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# Biflorin induces cytotoxicity by DNA interaction in genetically different human melanoma cell lines

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ABSTRACT

Cancer is a public health problem and the second leading cause of death worldwide. The incidence of cutaneous melanoma has been notably increasing, resulting in high aggressiveness and poor survival rates. Taking into account the antitumor activity of biflorin, a substance isolated from *Capraria biflora* L. roots that is cytotoxic in vitro and in vivo, this study aimed to demonstrate the action of biflorin against three established human melanoma cell lines that recapitulate the molecular landscape of the disease in terms of genetic alterations and mutations, such as the *TP53*, *NRAS* and *BRAF* genes. The results presented here indicate that biflorin reduces the viability of melanoma cell lines by DNA interactions. Biflorin causes single and double DNA strand breaks, consequently inhibiting cell cycle progression, replication and DNA repair and promoting apoptosis. Our data suggest that biflorin could be considered as a future therapeutic option for managing melanoma.



## 1. Introduction

Melanomas are tumors that arise from the malignant transformation of melanocytes, and they are the most frequent and aggressive form of skin cancer (Gray-Schopfer et al., 2007; Nikolaou and Stratigos, 2014). This type of cancer is notable because of its high metastatic potential and resistance to conventional treatments, both of which contribute to its high mortality and low survival rates (Siegel et al., 2015; Tentori et al., 2013). These characteristics justify the numerous studies that have been performed to attempt to understand its genetic and molecular behaviors and to identify the critical signaling pathways that are involved in the initiation and progression of these malignancies. These studies have resulted in the development of new therapeutic targets for melanoma treatment (Flaherty et al., 2010).

Signaling pathways involving MAPK, AKT, ERK and PI3K have been targeted by various drugs, such as the $BRAF^{V600E}$ inhibitors vemurafenib (Zelboraf) and dabrafenib (Tafinlar) and the MEK inhibitor trametinib. These drugs have changed the management of stages III and IV melanoma by decreasing tumor progression and increasing the life expectancy of patients (Flaherty, 2006; Homet and Ribas, 2014). Nevertheless, most patients develop reversible and adaptive resistance to these small molecule drugs because of feedback activation via other signaling pathways (Bollag et al., 2010; Eliades et al., 2015; Flaherty, 2012).

Considering the aggressiveness and debilitating nature of the chemotherapeutic regimens that are associated with this disease, new

*Abbreviations:* AKT, murine thymoma viral oncogene homolog 1; BRAF, v-raf murine sarcoma viral oncogene homolog B; DNMT, DNA methyltransferase; ERK, extracellular regulating kinase; MAPK, mitogenic activated protein kinase; MBP, methyl-CpG binding proteins; MELK, maternal embryonic leucine zipper kinase; MGMT, O6-methyl-guanine-DNA-methyl-transferase; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; RAD, repair DNA ATPase; RAS, rat sarcoma; TYMS, thymidylate synthase.

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therapeutic strategies aim to develop molecules with greater therapeutic efficacy and a reduced incidence of adverse effects along with increased target specificity. Biflorin, an *o*-naphthoquinone isolated from *Capraria biflora* roots, has been demonstrated to be a potent antitumor drug both in vitro and in vivo. In addition, biflorin does not present clastogenic or aneuploidogenic activity (Montenegro et al., 2013a,b; Vasconcellos et al., 2005, 2007, 2010, 2011, 2015).

We evaluated biflorin's cytotoxicity, morphological changes, type of cell death, ability to induce DNA damage and alterations in the expression of genes related to cell cycle progression (*MELK*) (Ganguly et al., 2015), DNA replication (*TYMS*) (Kotoula et al., 2012), and repair (*MGMT*, *RAD*) (Christmann et al., 2011; Wu et al., 2008) along with the oncogene *BRAF*. We evaluated the cytotoxicity of biflorin in different genetic cell models and established its role as an important inducer of apoptosis. This ability is critical for drugs aimed at regulating cancer cell proliferation via processes that are either dependent or independent of mutations in *TP53*, *NRAS* and *BRAF*.

## 2. Materials and methods

### 2.1. Chemicals

Resazurin (CAS n. 62758-13-8), crystal violet (CAS n. 548-62-9), acridine orange (CAS n. 10127-02-3), ethidium bromide (CAS n. 1239-45-8), agarose (CAS n. 39346-81-1), sodium chloride (CAS n. 7647-14-5), ethylene diamine tetra acetic acid (CAS n. 6381-92-6) and trypan blue (CAS n. 72-57-1) were obtained from Sigma Chemical, Co. (St. Louis, MO, USA).

### 2.2. Isolation of biflorin

*C. biflora* was collected from a plantation that is located in Fortaleza (Ceará, Brazil) and identified by Dr. Edson Paula Nunes (Botany Professor of Federal University of Ceará, Biology Department, Prisco Bezerra Herbarium). A voucher specimen (number: 30848) was deposited in the Herbarium Prisco Bezerra of the Biology Department of the Federal University of Ceará. The extraction, isolation and purity were confirmed as previously described in a study by Fonseca et al. (2003).

Pure biflorin was dissolved in 100% DMSO and stored at 20 °C at a concentration of 1.0 mM. The DMSO concentrations never exceeded 0.1% in culture and resulted in 100% cell viability when used as a control (Fonseca et al., 2003).

### 2.3. Cell lines and culture conditions

Human malignant melanoma cells SK-Mel 19, 28 and 103 were kindly provided by Sylvia Stuch-Engler (Department of Pharmaceutical Sciences, School of Pharmaceutical Sciences, University of Sao Paulo). Cells were grown in 75-cm$^2$ culture flasks as adherent monolayer cultures in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, Foster City, CA) supplemented with 10% fetal bovine serum (FBS, Vitrocell), 100 μg/mL streptomycin and 100 U/mL penicillin, and incubated at 37 °C with 5% $CO_2$ atmosphere. The genetic differences in the cell models are presented in Table 1.

