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Inhibition of metastatic potential of B16-F10 melanoma cell line in vivo and in vitro by biflorin

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ABSTRACT

Aim: The aim of this study was to determine the antimetastatic potential of biflorin using in vivo and in vitro approaches.
Main methods: Biflorin was isolated from *Capraria biflora* collected in Fortaleza, Ceará, Brazil. Adhesion, migration and invasion assays were performed to avail of the antimetastatic potential of this quinone. Experimental metastasis was performed to avail of the antimetastatic potential of bilflorin using in vivo assay.
Key findings: Treatment with biflorin (25 and 50 mg/kg/day) was shown to be effective in reducing B16-F10 melanoma metastasis in C57BL/6 mice. The administration of biflorin at 25 mg/kg/day intraperitoneally inhibited the formation of metastases by about 57% compared to untreated control animals. When the animals were treated with 50 mg/kg/day intraperitoneally, there was a 71% decrease in the number of lung metastases. Morphological assays showed the presence of hemosiderin and erythrocytes in the lung parenchyma, indicating the occurrence of hemorrhage, probably a side effect of biflorin. Biflorin at non-toxic concentrations (0.5, 1.0 and 1.5 g/mL) was tested directly on B16-F10 cells in vitro, and it inhibited cell adhesion to type I collagen and cell motility using the wound-healing assay.
Significance: These data suggest that biflorin has a promising antimetastatic potential, as shown by its anti-adhesion, anti-migration and anti-invasion properties against a metastatic melanoma cell line. However, further studies are essential to elucidate its mechanism of action.
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Introduction

The spread of cancer cells from the primary tumor to distant locations is known as metastasis. The occurrence of metastasis is the major cause of mortality in cancer patients (Lee et al., 2003; Weiss, 1990), and the treatment of metastasis is still far from satisfactory (Han et al., 2009). Metastasis of cancer cells involves multiple processes including inhibition of cell-to-cell adhesion, enhancement of cell-to-extracellular matrix (ECM) adhesion, and invasion, which involves the degradation of the ECM (Lee et al., 2003, 2006; Cavallaro and Christofori, 2001). Tumor invasion of tissues by penetrating the basement membrane is also an important step, which involves the adhesion of tumor cells to ECM components followed by its degradation (Cavallaro and Christofori, 2001).

Natural products are a rich source of pharmacologically active compounds, in which plant materials hold an important position. One such plant *Capraria biflora* L., a perennial shrub of the family Schrophulariaceae, is used to treat various symptoms such as pain, fever, flu, vomiting, childbirth recovery, diarrhea, hemorrhoids, rheumatism, and swelling. The roots of this plant have antibacterial properties (Vasconcellos et al., 2007) and its aqueous extract has demonstrated both peripheral and central analgesic effects (Acosta et al., 2003).

Biflorin is a natural product that can be isolated from the roots of *C. biflora* L., a substance with an o-quinone structure. This quinone has antibiotic activity against Gram-positive bacteria, yeasts and fungi (Aquino et al., 2007). Moreover, biflorin has antifungal and antitumor effects, such as in the melanoma model (Vasconcellos et al., 2011).

Several studies have shown that some compounds isolated from plants can prevent tumor metastasis through inhibition of tumor adhesion and migration (Lee et al., 2006; Yang et al., 2007; Huey-Chun et al., 2003). Thus, the aim of this study was to determine the antimetastatic potential of biflorin using in vivo and in vitro approaches.

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Material and methods

Plant material

C. biflora L. (Scrophulariaceae) was collected at a plantation located in Fortaleza, Ceará, Brazil in 2008 and identified by Dr. Edson Nunes. A voucher specimen (No. 30848) was deposited in the Herbarium Prisco Bezerra of the Biology Department of the Federal University of Ceará.

