triplicate, and subsequently quantified as a percentage of the total number of cells.

Statistical analyses

The results are reported as means \pm standard deviation (SD) of each group. One-way analysis of variance (ANOVA) followed by the Tukey *post hoc* test was used to evaluate differences among the treatment groups. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS 19.0) software (IBM, Armonk, NY, USA). The level of significance was uniformly set at $P < 0.05$.

RESULTS

Isolation and chemical characterization of biflorin

In this study, ESI (+)-MS and tandem ESI (+)-MS/MS were acquired using a microTOF-Q II (Q-TOF), and the spectra from biflorin is displayed in Figure 1. Accordingly, the m/z 309.1483 $[\text{M} + \text{H}]^+$ and 331.1286 $[\text{M} + \text{Na}]^+$ matched the molecular formula $\text{C}_{20}\text{H}_{21}\text{O}_3$ and $\text{C}_{20}\text{H}_{21}\text{O}_3\text{Na}$ with the experimental difference 2.49 and 7.29 ppm, respectively (Figure 1). The isotopic ratio of $[\text{M} + \text{H}]^+$ is confirmatory with the m/z ratio of 310.1511 (22.85%) and 311.1530 (2.67%). There were no ions in the mass spectra in positive mode ESI (+) beyond those reported (Figure 1). In negative mode ESI (-) wherein ammonium hydroxide (0.1%) was used in solution, no answer was expected because of the poor ionization of this functional group. This set of results confirmed the purity of the compound.

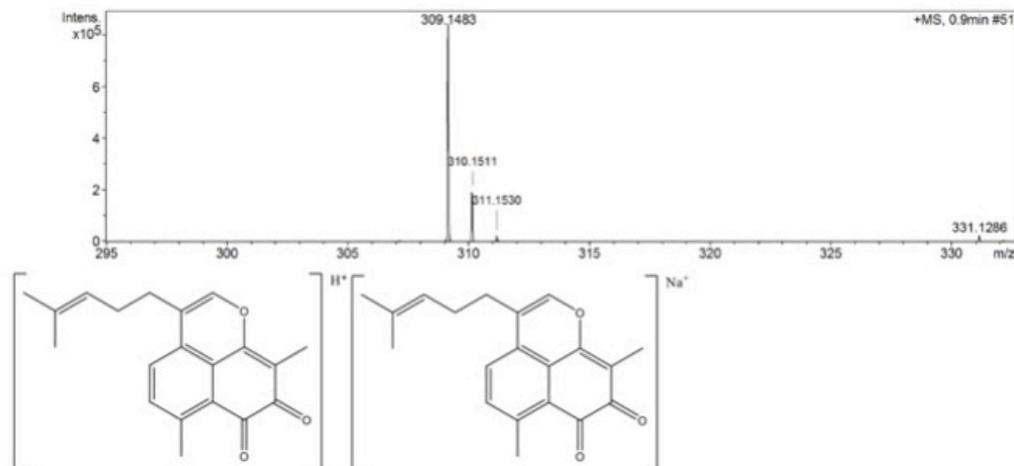


Figure 1. ESI (+) mass spectra of biflorin (6,9-dimethyl-3-(4-methyl-3-pentenyl) naphtha[1,8-bc]-pyran-7,8-dione), highlighting adducts chemical structures $[M + H]^+$ and $[M + Na]^+$ at m/z 309.1483 and 331.1286, respectively.

Cytotoxicity assay

Initially, the tumor line HeLa and non-tumor cell line HEK-293 were treated with biflorin at a range of 5-50 $\mu\text{g/mL}$ for 24, 48, and 72 h (Figure 2). A selective cytotoxicity was observed in HeLa (Figure 2A), compared to the HEK-293 (Figure 2B) cells. The cytotoxicity was further evaluated exclusively at 48 h of treatment on six different cell lines (Hep-2, HeLa, HT-29, A-375, A-549, and HEK-293) and the half-maximal inhibitory concentration (IC_{50}) is presented in Figure 3. HeLa cells were more susceptible to biflorin, followed by HT-29, A-549, A-375, and Hep-2 at all tested concentrations (5-50 $\mu\text{g/mL}$); the highest IC_{50} value ($56.01 \pm 1.17 \mu\text{g/mL}$) was exhibited by the HEK-293 cell line. Given the increased susceptibility of HeLa to the cytotoxic effect of biflorin, this cell line was selected for further morphological analyses using Giemsa staining. Substantial morphological changes in HeLa cells were observed after 48 h of treatment with increasing concentrations of biflorin (5-50 $\mu\text{g/mL}$; Figure 4).