**Table 1**
Genetic characteristics of the SK-Mel 19, SK-Mel 28 and SK-Mel 103 cell lines. Wt — wild-type; Q61R — amino acid substitution at position 61 of glutamine to arginine; *TP53*–R273H: hot spot mutation of the protein, resulting in substitution of arginine to histidine at codon 273; V600E — substitution of valine for glutamic acid at position 599.
Adapted from Brohem et al., 2012.

| Gene | Cell line | | |
|---|---|---|---|
| | SK-Mel 19 | SK-Mel 28 | SK-Mel 103 |
| *TP53* | Wt | R273H | Wt |
| *NRAS* | Wt | Wt | Q61R |
| *BRAF* | V600E | V600E | Wt |

### 2.4. Viability of human melanoma cell lines

The 50% inhibitory concentration ($IC_{50}$) of SK-Mel cells was determined by colorimetric Alamar blue (AB) assay (resazurin 7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide) according to Ahmed et al. (1994). A total of $0.5 \times 10^4$ cells from each cell line were plated and allowed to attach for 24 h. Biflorin (50–0.78 μM) was then added at 100 μL/well. After 24, 48 and 72 h of biflorin exposure, 10 μL/well of AB solution in 0.02% phosphate-buffered saline was added. After the cells were incubated for an additional 2 h, fluorescence was measured at 465 nm and 540 nm using a microplate reader (DTX 800 Beckman Coulter Multimode Detector) (Ahmed et al., 1994).

Cell viability was assessed by trypan blue exclusion assay according to Strober (2001). A total of $5.0 \times 10^4$ cells from each cell line were plated for a 24-h attachment period This step was followed by the addition of 1.0, 2.5 and 5.0 μM biflorin at 1.0 mL/well. After incubation periods of 24, 48 and 72 h with biflorin, SK-Mel cells were harvested to count live versus dead cells on a hemocytometer chamber using trypan blue solution 0.4% (Strober, 2001).

### 2.5. Morphological analysis with crystal violet staining

To evaluate morphological changes in the cytoplasm and nucleus, we performed crystal violet staining. SK-Mel human melanoma cells ($2.0 \times 10^5$ cells/well) were treated with 1.0, 2.5 and 5.0 μM biflorin for 24 h. Cells were then fixed with paraformaldehyde 4% for 30 min at 25 °C. Subsequently, the cells were washed three times with distilled water and stained with 0.1% crystal violet solution for 20 min at 25 °C in the dark. The wells were photographed for posterior analysis using light microscopy (Leica Microsystems).

### 2.6. Fluorescence microscopic analysis of cell death

To determine the type of cell death caused by biflorin, we performed acridine orange/ethidium bromide staining according to Kroemer et al. (2005). SK-Mel cells ($3 \times 10^4$ cells/well) were treated with 1.0, 2.5 and 5.0 μM biflorin for 24, 48 and 72 h. Then, cell aliquots were suspended in 20 μL phosphate buffered saline (PBS) and stained with 1 μL of an aqueous solution of acridine orange/ethidium bromide (100 μg/mL; AO/EB). The cell suspension was immediately examined using fluorescence microscopy (Leica Microsystems) at 40× magnification. Three hundred cells were counted per sample and classified as viable (uniform bright green nuclei with an organized structure), apoptotic (orange to red nuclei with condensed to fragmented chromatin and green cytoplasm) or necrotic (uniformly orange to red nuclei with an organized structure and red cytoplasm) (Kroemer et al., 2005).

### 2.7. DNA fragmentation by alkaline and neutral comet assay

To determine the capacity of biflorin to induce DNA fragmentation, an alkaline comet assay was performed according to Singh et al. (1988) with modifications. SK-Mel cells ($1.0 \times 10^6$ cells/well) were treated with 5.0 μM biflorin for 3 h. The slides were prepared by mixing 50 μL of the treated cell suspension in 100 μL of 1% low melting point (LMP) agarose and layering this mixture on microscope slides that were previously covered with normal melting point (NMP) agarose. The microscope slides were then placed in lysis solution (2.5 M sodium chloride, 100 mM ethylene diamine tetra acetic acid, 10 mM Tris, 10% dimethylsulfoxide and 1% Triton X-100) at 4 °C overnight to remove cellular proteins. The slides were then immersed in electrophoresis buffer [300 mM NaOH, 1 mM EDTA (pH > 13)] for 20 min to unwind the DNA, and they were then subjected to electrophoresis (20 V; 300 mA) for 20 min at 4 °C in the dark (Singh et al., 1988). After electrophoresis, the slides were neutralized with 0.4 M Tris–HCl pH 7.5 and dried overnight at room temperature. Neutralized and dehydrated slides were stained with ethidium bromide (2 ng/mL) before scoring. One hundred

**Table 2**
Cytotoxic activity of biflorin on human melanoma cells SK-Mel 19, 28 and 103 after 24, 48 and 72 h of treatment using Alamar blue assay. Dacarbazine and doxorubicin were used as positive control. Data are presented as half maximal inhibitory concentration ($IC_{50}$) value and 95% confidence intervals (CI 95%) from three independent experiments performed in triplicate.