Isolation of biflorin

The isolation of biflorin was performed as described by Fonseca et al. (2003). Air-dried powdered roots (4.5 kg) was extracted with light petroleum (4 L) for 2 days and solvent was evaporated under reduce pressure to yield 2.3 g. The extract was then dissolved in petroleum light 10 mL and kept in a refrigerator for 2 h until precipitating a solid material with a purple color. It was later filtered under vacuum and yielded a purple solid material (2 g). The purple solid material was chromatographed on silica gel by isocratic elution using a binary mixture of light petroleum/ethyl acetate, 9:1 (v/v). Fractions were pooled according to thin-layer chromatographic (TLC) analysis. Combined fractions containing purified biflorin yielded 335.5 mg (purity: 99%). Biflorin was analyzed in high performance liquid chromatography (HPLC) on a Shimadzu SPD-M20A instrument with UV–Vis detector using a 254 nm, C18 column. The eluent was methanol/water (90:10) using isocratic elution and showed a retention time of 6.65 min. Structure determination of biflorin was determined by spectroscopic analysis, including one- and two-dimensional nuclear magnetic resonance spectral data, physical properties and comparison with data from the literature (Fonseca et al., 2003). Biflorin is a red intense solid m.p. 154–157 °C, IR (KBr)$^{cm-1}$ 2921, 1684, 1592, 1438, 1236, 1023; ^{13}C NMR (500 MHz, $CDCl_3$) 7.8(C-17); 18.0(C-14); 22.6 (C-16); 25.2 (C-15); 26.7(C-11); 26.7(C-10); 113.5(C-9); 116.1(C-3); 122.0(C-12); 124.2(C-9b); 126.6(C-6a); 127.8(C-4); 129.0(C-3a); 133.6(C-13); 135.9(C-5); 140.2(C-2); 146.7(C-6); 161.9(C-9a); 178.0(C-8); 182.0(C-7) and ^{1}H NMR agree with literature. Melting points were determined by a digital Mettler Toledo FP90 apparatus. IR spectra were obtained on a Perkin-Elmer Ft-IR Spectrum 1000. Nuclear magnetic resonance (NMR) spectra were obtained using $CDCl_3$ on a Bruker Avance DRX-500 (500 MHz) spectrometer.

Cell conditions

Mouse melanoma B16-F10 was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1.5% MEM vitamin solution, 1% sodium pyruvate, 1% non-essential amino acids and 1% 100× penicillin and streptomycin (Pen-Strep) at 37 °C in a humidified atmosphere of 5% CO_2.

Animals

A total of 48 C57BL/6 mice (female, 25–30 g), obtained from the central animal house of the Federal University of Ceará, Brazil, were used. The animals were housed in cages with free access to food and water. All animals were kept under a 12 h:12 h light–dark cycle (lights on at 6:00 a.m.). The animals were treated according to the ethical principles of animal experimentation of SBCAL (Sociedade Brasileira de Ciências em Animais de Laboratório), Brazil. The Animal Studies Committee of the Federal University of Ceará approved the experimental protocols (No. 52/08).

Drugs and reagents

RPMI 1640 media, fetal bovine serum (FBS) were purchased from Cultilab, Pen-Strep, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium (MTT), DMSO, Doxorubicin hydrochloride, bovine serum albumin (BSA), mitomycin C and collagen type I were purchased from Sigma.

In vivo experimental lung metastasis and survival assay

B16-F10 cells were harvested, washed with serum-free RPMI 1640 and resuspended to give the appropriate concentrations in phosphate-buffered saline (PBS). An amount of 0.5 mL of the resultant B16-F10 cell suspension (1×10^6 cells) was injected into the mice via the tail vein. On the following day (Day 1), mice were randomly divided into three groups ($n = 16$ for each group). In the control group, the animals received 10% DMSO i.p., and the treatment groups received biflorin (25 mg/kg and 50 mg/kg i.p.) suspended in sterile 10% DMSO in distillate water. The treatment was continued daily for 21 days, and afterwards, 8 animals from each group were sacrificed. The lungs were excised and fixed in 10% formaldehyde. The number of B16-F10 colonies present on the surface of each set of lungs was determined by visual inspection using a magnifying glass. The other 8 animals in each group were observed for no more than 2 months for a determination of survival rate.

Histopathology and morphological observations

After being fixed in formaldehyde, the lungs were examined for size or color changes and hemorrhage. Portions of the lungs were then cut into small pieces, and histological sections were stained with hematoxylin and eosin. Histological analyses were performed by light microscopy.

Adhesion assay

Adhesion studies were performed as described previously by Hatai et al. (1993) with modifications. Briefly, type I collagen (0.1 mg/mL) was immobilized in 96-well plates containing 0.1% acetic acid, overnight at 4 °C. After immobilization, any uncoated surface was blocked with 1% BSA at 37 °C for 2 h. The cells were trypsinized and suspended in culture medium, pelleted, and resuspended at 2×10^5 cells/mL in PBS, and 100 μL of the cell suspension was added to each well in the presence or absence of biflorin (0.5, 1.0 and 1.5 μg/mL) at 37 °C for 1 h. After incubation, the wells were washed twice with PBS. Next, 200 μL of MTT solution (5 mg/mL) was added to each well, and the plates were incubated for 4 h at 37 °C and 5% CO_2. After incubation, supernatants were removed and DMSO was added. The absorbance was determined at 595 nm (Mosmann, 1983).

