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Bauhinia forficata lectin (BfL) induces cell death and inhibits integrin-mediated adhesion on MCF7 human breast cancer cells

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ABSTRACT

Background: Plant lectins have attracted great interest in cancer studies due to their antitumor activities. These proteins or glycoproteins specifically and reversibly bind to different types of carbohydrates or glycoproteins. Breast cancer, which presents altered glycosylation of cell surface glycoproteins, is one of the most frequent malignant diseases in women. In this work, we describe the effect of the lectin *Bauhinia forficata* lectin (BfL), which was purified from *B. forficata* Link subsp. *forficata* seeds, on the MCF7 human breast cancer cellular line, investigating the mechanisms involved in its antiproliferative activity.
Methods: MCF7 cells were treated with BfL. Viability and adhesion alterations were evaluated using flow cytometry and western blotting.
Results: BfL inhibited the viability of the MCF7 cell line but was ineffective on MDA-MB-231 and MCF 10A cells. It inhibits MCF7 adhesion on laminin, collagen I and fibronectin, decreases α_1, α_6 and β_1 integrin subunit expression, and increases α_5 subunit expression. BfL triggers necrosis and secondary necrosis, with caspase-9 inhibition. It also causes deoxyribonucleic acid (DNA) fragmentation, which leads to cell cycle arrest in the G2/M phase and a decrease in the expression of the regulatory proteins pRb and p21.
Conclusion: BfL shows selective cytotoxic effect and adhesion inhibition on MCF7 breast cancer cells.
General significance: Cell death induction and inhibition of cell adhesion may contribute to understanding the action of lectins in breast cancer.

1. Introduction

An ideal strategy for cancer chemotherapy is the induction of cell death in tumor cells [1,2], and the use of agents with this ability has attracted considerable interest in recent years [2,3]. Plant lectins have a great potential in cancer treatment [4,5] and have been used to differentiate malignant tumor from benign and the degree of glycosylation, which is associated with tumor metastasis [6,7]. Lectins are proteins or glycoproteins of non-immune origin [8–10] that possess at least one non-catalytic domain that reversibly binds to a specific mono- or oligosaccharide [11,12]. Some plant lectins have been used as alternative therapies in breast cancer patients, such as mistletoe lectin [5,13].

Breast tumors are the most common malignancy in women, and the two most effective means to reduce mortalities are early detection and better treatment strategies [14]. Altered glycosylation of cell surface glycoproteins occurs in malignant transformation [15], and these changes can be identified using lectins [15–19]. The altered glycoproteins can act as biomarkers for monitoring the metastatic spread of breast cancer [15].

The metastatic potential of cancerous cells is attributed to their adhesive properties [20], and the adhesion process is enhanced by altered glycosylated structures, which are different from those found on the surface of normal cells [20–23]. Cell adhesion is partly mediated by integrins, receptors that are composed by the noncovalent association of glycoprotein α and β subunits [24] with large extracellular domains and short cytoplasmic domains [25]. The β_1 integrin subunit is expressed in tumor cells, and its interaction with matrix ligands (collagen, fibronectin or laminin) depends on the integrin α subunit partner [26,27]. Kato et al. [27] reported that the increased expression of β_1 integrins, such as $\alpha_5\beta_1$ [27–29] or $\alpha_6\beta_1$ [27,30], in some human solid tumors is associated with increased metastatic potential.

Previously, we isolated and characterized a novel lectin, BfL, from the seeds of *Bauhinia forficata* Link subsp. *forficata. B. forficata* is a tree that

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BfL, *Bauhinia forficata* lectin; PI, propidium iodide; ANOVA, analysis of variance; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; HRP, horseradish peroxidase; NaF, sodium fluoride

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grows in the tropical regions of countries of South America [31]. In Brazil, this plant has been used as a hypoglycemic agent [32]. BfL is a monomeric glycoprotein with a molecular mass of 27.8 kDa, has two N-glycosylation sites and is inhibited by azocasein and thyroglobulin. BfL belongs to the legume lectin domain family and has similarities to other reported *Bauhinia* lectins [32].

In this work, we report the antitumor property of the lectin BfL on MCF7 human breast cancer cells.

2. Material and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), laminin, fibronectin, collagen IV, collagen I, phosphatase inhibitor cocktail (sodium orthovanadate, sodium molybdate, sodium tartrate and imidazole), protease inhibitor cocktail [104 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 80 μM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin and 1.5 mM pepstatin A] and Con A lectin (positive control) were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), penicillin and streptomycin were obtained from Gibco® (Gaithersburg, USA). Trypsin 2.5% (v/v) was purchased from Cultilab (São Paulo, Brazil). Antibodies from rabbit against integrin α_6, integrin α_5, integrin β_1, p21 Waf1/Cip1, pRb (Ser^{780}), β-actin and IgG conjugated with horseradish peroxidase from rabbit or mouse were obtained from Cell Signaling Technology (Danvers, USA). Antibodies from mouse against integrin α_1 and integrin α_2 were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). The Ac-Leu-Glu-His-Asp-AFC substrate was from Calbiochem (Darmstadt, Germany). The fluorescein isothiocyanate (FITC) annexin V apoptosis detection kit was obtained from BD Biosciences (California, USA), the super signal® west pico chemiluminescent substrate and micro BCA™ were purchased from Thermo Scientific (Rockford, Illinois, USA) and the APO-BrdU™ TUNEL assay kit was purchased from Invitrogen (Eugene, Oregon, USA).