| Cell line | SKMEL 19 IC50 (μM) (CI 95%) | | | SKMEL 28 IC50 (μM) (CI 95%) | | | SKMEL 103 IC50 (μM) (CI 95%) | | |
|---|---|---|---|---|---|---|---|---|---|
| Time (h) | 24 | 48 | 72 | 24 | 48 | 72 | 24 | 48 | 72 |
| Biflorin | 2,32 (1.94–2.77) | 1.97 (1.77–2.19) | 1.6 (1.33–1.89) | 9.2 (6.89–12.36) | 3.73 (2.8–4.96) | 4.8 (4.09–5.64) | 1.85 (1.62–2.10) | 1.54 (1.38–1.72) | 2.10 (1.94–2.28) |
| Doxorubicin | 4.19 (3.28–5.36) | 0.14 (0.06–0.28) | 0.14 (0.07–0.27) | 5.68 (4.73–6.83) | 1.08 (0.90–1.29) | 1.06 (0.94–1.19) | 4.16 (3.48–4.98) | 1.75 (1.14–2.68) | 1.31 (1.01–1.26) |
| Dacarbazine | 56.59 (49.88–64.20) | 40.96 (37.36–44.90) | 30.46 (25.76–36.02) | 21.73 (18.15–26.02) | 21.51 (16.89–27.40) | 21.45 (20.76–22.81) | 78.85 (63.09–98.54) | 34.05 (27.37–42.35) | 28.64 (26.38–31.10) |

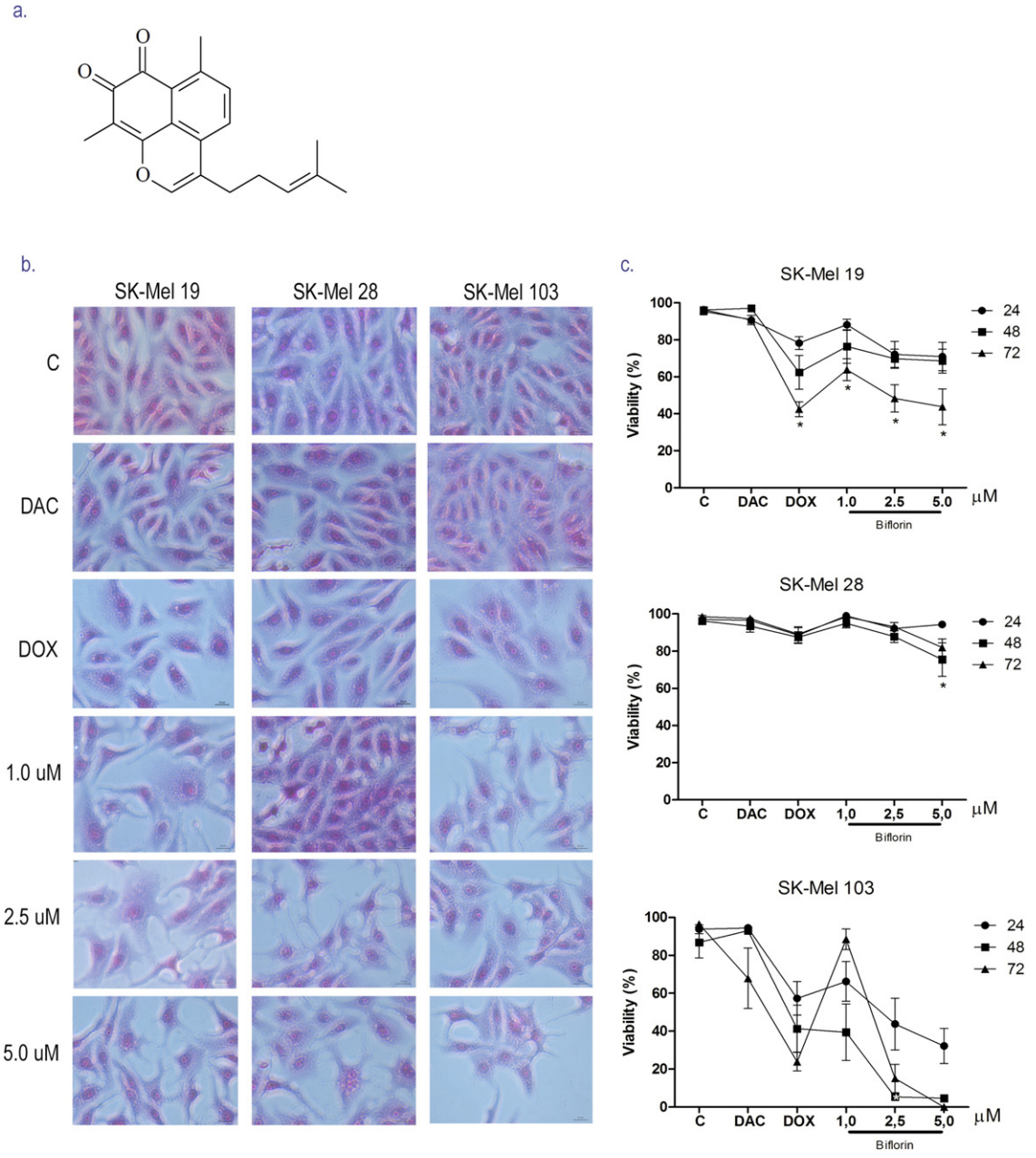


**Fig. 1.** Cell viability in the human melanoma cell lines SK-Mel 19, 28 and 103 after treatment with biflorin for 24, 48 and 72 h. a. biflorin molecular configuration; b. SK-Mel cell lines stained with crystal violet; c. viability of cells performed by trypan blue staining. The vehicle 0.1% dimethylsulfoxide (C) was used as the negative control. Dacarbazine (DAC) and doxorubicin (DOX) 5.0 μM were used as positive control. Data are presented as mean values ± SE, from three independent experiments performed in independent biological replicates in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to negative control; ANOVA + Bonferroni posttest.

cells were scored for each sample (50 per slide), and DNA damage was classified using a visual scoring method described by Collins et al. (2001). Neutral comet assay was performed according to Wojewódzka et al. (2002). The procedures for the cell treatments, slide preparations, lysis, neutralization and staining were identical to those used to perform the alkaline assay. Nevertheless, the electrophoresis buffer was 300 mM sodium acetate and 100 mM Tris–HCl pH 8.5 and the electrophoresis was run at 25 V and 300 mA for 20 min in the dark (Collins et al., 2001; Singh et al., 1988; Wojewódzka et al., 2002).