Migration assay

The migration assay was performed by the razor wound method described by Bürk (1973), with modifications. Briefly, 5×10^5 B16-F10 cells were seeded in 2 mL of RPMI medium with 10% calf serum (FBS) in 6-well plates. After 48 h, a wound was made in the confluent monolayer by pressing a razor blade down onto the plate to cut the cell sheet and to mark the plate; the blade was then gently moved to one side to remove part of the sheet. The wells were washed twice with PBS and the medium was replaced. Afterwards, the medium was removed and replaced with 2 mL of RPMI medium without serum. At this moment, biflorin (0.5, 1.0 and 1.5 μg/mL) was added to the wells and the plates incubated for 3, 6, 12 and 24 h at 37 °C and 5% CO_2. After incubation, the cells were observed to see if they had crossed the starting line marked on the plate. To discard a proliferation component of cell migration, mitomycin C was added to a concentration of 10 μg/mL 15–20 min prior to scraping the monolayer to inhibit DNA synthesis and cell replication. All samples were assayed at least in duplicate.

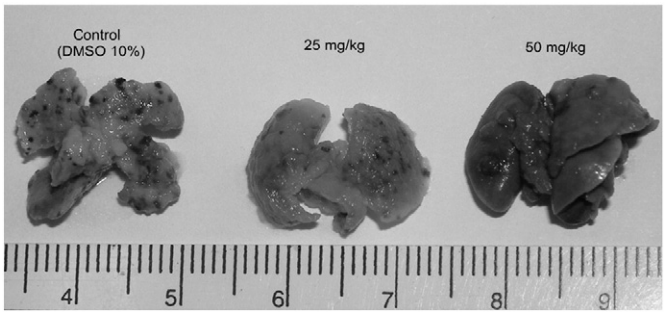

Fig. 1. Photography of the lung of animals inoculated with B16-F10 melanoma (1×10^6 cells/500 μL) via tail vein. In the photograph on the left, the animal received only the vehicle used to dilute the drug (DMSO 10%); in the photograph on the right, the animal received biflorin at 25 mg/kg i.p.

Invasion assay

Tumor cell invasion was assayed using transwell chambers coated with Matrigel accordingly as previously described in (Lee et al., 2003, 2006) with some modifications. Briefly, transwell chambers with 24-Multiwell of 8-μm pore size coated with Matrigel (BD BioCoat™, Bioscience) were used. B16-F10 cells (2×10^5 cells/well) were treated previously with biflorin (0.5, 1.0 and 1.5 μg/mL) during 16 h. After the treatment, the cells were carefully transferred into the upper chambers top well with biflorin (0.5, 1.0 and 1.5 μg/mL). Lower chambers were filled with 10% FBS medium to attract cells. Matrigel chambers were incubated for 8 h at 37 °C with 5% CO_2. After 8 h incubation, the non-invaded cells were completely wiped out with a cotton swab, and the lower surface of the filter was fixed with 30% methanol, stained with 0.5% crystal violet in 20% methanol. Experiments were performed independently three times. For each replicate, the cells were counted under a microscope at a magnification of 400× in 5 randomly selected fields and the counts were averaged.

Cell proliferation assay

The cell proliferation assay was performed to test the effect of biflorin against B16-F10 cells using the concentrations used in the adhesion and migration assays. Doxorubicin hydrochloride was used as the positive control. Briefly, cells were grown in RPMI-1640 medium supplemented with 10% FBS, 2 mM glutamine and Pen-Strep, and incubated at 37 °C in 5% CO_2 atmosphere. For experiments, the cells were plated in 96-well plates (0.6×10^5 cells/well in 100 μL of medium), biflorin was added to each well (final concentrations of 0.5, 1.0 and 1.5 μg/mL and volume of 200 μL), and cells were incubated for 24 h. Control groups received the same amount of sterile DMSO. Tumor cell growth was quantified by the ability of living cells to reduce MTT to a purple formazan product. At the end of the incubation period, plates were centrifuged, the medium was replaced by 150 μL of fresh medium containing 0.5 mg/mL MTT, and the cells were re-incubated for 3 h. The formazan produced was dissolved in 150 μL of DMSO, and the absorbance was measured using a multiplate reader (Multimode Detector DTX 880, Beckman Coulter) [14, 16]. The growth-inhibitory effect of biflorin was determined as the percentage of control absorbance of reduced dye at 595 nm.