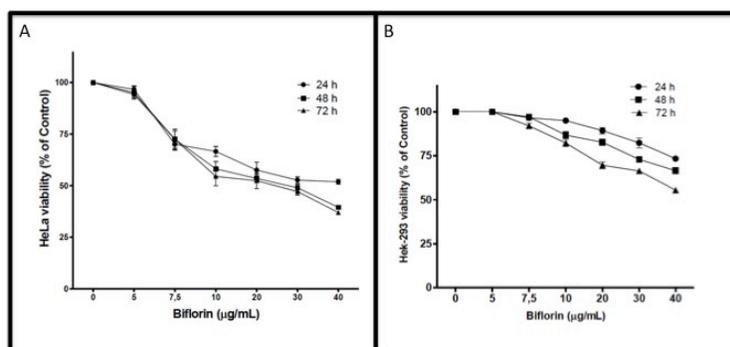


Figure 2. Cytotoxic effect of biflorin extracts on the viability of HeLa (A) and HEK-293 (B) cell lines. Cells were treated for 24, 48, and 72 h with 5, 7.5, 10, 20, 30, and 40 $\mu\text{g/mL}$ biflorin. Results are reported as means \pm standard deviation (SD) for triplicate experiments.

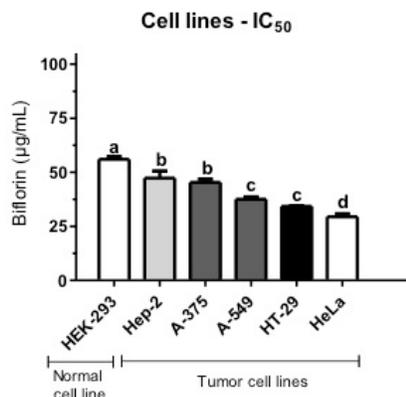


Figure 3. Half maximal inhibitory concentration (IC₅₀) of biflorin (after 48 h treatment) in different cell lines; IC₅₀ (µg/mL) is reported as means ± SD. Different letters indicate the level of significance determined by one-way ANOVA followed by Tukey *post hoc* analysis ($P < 0.05$) used to examine the difference between treatment groups against the appropriate control (HEK-293).

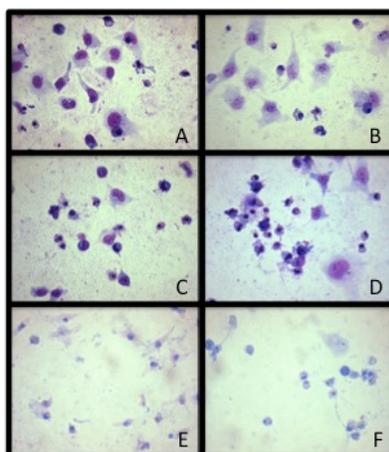


Figure 4. Morphological changes in HeLa cells treated with biflorin (5-50 µg/mL) for 48 h. Photomicrographs were taken under a light microscope (200X). Cells treated with different concentrations of biflorin: **A.** 5 µg/mL; **B.** 7.5 µg/mL; **C.** 10 µg/mL; **D.** 20 µg/mL; **E.** 30 µg/mL; and **F.** 40 µg/mL were stained with Giemsa.

Detection of late apoptosis by *in situ* analysis

Biflorin induced apoptosis in Hep-2, A-375, A-549, HT-29, and HeLa tumor cells. *In situ* Annexin V immunostaining showed that a majority of the cells from all cell lines were at a late apoptotic stage in a biflorin dose-dependent manner. Annexin V+/PI- cells indicate phosphatidylserine detection on the external surface of an intact plasma membrane (green fluorescence). PI+ (red fluorescence) in conjunction with Annexin V+ (Merge) labeling in cells indicate a compromised membrane that may be a result of either late apoptotic or necrotic activation. Late apoptosis/necrosis events varied according to the lines analyzed. In general, all cells presented an increase in late apoptotic activation when treated with higher doses of biflorin. Interestingly, late apoptosis events

started at lower concentrations of biflorin (10 $\mu\text{g}/\text{mL}$) in all tumor lines (Figure 5). Hep-2 cells showed greater values when treated with higher concentrations (40 $\mu\text{g}/\text{mL}$) of biflorin ($69.63 \pm 2.28\%$). The non-tumor HEK-293 cell line showed more resistance to apoptosis activation; however, late apoptotic events were visible after treatment with higher concentrations of biflorin ($77.69 \pm 6.68\%$).