### 2.8. Quantitative reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed to evaluate the expression levels of the following genes: *MELK*, *TYMS*, *MGM*, *RAD*, and *BRAF*. Total RNA was extracted from the treated (biflorin 1.0, 2.5 and 5.0 μM) human melanoma cell lines after 24 h using Trizol (Invitrogen) according to the manufacturer's instructions. The concentration and quality of the extracted RNA were measured using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE), and the integrity of the samples was determined using gel electrophoresis. Complementary DNA was synthesized using a High-Capacity® cDNA Reverse Transcription kit (Life Technologies, Foster City, CA) according to the manufacturer's instructions. The reaction to detect the expression range of the 5 candidate reference genes was performed in triplicate using TaqMan® inventoried assays-on-demand probes (Life Technologies, Foster City, CA) and the Applied Biosystems 7500 fast real-time PCR system. We quantified the mRNA expression levels of the target genes *MELK* (Hs01106440_m1), *TYMS* (Hs00426586_m1), *MGMT* (Hs00172470_m1), *RAD* (Hs00269177_m1), and *BRAF* (Hs00269944_m1) using the reference gene *B2 M*. Relative quantifications (RQs) were calculated according to the Livak method (Livak and Schmittgen, 2001).

### 2.9. Statistical analysis

Data were presented as the mean ± S.E.M. (standard error of the mean) using GraphPad Prism 5.0 for Windows (Institute Software for Science, San Diego, CA). Viability was expressed as $IC_{50}$ and was obtained using nonlinear regressions based on three replicates per concentration level. The percentage of viable cells in the trypan blue assay, the percentage of apoptotic cells in the AO/EB stain, the damage frequency in comet assay, and the RQ of mRNA expression in quantitative RT-PCR were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. The damage index in the normal and modified comet assay was analyzed by one-way ANOVA followed by Tukey's multiple comparison test. The data are presented as mean values ± standard error (SE) from three independent experiments performed in triplicate.

## 3. Results

### 3.1. Biflorin cytotoxicity in human melanoma cells

Assessment of cell viability was performed to test the potential cytotoxic effects of biflorin on the human melanoma cell lines SK-Mel 19, 28 and 103. The AB assay was utilized, and the effects of different concentrations of biflorin (50 μM to 0.0315 μM) were evaluated after 24, 48 and 72 h of exposure (Table 2). Biflorin and the chemotherapeutic agents doxorubicin and dacarbazine caused cell death of the three human melanoma cell lines studied. Considering the $IC_{50}$ values shown in Table 2, the concentrations of 1.0, 2.5 and 5.0 μM of biflorin and 5.0 μM of doxorubicin and dacarbazine were used for subsequent experiments.

Biflorin exerts a concentration-dependent cytotoxic effect according to the results of trypan blue exclusion and crystal violet stain assays (Fig. 1). After 24 h of biflorin exposure, cell viability was greater than 60% in all cell lines. In SK-Mel 28, biflorin caused significant cell death just after 48 and 72 h of treatment, demonstrating that this cell line was more resistant to biflorin. Otherwise, SK-Mel 103 was the most sensitive, and the second most sensitive was SK-Mel 19 at all times tested. Biflorin also causes a decrease in the cell density which is directly proportional to its increase in concentration, as presented in Fig. 1b. Additionally, the adhered cells presented important morphology alterations, which were detailed in Fig. 2.

To verify the cell morphology after 24 h of treatment with 1.0, 2.5 and 5.0 μM biflorin, we performed crystal violet staining of monolayered cells in 6-well plates. The untreated cells (Fig. 2a) morphologically presented malignancy criteria, such as an increased nuclear/cytoplasmic ratio, nuclear hyperchromasia, irregular nuclear

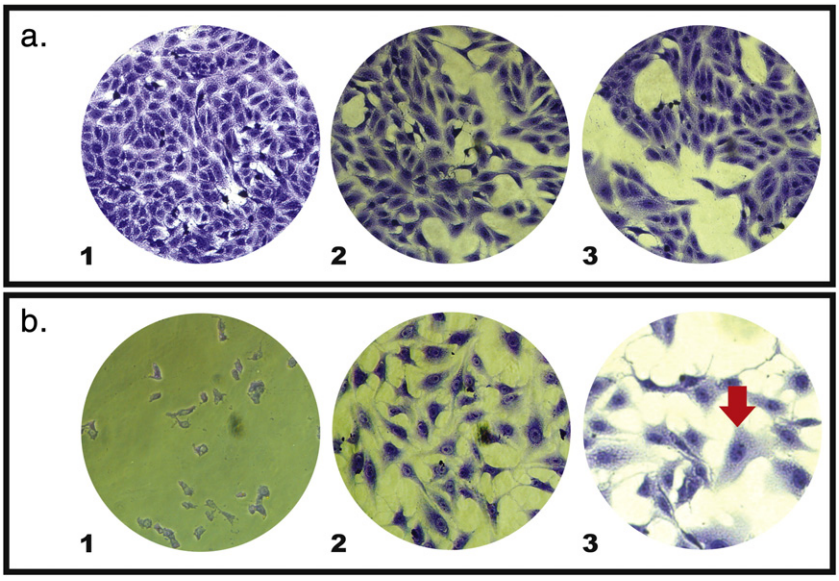


**Fig. 2.** Morphological alterations in SK-Mel cells treated with biflorin for 24 h performed by crystal violet staining. Figures are representative of three independent experiments. a — negative control on: a.1 SK-Mel 19, a.2 SK-Mel 28, and a.3 SK-Mel 103, 40×. b — cells treated with biflorin 5.0 μM during 24 h. b.1 SK-Mel 19; b.2 SK-Mel 28; b.3 SK-Mel 103. b.1 and b.2 — 20×; b.3 — 40× The red arrow presents nucleus fragmentation.