Table 1
Body weights expressed as grams of the weight of mice on day 0 (before the inoculation of B16-F10 melanoma via tail vein) and after 21 days of treatment with biflorin (25 and 50 mg/kg i.p.) or DMSO 10%. Values are mean ± SE, n = 10.

Treatment	Day 0	Day 21st
DMSO 10%	25 ± 2.1	23 ± 1.9
Biflorin 25 mg/kg	26 ± 1.4	25 ± 1.2
Biflorin 50 mg/kg	24 ± 2.2	25 ± 1.4

Statistical analysis

Data are presented as a mean ± SEM. The differences between the experimental groups were compared by analysis of variance (ANOVA) followed by Dunnett's multiple comparison test ($P < 0.05$) using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA). Kaplan–Meier survival curves were plotted to compare survival rates.

Results

Effect of biflorin on experimental lung metastasis and survival

Long-term treatment with biflorin with 25 or 50 mg/kg/day reduced by 57 and 71%, respectively, the number of lung metastases in comparison to control group (Figs. 1 and 2). At these dose levels, there was no effect on body weight, nor were there any other clinical signs of toxicity (Table 1).

In addition, the life span of these animals was significantly increased by biflorin treatment (Fig. 3). The control animals survived for 22.3 ± 1.9 days after inoculation of mice with B16-F10 melanoma. Animals treated with biflorin survived for 24.4 ± 0.5 days (25 mg/kg/day) and 25.3 ± 0.42 days (50 mg/kg/day) ($P < 0.05$).

Histopathological analysis

Histopathological analysis showed the presence of melanoma cells in the lung parenchyma, which proved the presence of metastasis (Fig. 4). In addition, fewer numbers of melanoma cells were seen after

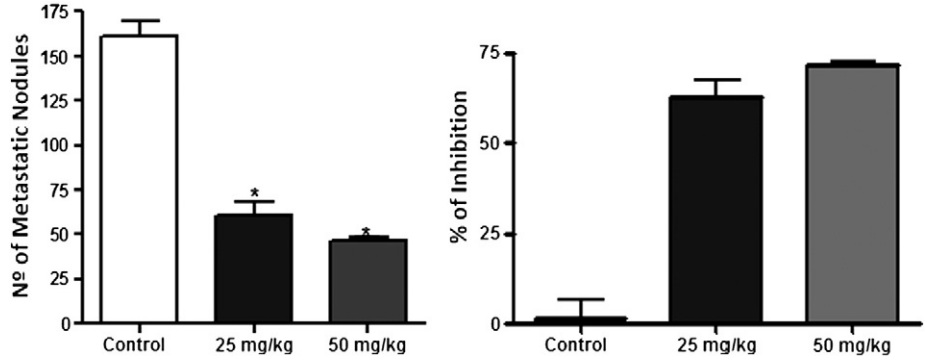

Fig. 2. The lungs were dissected and examined for any metastases on the 21st day after inducing a B16-F10 melanoma. Administration of biflorin (25 mg/kg and 50 mg/kg, i.p.) was started 24 h after injection of the tumor cells through the lateral tail vein (n = 08) * $p < 0.001$ compared with the negative control by ANOVA followed by Student–Newman–Keuls test.

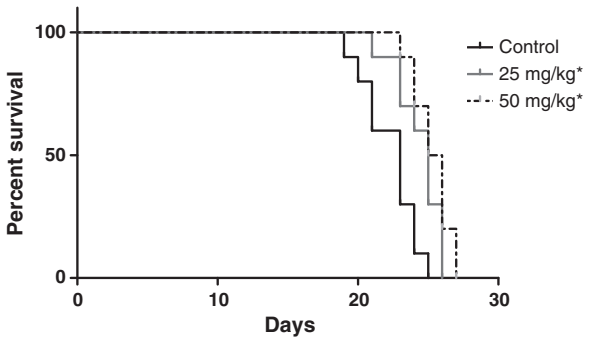

Fig. 3. Effect of biflorin on the lifespan of experimental metastasis. Mice (C57BL/6) were injected with B16-F10 cells (10^6 cells/500 ml, i.v.). Biflorin was administered at 25 mg/kg and 50 mg/kg i.p., starting 1 day after tumor implantation, for 21 days (n = 08). Control mice received vehicle (10% dimethyl sulfoxide). Data are representative of 08 animals per group. The analyses were performed using Kaplan–Meier curves, and the controls and treated groups were compared by the $\chi 2$ test. *P < 0.01 compared with the control.