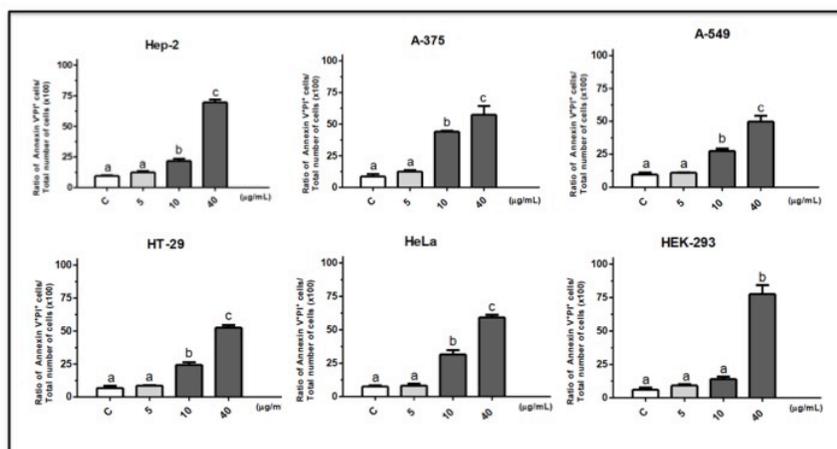


Figure 5. Detection of apoptosis by *in situ* analysis with PS-binding Annexin V in biflorin-treated Hep-2, A-375, A-549, HT-29, HeLa, and HEK-293 cells (48 h). The letter C represents the negative solvent control. Different letters indicate the level of significance, as tested by one-way ANOVA followed by the Tukey *post hoc* test ($P < 0.05$), compared to the control.

DISCUSSION

Natural products have played an important role in the treatment and prevention of human diseases. Furthermore, 60% of the drugs currently used have their origin related to natural sources. Among the sources found in nature, plants have made the single largest contribution to the development of therapeutic substances (Kirkpatrick, 2002). Quinones are secondary metabolites found in several plants and present a variety of biological effects, including anticancer, antibacterial, and antimalarial effects. In particular, naphthoquinones, which include biflorin, belong to a promising group of compounds with antitumor properties (Asche, 2005). Moreover, quinone moieties are present in many drugs, such as anthracyclines, daunorubicin, doxorubicin, mitomycin, mitoxantrone, and saintopin, which are clinically used in the treatment of solid cancers (Verma, 2006).

Despite previous studies using *in vitro* and *in vivo* models indicating that biflorin presents potential antitumor activity, there have been no reports on its selective cytotoxicity. Additionally, its effects on the morphological changes and induction of apoptosis in tumor cell lines have not been described. In this study, the cytotoxic activity of biflorin was evaluated in five tumor lines (Hep-2, HeLa, HT-29, A-375, and A-549), and compared these effects to those seen in non-tumor Hek-293 cells. The morphological aspects of biflorin-treated HeLa cells were also investigated, and the induction of apoptosis was evaluated in all cell lines by *in situ* analysis. We observed that the same concentration of biflorin was able to inhibit cell proliferation more effectively in tumor cells (5-50 $\mu\text{g}/\text{mL}$) than in the Hek-293 cells (56.01 $\mu\text{g}/\text{mL}$).

The effect of biflorin on different tumor cell lines have been previously screened at a range of biflorin concentrations (0.39-25 $\mu\text{g}/\text{mL}$) similar to the one reported in this study (5-50 $\mu\text{g}/\text{mL}$) (Vasconcellos et al., 2005, 2007, 2011). Recently, Montenegro et al. (2013a) revealed that biflorin

(1-20 μM) exerted cytotoxic effects on breast cancer SK-Be3 cells; additionally, biflorin treatment was seen to exert selective cytotoxicity against cancer cells in relation to normal cells, as observed in this study. The authors also demonstrated that the inhibition of cell proliferation by biflorin could be mediated through the down-regulation of the EGFR signaling pathway, as biflorin decreases the total EGFR expression (Montenegro et al., 2013a).