contours, multinucleation, granular chromatin, and perinuclear halo. After treatment with biflorin, we observed cell lysis with an increase of scattered cellular debris, cytoplasm vacuolization, disruption of the nucleus and chromatin fragmentation. These characteristics indicated cell death by apoptosis and reflected the cytotoxic capacity of biflorin for 24 h of treatment in all three of the SK-Mel cell lines tested, despite a lack of extensive cell death in SK-Mel 28.

### 3.2. Apoptotic cell death after biflorin treatment in AO/EB staining

To investigate whether biflorin-induced cytotoxicity is attributable to apoptosis or necrosis, acridine-orange ethidium bromide assay was performed (Fig. 3). We observed a concentration-dependent increase in the number of apoptotic cells, with significant differences observed at all concentrations tested. The number of necrotic cells was not significantly different across any of the treatments. In contrast, even the highest concentration of biflorin (5.0 μM) caused less than 80% of apoptosis in SK-Mel-28 cells at 24 h, thus corroborating the cytotoxicity results.

### 3.3. DNA fragmentation after biflorin treatment in alkaline and neutral comet assays

To determine and compare the genotoxicity of biflorin in the human melanoma cell lines SK-Mel 19, 28 and 103, we determined the damage index using alkaline and neutral comet assays (Fig. 4). First, we analyzed biflorin's potency to induce single-strand breaks, double-strand breaks and alkali-labile sites using alkaline comet assay. Second, we determined the particular induction of double-strand breaks using neutral comet assay. After a 3 h incubation period, biflorin induced DNA strand breaks that led to a significantly higher damage index than what was observed in the negative control in all three SK-Mel cell lines. In the alkaline comet assay, 5.0 μM biflorin induced a damage index (DI) that was approximately three times greater than the index of the negative control.

No significant difference was observed in the alkaline and neutral damage index across the SK-Mel cell lines except between SK-Mel 19 and 103 under alkaline conditions. Therefore, 5.0 μM biflorin induced