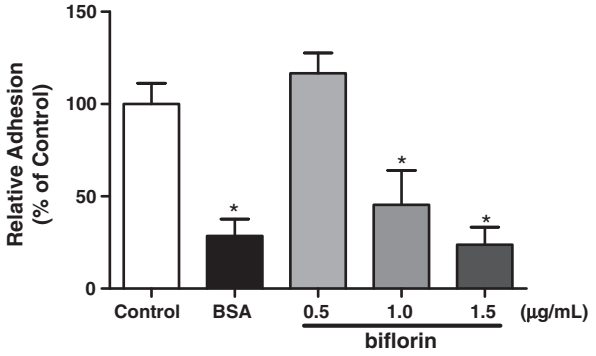

Fig. 5. Effect of biflorin on the adhesion of B16-F10 melanoma cells to the collagen. B16-F10 melanoma cells were added to the microculture wells precoated with collagen in the presence or absence of biflorin. After 1 h incubation, the non-adherent cells were washed away and the adherent cells were stained with MTT assay. Tumor cell adhesion is expressed as % of the untreated control. Each bar shows the mean ± SD of three independent experiments that were performed in triplicate. *P < 0.01 compared with the control.

drug treatment. The presence of hemosiderin and erythrocytes was also evident, indicating the occurrence of hemorrhage.

Adhesion assay

Biflorin (0.5, 1.0 and 1.5 μg/mL) inhibited the adhesion of these cells to type I collagen in a concentration-dependent manner by 45.49 ± 18.51, 23.80 ± 9.50 and 28.57 ± 9.17%, respectively (Fig. 5).

Migration and invasion assays

In migration assay, B16-F10 cells rapidly moved from the edge of the wound into the open area (Fig. 6). The biflorin-induced inhibition of cell movement occurred at all doses (0.5, 1.0 and 1.5 μg/mL). In addition, biflorin showed a dose-dependent inhibitory effect on cell invasion through the Matrigel chamber (Fig. 7).

Cytotoxic effect of biflorin on B16-F10 melanoma cells

The cytotoxicity of biflorin was determined using an MTT assay. This assay was performed to determine if the anti-adhesion and anti-migration effect of biflorin was caused by a false positive due to cytotoxicity. Fig. 8 shows that biflorin had no inhibitory effect on the growth of B16-F10 cells after 24 h incubation at the concentrations used in this study (0.5, 1.0 and 1.5 μg/mL).

Discussion

Initially, a previous study in our laboratory by Vasconcellos et al. (2011) demonstrated in vitro and in vivo anticancer activities of biflorin against a murine melanoma cell line. At 25 mg/kg/day i.p., this quinone improved the lifespan and also reduced tumor growth in animals bearing B16 melanoma tumors (Vasconcellos et al., 2011).

It is well known that B16-F10 melanoma cells are highly metastatic and form tumor nodules in the parenchyma of the lungs when