The selective induction of cell death plays a major role in decreasing the possible side effects of chemotherapy (Reichert and Wenger, 2008; Parkinson et al., 2013). The relation between cancer and apoptosis has been emphasized for a long time, suggesting that tumor progression involves the inhibition of apoptotic stages in tumor cells (Yang et al., 2006; Vasconcellos et al., 2011). Apoptosis, autophagy, and necrosis are the three main forms of cell death, each one presenting a unique set of biochemical and morphological changes that occur within the dying cell (Elmore, 2007; Ouyang et al., 2012). Moreover, apoptosis is a programmed form of cell death observed in tissues during development, and maintains tissue homeostasis through the elimination of excessive or injured cells (Ouyang et al., 2012; Solá et al., 2013).

Substantial morphological changes in HeLa cells were observed when treated with increasing concentrations (5-50 $\mu\text{g/mL}$) of biflorin for 48 h, through Giemsa staining. *In situ* immunostaining with Annexin V showed that the cells were mainly at the late apoptotic stages. Late apoptosis was more evident ($77.69 \pm 6.68\%$) in the non-tumor line HEK-293 treated with higher concentrations of biflorin, compared to the tested tumor cell lines, which may indicate the resistance of non-tumor cells and a specific selectivity of the compound. Exposure of PS anionic phospholipids at the outer leaflet of the plasma membrane is correlated with the loss of asymmetry in the cell membrane, and plays a physiological role in the recognition and subsequent removal of the dying cell via phagocytosis (Poon et al., 2010). This phenomenon permits the detection and differentiation of apoptotic cell death from necrosis. The staining of cells by membrane impermeable dyes such PI allows one to differentiate between early apoptosis and late apoptosis/necrosis (Elmore, 2007).

Evidence suggests that tumor cells with changes in apoptotic function may diverge to another means of cell death such as necrosis, thereby promoting an alternative therapeutic approach (Zong et al., 2004; Degenhardt et al., 2006; Zong and Thompson, 2006). Necrosis may arise as a result of errors in apoptotic function, which could also induce alterations in the glucose metabolism. Although necrosis can be considered a less efficient mechanism of cell death compared to apoptosis, defective apoptotic tumor cells may be more susceptible to therapy despite the possible consequences, such as inflammation (Degenhardt et al., 2006).

Many therapeutic agents for cancer, such as cisplatin, paclitaxel, isothiocyanate, and adriamycin, eliminate tumor cells by inducing apoptotic cell death (Tan et al., 2009; Hanahan and Weinberg, 2011). Tumor growth can be determined by the speed with which tumor cells progress towards death and elimination, in addition to the progression of malignant cells (Mester and Redeuilh, 2008). Therefore, the inhibition of tumor growth and induction of tumor cell death are elective therapeutic strategies for the control of tumor progression (Yang et al., 2006). The activation of programmed cell death should specifically target tumor cells, minimizing the side effects of inappropriate apoptosis induction in normal cells. Therefore, the development of new treatment strategies for cancer is crucial. Specifically, multiple pathways of cell death and the molecular mechanisms involved in carcinogenesis must be thoroughly elucidated.

These are preliminary results and should be interpreted in light of their limitations. Although cancer cell lines are an established model for the study of molecular mechanisms of anti-cancer phytometabolites, the use of cancer stem cells may help in better understanding the regulation of

cell dynamics by the phytometabolite biflorin. Secondly, this study used imaging techniques and immunocytochemistry to verify and quantify the death pathway activation. Necessary parameters were taken to ensure the consistency of acquisition measurements; however, immunofluorescence is a limited and semi-quantitative method. The use of more sensitive techniques, such as real-time polymerase chain reaction (RT-PCR), is strongly advocated for future studies to confirm these findings. Finally, exploratory analyses of a broader range of factors, including the changes in expression patterns of specific proteins and alterations in DNA, such as locus-specific methylation and demethylation changes, would help further clarify the mechanism of biflorin. Future studies that explore these factors may allow for the validation of biflorin as a potential therapeutic agent.

Conflicts of interest

The authors declare no conflict of interest.

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