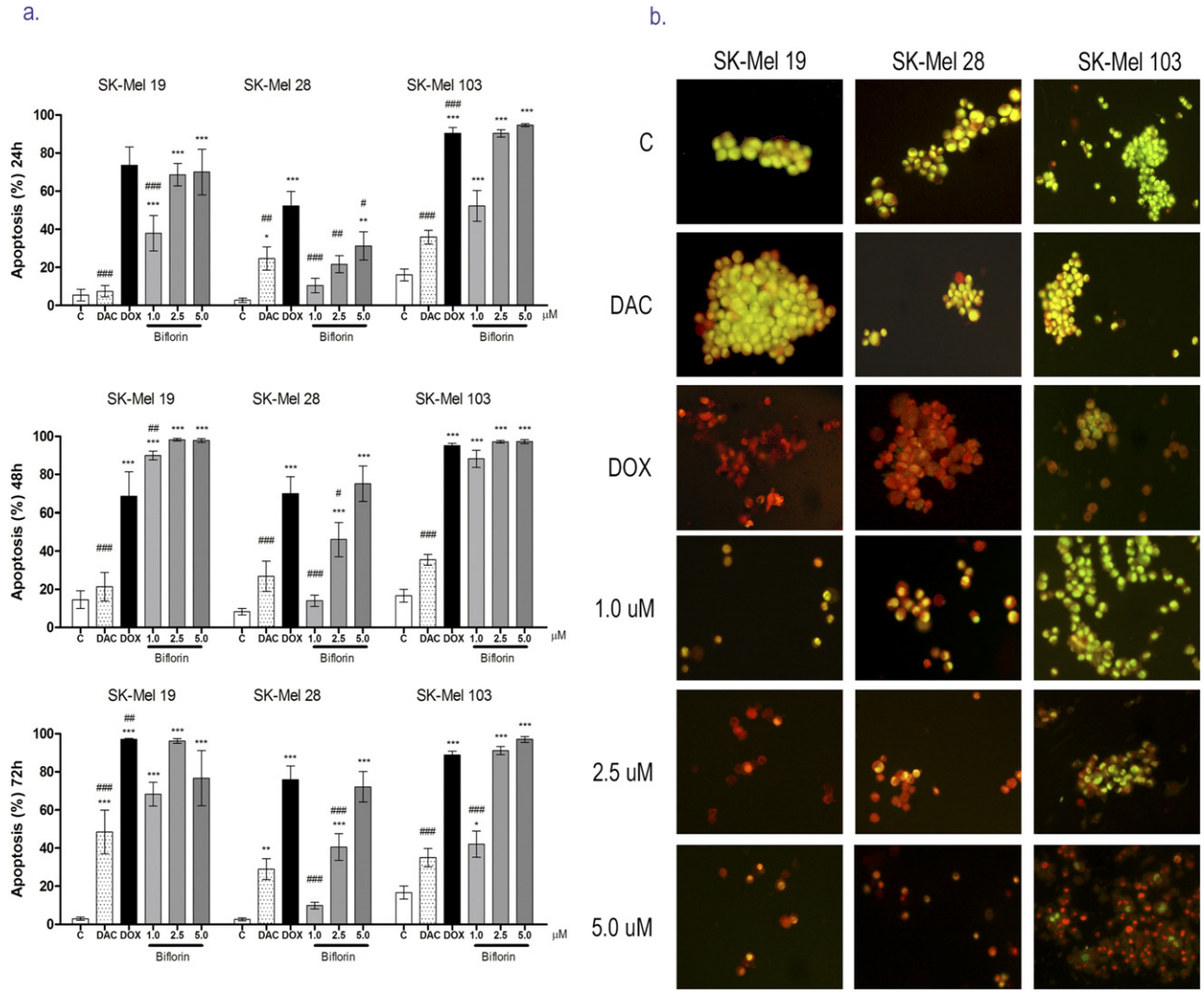


**Fig. 3.** Time-dependent induction of apoptosis in human melanoma cells SK-Mel 19, 28 and 103 after 24, 48 and 72 h of treatment with biflorin determined by acridine orange and ethidium bromide staining. a. Graphical representation; b. photographs of cells stained with AO/EB. Viable cells — green cytoplasm and nucleus; apoptosis — red nucleus with green cytoplasm; necrosis — red nucleus and cytoplasm. The vehicle 0.1% dimethylsulfoxide (C) was used as the negative control. Dacarbazine (DAC) and doxorubicin (DOX) 5.0 μM were used as positive controls. Data are presented as mean values ± SE from three independent experiments performed in triplicate using independent biological replicates. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to negative control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared to doxorubicin. ANOVA + Bonferroni post-test.

an equivalent genotoxic capacity as doxorubicin in all cell lines studied. In addition, when we compared the effects of biflorin and doxorubicin, biflorin caused fewer double strand breaks (neutral comet assay) than global points of damage (alkaline comet assay) except in the SK-Mel 103 cells. Based on these results, biflorin has genotoxic activity in human melanoma cells.

### 3.4. RT-PCR

#### 3.4.1. mRNA expression of cell cycle progression, DNA replication and repair genes

To explore the complementary actions of biflorin during proliferation and DNA interactions, we performed RT-PCR to analyze genes

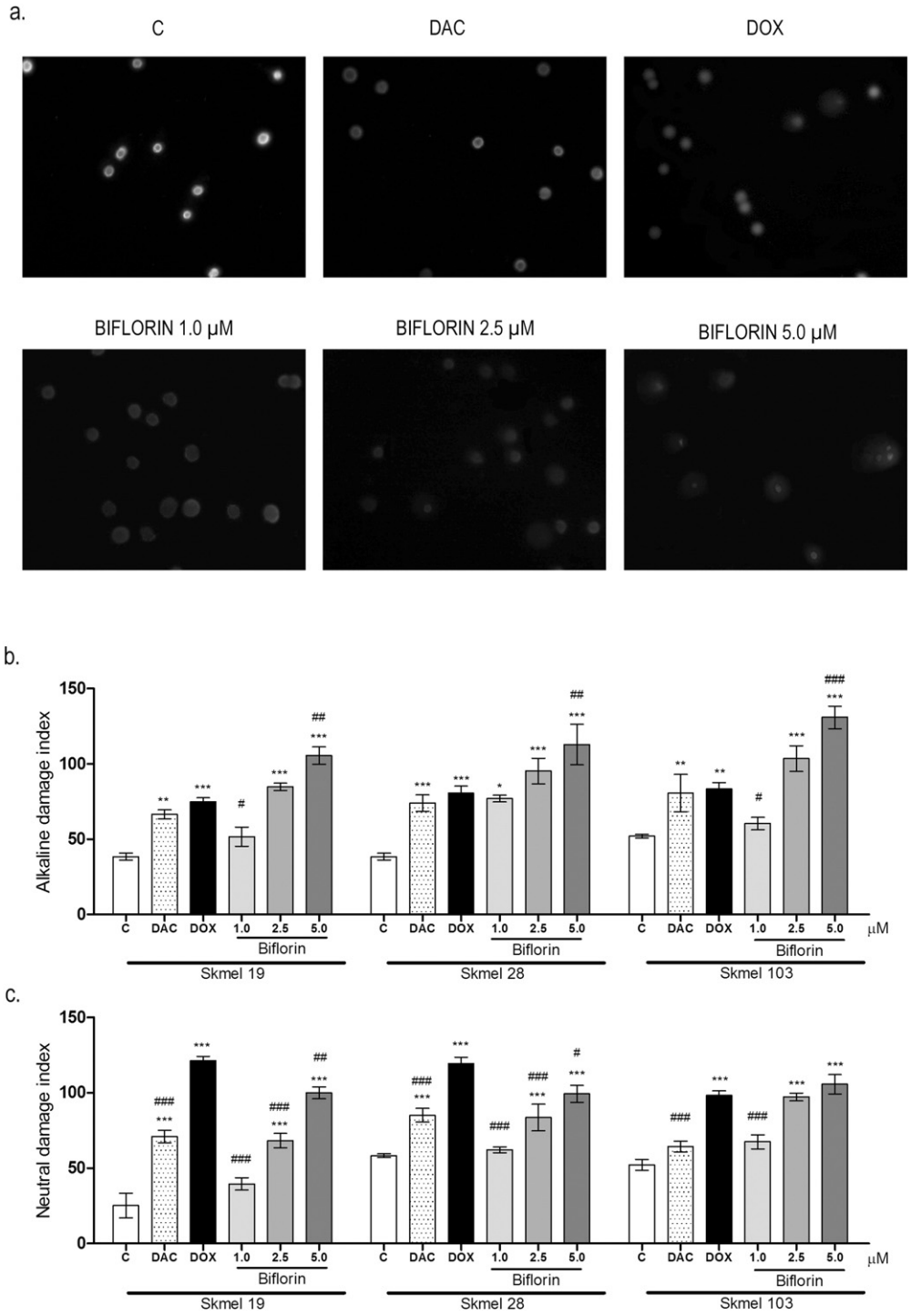


**Fig. 4.** Damage indexes in human melanoma cell lines SK-Mel 19, 28 and 103 after 3 h of treatment with biflorin 1.0, 2.5 and 5.0 μM. Results were determined using alkaline and neutral comet assays. a. photographs of SK-Mel 19 comets after alkaline comet assay; b. graphical representation of alkaline damage index; c. graphical representation of neutral damage index. The vehicle, 0.1% dimethylsulfoxide (C), was used as negative control. Dacarbazine (DAC) and doxorubicin (DOX) 5.0 μM were used as positive control. Data are presented as mean values ± SE from three independent experiments that were performed in triplicate using independent biological replicates. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to negative control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared to doxorubicin. ANOVA + Bonferroni post-test.