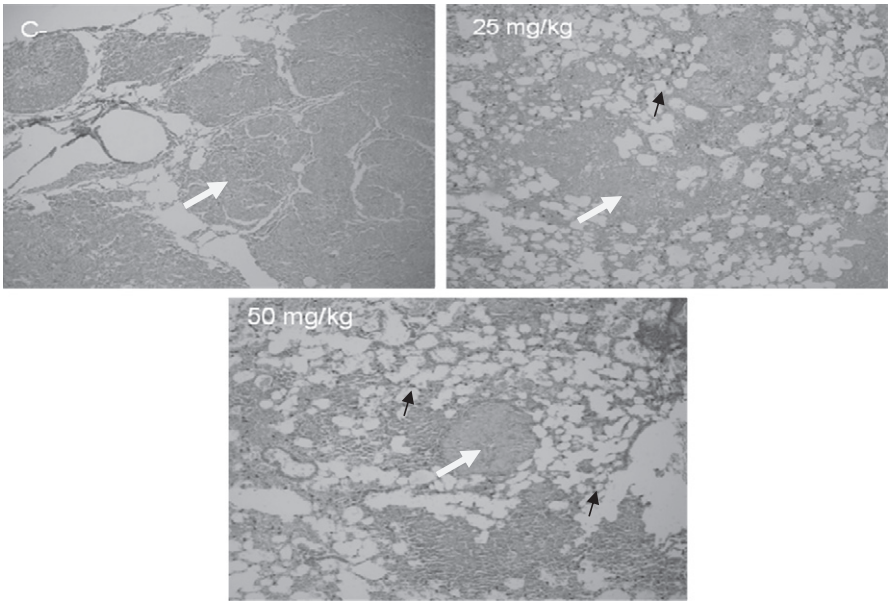

Fig. 4. Mice (C57BL/6) were inoculated with B16-F10 cells (10^6 cells/500 ml, i.v.). Biflorin was administered intraperitoneally at a dose of 25 mg/kg and 50 mg/kg, starting 1 day after tumor implant, for 21 days (n = 08). Control mice (n = 08) received vehicle (10% DMSO). After the treatment, the lungs were dissected and observed for any metastases. Black arrows: hemosiderin, white arrows: melanoma cells. Magnification = 400×.