known to be involved in cell cycle progression (*MELK*), DNA replication (*TYMS*), and repair (*MGMT* and *RAD*) in addition to the oncogene *BRAF* (Fig. 5). Considering that all of three cell lines were almost 100% viable in 1.0 μM biflorin, we choose this concentration to discuss the mRNA expression. In SK-Mel 28 cells, *TYMS*, *RAD* and *MGMT* presented overexpression (RQ 1.792 ± 0.115, RQ 2.294 ± 0.141, RQ 1.935 ± 0.132, respectively). In SK-Mel 103 cells, the *MELK*, *RAD* and *TYMS* genes exhibited reduced gene expression after treatment with 1.0 μM (RQ 0.403 ± 0.034; RQ 0.328 ± 0.020, RQ 0.312 ± 0.020, respectively). In SK-Mel 19 and SK-Mel 28 ($BRAF^{V600E}$) cells, the expression of the oncogene *BRAF* presented decreased and increased expression (RQ 0.582 ± 0.020, RQ 4.386 ± 0.234), respectively. However, no significant difference was observed in *BRAF* expression compared to its expression in SK-Mel 103 (*wtBRAF*) cells (RQ 1.052 ± 0.049).

## 4. Discussion

Biflorin has been shown to be a promising antitumor drug lead for different types of cancer (Vasconcellos et al., 2005, 2007, 2011; Montenegro et al., 2013b). Polycyclic quinoid compounds, such as naphthoquinones, like biflorin, and anthraquinones, commonly produce DNA strand breaks, and this DNA interaction (e.g., intercalated DNA or alkylation) can contribute to cytotoxicity (Vasconcellos et al., 2010, 2015; Neves et al., 2013). In the present study, biflorin induced cell death in melanoma cell lines mainly due to DNA interaction leading to apoptosis with different efficacy between genetically modified cell lines, such as SK-Mel 19 (BRAFV600E and wtNRAS), SK-Mel 28 (TP53R273H and BRAFV600E) and SK-Mel 103 (wtBRAF and NRASQ61R). Previously, Montenegro et al. (2013b) and Vasconcellos et al. (2011) demonstrated that biflorin is cytotoxic to melanoma cell lines, but not to normal cells, which is in accordance to our findings. However, these cancer cell lines were not genetically modified, being the first time that biflorin activity is related to a genetic background.

The components of the RAS-RAF-MEK-ERK pathway are highly conserved and play important roles in melanocytic cancers. This pathway can be activated in cutaneous melanocytes via several mechanisms, such as the stimulation of autocrine growth factors and mutations in the oncogenes NRAS and BRAF, causing the activation of numerous signaling cascades that are involved in controlling cell growth. Furthermore, BRAF mutation exerts many oncogenic effects, leading to the uncontrolled growth in melanoma cells (Fedorenko et al., 2011). Interestingly, SK-Mel 19 cell line (BRAFV600E/wtNRAS) and SK-Mel 103 cell line (wtBRAF/NRASQ61R) present different profiles of mutation and thus biflorin exerts its cytotoxic activity on both cell lines which might provide it with a therapeutic advantage to treat different types of tumors where these mutations are involved (Homet and Ribas, 2014).

SK-Mel 28 cell line was the most resistant to toxicity. This may be due to its mutation on TP53R273H, since the mutation on BRAF and/or NRAS does not significantly alter the induction of cytotoxicity by biflorin. The point mutation in codon 145 (p53-L145R) of the TP53 gene causes the overexpression of a mutant form of the protein (Haapajärvi et al., 1999), which confers a growth advantage in the absence of endogenous wild-type p53 protein (Dittmer et al., 1993), leading this cells to be more resistant to chemotherapy (Alam et al., 2015). The fact that SK-Mel-28 cell line possesses a gain of function mutation in p53 this may lead to an increase in the accumulation of ROS due to

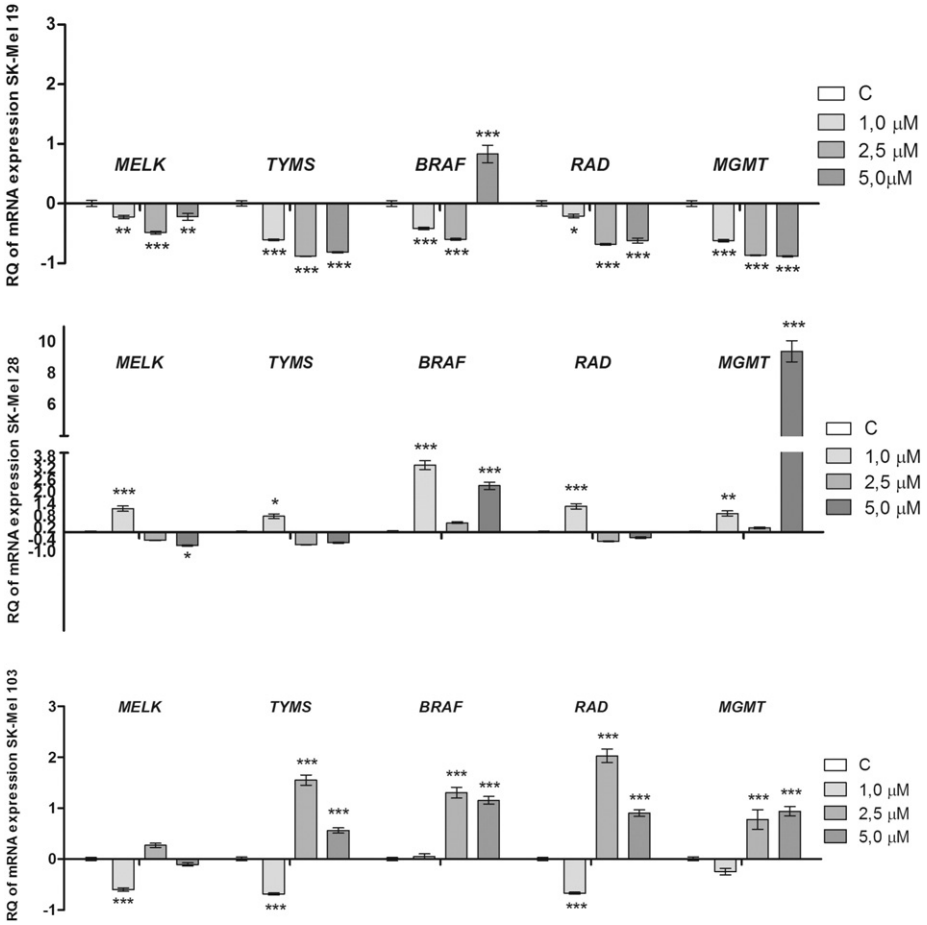


**Fig. 5.** Relative mRNA quantification of *MELK*, *TYMS*, *BRAF*, *RAD*, and *MGMT* gene expression levels in human melanoma SK-Mel cells after treatment with biflorin 1.0, 2.5 and 5.0 μM. Data are presented as mean values ± SE, from three independent experiments that were performed in triplicate using independent biological replicates. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to negative control; ANOVA + Bonferroni post-test.

the induction of mitochondrial oxidative stress (Brohem et al., 2012; Chen et al., 2003), which may lead to DNA damage as observed in our study. In the contrary, SK-Mel 19 and 103 cell lines present a wild-type TP53 (wtTP53), which makes the cells more sensitive to DNA damage and apoptosis as observed.

To maintain the balance between proliferation and survival, the MELK gene acts during cell cycle regulation, cell proliferation and apoptosis. Additionally, the MELK protein physically interacts with p53 and contributes to cell cycle arrest and p53-dependent apoptosis (Jiang and Zhang, 2013). In a complementary manner, the TYMS gene is also related to proliferation and cell cycle regulation (Kotoula et al., 2012). Biflorin decreased MELK and TYMS expression on SK-Mel 19 and 103 cell lines, whereas the opposite effect was observed on SK-Mel 28 (TP53R273H) cells, reinforcing the involvement of the p53-mediated cytotoxicity by biflorin.

The p53 works through several mechanisms of anticancer properties and plays a role in apoptosis, genomic stability, angiogenesis, cell cycle arrest, and DNA repair when DNA has sustained damage (Dittmer et al., 1993; Alam et al., 2015). MGMT gene is critical for protecting cells against alkylating agents, which commonly cause an increase in its levels (Kaina et al., 2007). However, the presence of mutations in the KRAS gene in malignant tumors has been correlated with the epigenetic inactivation of MGMT. A strong association between MGMT inactivation by hypermethylation and the appearance of G–A mutations in KRAS has been noted, which reinforces the notion that the hypermethylation/inactivation of MGMT might be related to controlling cancer via an increase in partial responses and disease stabilization (Amatu et al., 2013; Esteller et al., 2000). Moreover, loss of RAD-dependent repair pathways reduces repair efficiency and the deletion of RAD51, RAD54, RAD55 and RAD57 results in single-strand DNA annealing (Wu et al., 2008). According to the results obtained in MGMT and RAD repair gene expression, MGMT expression levels were lower in SK-Mel 19 cells, and RAD expression was lower in SK-Mel 103 cells, which supports its increased susceptibility to cell death. Biflorin appears to be an improvement compared to traditional chemotherapy, considering that the downregulation of MGMT expression is accompanied by the inhibition of acquired resistance to anticancer drugs in melanoma cells (Sarkar et al., 2015). In contrast, significant increases in RAD and MGMT expression were observed in SK-Mel 28 cells. The overexpression of MGMT in SK-Mel 28 (TP53R273H) cells works against the high methylation frequency of the MGMT promoter in TP53-mutated tumors (Lai et al., 2009). Thus, these targeted gene alterations might be a result of biflorin's effects on DNA repair, DNA damage and cytotoxicity by induction of the p53 pathway. In addition, biflorin's cytotoxicity against NRAS- and BRAF-mutated human melanoma cells in vivo along with the induction of MGMT may improve responses to melanoma therapy thus increasing survival rates and limiting drug toxicity.

In conclusion, we have demonstrated that biflorin, a naphthoquinone isolated from *C. biflora*, has the ability to overcome the resistance of melanoma cell lines, despite the presence of distinctive mutations on BRAF, NRAS and TP53 genes. Biflorin induces cytotoxicity via apoptosis by the induction of DNA damage thereby decreasing cell cycle progression, replication, DNA repair and methylation. The present study contributes to a better understanding of the mechanism of action that underlie the effects of biflorin as a way to promote its future use in cancer therapy. Furthermore, our results raise further questions regarding the apoptotic mechanisms and the proteins involved in this process.

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