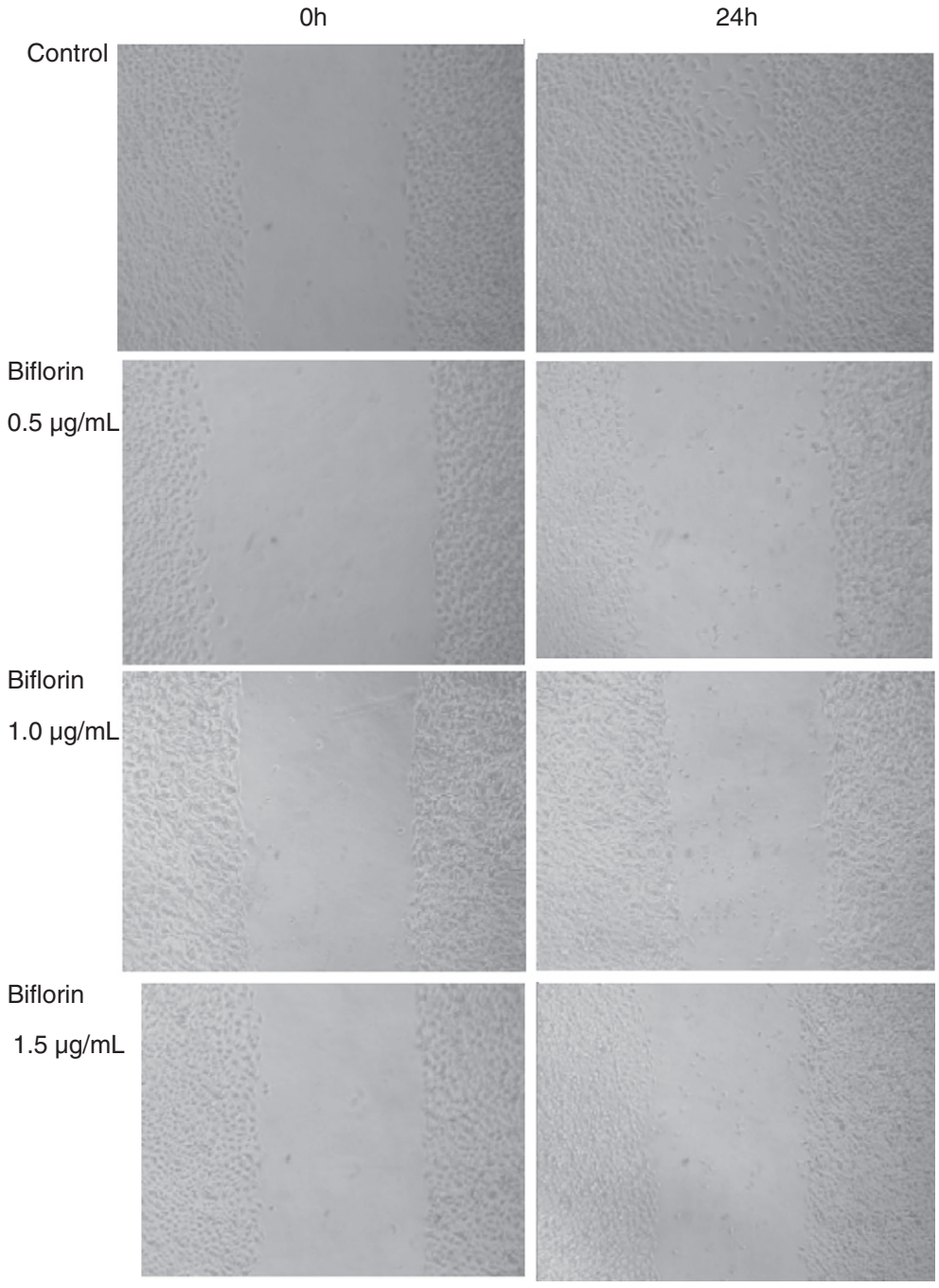

Fig. 6. Photographs of wounds in monolayers of cells. Cell layers were wounded 2 days after seeding. After 24 h without serum, the cultures were fixed with methanol. Magnification, 100×.

administered through the tail vein (Fidler, 1970). Since biflorin can enhance the lifespan of melanoma tumor-bearing animals, it was decided to determine the effect of biflorin on the metastatic process using in vitro and in vivo approaches.

First, in the present study, the anti-metastatic activity of biflorin was assessed using the experimental metastasis assay. The lungs with metastatic melanoma nodules produced after B16-F10 cell infection can be seen in Fig. 1. Melanoma nodules occurred in untreated control but the presence of these nodules was reduced on the lungs of the animals treated with biflorin (25 and 50 mg/kg/day). As seen in Fig. 2, the number of lung metastases was significantly reduced after administration of biflorin (57 and 71% for 25 and 50 mg/kg/day, respectively) with an increase of lifespan (22.3 ± 1.9, 24.4 ± 0.5 and 25.3 ± 0.42 days for control and 25 and 50 mg/kg/day, respectively) (Fig. 3).

Some drugs, in the literature, have demonstrated similar anti-metastatic activity as biflorin using this experimental melanoma metastasis assay. Lapachol, for example, is also a naphthoquinone and also has antitumor activity. When this drug was tested by serial oral administration of low non-toxic doses (5–20 mg/kg), it produced a weak but significant suppression of metastasis after injection of murine B16-BL6 melanoma cells into the tail vein (Lee et al., 2006). The mechanism of action of this drug probably involves an alteration in protein profile and inhibition of cellular invasiveness, thus representing an important antimetastatic activity (Almeida, 2009).

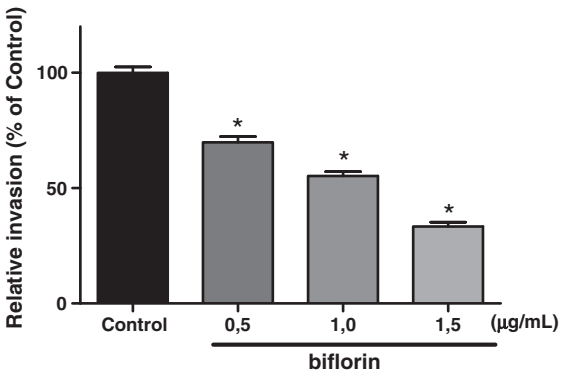

Fig. 7. Effects of biflorin on invasion in B16-F10 cells. The cells were treated with various concentrations of biflorin for 16 h. After this treatment, B16-F10 cells were cultured with biflorin within a Matrigel invasion chamber. Quantitative analysis of the Matrigel chamber invasion assay is shown. Invasion was expressed as a percentage of control (DMSO). Each bar represents the mean ± S.D. calculated from three independent experiments. * $p < 0.001$ compared with the negative control by ANOVA followed by the Student–Newman–Keuls test.

However, the histological analyses of the lungs of the tumor-bearing animals after administration of biflorin (25 and 50 mg/kg/day), showed the presence of hemosiderin and erythrocytes in the lung parenchyma, which is evidence of hemorrhage and probably an adverse effect. Nevertheless, more experiments are necessary to confirm this untoward effect.

It is well known that the metastasis occurs through several steps, including dissociation of cancer cells from the primary site, change in adhesion capability between cells and extracellular matrix (ECM), migration and invasion through the extracellular matrix (ECM) and circulation in the blood and lymph (Han et al., 2009; Lee et al., 2006; Chu et al., 2007; Zhao et al., 2008; Kurschat and Mauch, 2000).

The invasion of tumor cells into adjacent tissues, a crucial event in metastasis, involves cell–cell and cell–ECM interactions (Zhao et al., 2008). These interactions involve a number of adhesive molecules on the cell surface, which have been described in detail (Kurschat and Mauch, 2000). Alterations in these interactions are one of the initial events in cancer invasion, permitting cellular detachment from the primary tumor. Drugs that can inhibit the adhesion of the cells to ECM may have antimetastatic potential.

It is well been known that B16-F10 cells can adhere to a wide variety of cell-adhesive protein, including collagen, the most common component of the ECM, and typical integrin ligands (Hatai et al., 1993; Zhou et al., 2008). In the present study, biflorin was found capable of inhibiting the adhesion of B16-F10 cells to type I collagen in a concentration-dependent manner (Fig. 5).

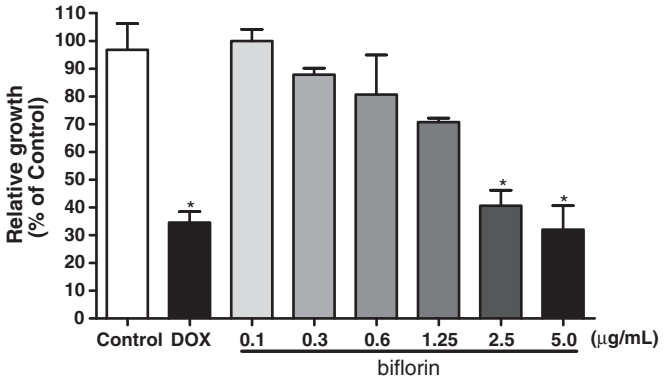

Fig. 8. Effect of biflorin on the growth of B16-F10 melanoma cells. The B16-F10 cells were incubated with biflorin (0.5, 1.0 and 1.5 μg/mL) or doxorubicin hydrochloride (DOX; 172 μM) for 24 h. MTT solution was added to each well and incubated for an additional 4 h. The absorbance at 450 nm was measured using a microplate reader. Tumor cell growth is expressed as % of the untreated control. Each bar shows the mean ± SD of two independent experiments that were performed in triplicate. *$P < 0.01$ compared with the control.

Salvicine, a diterpenoid quinone, identified as a non-intercalative topoisomerase II poison, has a broad range of antitumor and antimetastatic activity. Zhou et al. (2008) showed that salvicine down-regulates β1 integrin function and inhibits cell-ECM interaction, thereby providing further clues to the mechanism underlying the antimetastatic efficacy of salvicine.

Integrins are transmembrane glycoproteins composed of non-covalently linked α and β subunits. Integrins are essential for cell migration and invasion because they mediate the adhesion of cells to the ECM and regulate intracellular signaling pathways that control cytoskeletal organization, force generation, and survival (Zhou et al., 2008). Since these molecules are so important to cell adhesion, we can hypothesize that biflorin inhibits cell adhesion by blocking integrin function, like salvicine. However, this mechanism was not clear, and more experiments are necessary to confirm this hypothesis.

After adhesion, the migration process is another characteristic of cell invasion. The invasion of cells to ECM may be due to growth pressure, but it may be due to cell migration. Since this process is so important for the study of metastasis, it was decided to evaluate the effect of biflorin on the cell migration of B16-F10 cells using the method developed by Bürk (1973), with modifications. In this assay, biflorin was able to inhibit the migration of B16-F10 cells in a concentration-dependent manner (Fig. 6). Furthermore, tumor cell invasion to the ECM is an important step in the process of tumor metastasis, which involves the adhesion, migration and degradation of the ECM [1, 4]. In order to investigate the effect of biflorin on invasiveness of B16-F10 cell line, we performed a cell invasion assay chamber coated with Matrigel (Lee et al., 2003, 2006). As expected, biflorin was able to inhibit the invasion of B16-F10 cells in a concentration-dependent manner (Fig. 7), which demonstrates the anti-invasive potential of this quinone.

Taking together, these results showed that the antimetastatic effect of biflorin may be related to anti-migration, anti-adhesion and anti-invasion activities.

It was also demonstrated that biflorin did not show any cytotoxicity at the concentrations used in the in vitro studies after 24 h of incubation (Fig. 8), which suggests that the anti-adhesion, anti-migration and anti-invasion effects of biflorin were not due to cytotoxicity.

Conclusion

We demonstrated that biflorin exhibits antimetastatic action in the mouse melanoma lung metastasis model. Furthermore, the antimetastatic effect is suggested due to the inhibition of the adhesion, migration and invasion of melanoma cells. These results indicate that biflorin is a promising candidate for an antimetastatic agent. However, more experiments are necessary to elucidate its mechanism of action.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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