2.2. Lectin purification (BfL)

BfL was purified using the methodology reported by Silva et al. [32]. Briefly, *B. forficata* seeds (10% w/v) were powdered and homogenized for 16 h at 4 °C in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl. The homogenate was filtered, centrifuged at 5071 ×*g* for 15 min at 4 °C, and the supernatant proteins were precipitated using ammonium sulfate saturation (F0–40 and F40–80). The F40–80 fraction was solubilized in 0.1 M Tris–HCl buffer, pH 8.0, dialyzed in water for 24 h and applied to a DEAE-Sephadex column equilibrated in 0.1 M Tris–HCl buffer, pH 8.0. The unadsorbed proteins were eluted with the same equilibration buffer and applied to a Sepharose-4B column. The unadsorbed proteins in 0.1 M Tris–HCl buffer, pH 8.0, were subjected to a chitin column equilibrated with the same buffer. The adsorbed proteins were eluted with 1.0 M acetic acid, lyophilized, resuspended in water, filtered and subjected to size exclusion chromatography using a Superdex 75 10/300 GL column (GE Healthcare) that was equilibrated in 0.15 M NaCl at a flow rate of 0.5 ml/min, using an ÄKTA Purifier (GE Healthcare). The absorbance was monitored at 215 nm, and BfL was eluted in the first peak. The homogeneity was assessed by reversed-phase chromatography in a C_{18} column using a non-linear gradient of 90% acetonitrile in 0.1% trifluoroacetic acid (TFA) (5% at t = 5 min; 70% at t = 27 min; 80% at t = 60 min; and 100% at t = 65 min) for 65 min with a flow rate of 0.7 ml/min. For the cell experiments, BfL was concentrated and dialyzed by ultrafiltration (Amicon MILLIPORE Ultra-15, Mr 10,000 cut-off) to 7 mM Hepes, pH 7.4.

2.3. Tumor cell lines and culture conditions

The human breast cancer cell lines MCF7 (HTB-22™) and MDA-MB-231 (HTB-26™) were purchased from ATCC® and maintained in DMEM medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 μg/ml streptomycin and 100 IU/ml penicillin at 37 °C in an atmosphere of 5% (v/v) CO_2. The human breast non-malignant cell line MCF 10A (CRL-10317™ — ATCC®) was maintained in DMEM/F12 (1:1, v/v) medium, supplemented with 5% (v/v) horse serum, human epidermal growth factor (20 ng/ml), human insulin (10 μg/ml), hydrocortisone (100 ng/ml), cholera toxin (0.1 nM), 100 μg/ml streptomycin and 100 IU/ml penicillin at 37 °C in an atmosphere of 5% (v/v) CO_2. All the experiments were approved by the Ethics Committee of the Federal University of São Paulo, number CEP 1543/11.

2.4. Cell viability

The cell viability of the MCF7, MDA-MB-231 and MCF 10A lines was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [33,34]. Briefly, cells in DMEM containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), were seeded into 96-well plates (2×10^3 cells/100 μl/well) and incubated for 24 h at 37 °C under an atmosphere of 5% (v/v) CO_2. After incubation, the supernatant was removed, the medium replaced with DMEM supplemented with 0.2% FBS and the cells were incubated for 24 h at 37 °C under an atmosphere of 5% (v/v) CO_2. MCF 10A cells were seeded in the concentration of 5×10^3 cells/well without synchronization. After, the supernatant was removed, and the cells were incubated with BfL (2.5–15 μM) or Con A (2.5–15 μM) at a final volume of 200 μl with DMEM containing 2% FBS for 12, 24, 48 and 72 h, or with DMEM/F12 containing 5% horse serum for 48 h. After the selected period, 10 μl of MTT (5 mg/ml) was added into each well and the cells were incubated at 37 °C for 2 h. The supernatant was removed and 100 μl of dimethyl sulfoxide (DMSO) was added into each well to solubilize the formazan crystals formed. The absorbance was read at 540 nm in a microplate reader (Spectra Count™).

2.5. Cell adhesion

Ninety-six well plates were coated for 16 h at 4 °C with 100 μl of collagen type I or IV, diluted in 2% (v/v) acetic acid, and laminin or fibronectin, diluted in 0.01 M disodium phosphate containing 0.14 M NaCl, 0.0017 M KH_2PO_4 and 0.0027 M KCl, pH 7.4 (PBS), at a concentration of 4 μg per well. The wells were blocked with 1% heat-inactivated BSA (100 μl/well) in PBS for 1 h at 37 °C. After incubation, BSA was removed, and the wells were washed three times with PBS (200 μl/well). BfL (0.3–14 μM) was incubated with MCF7 cells in DMEM containing 2% FBS at a concentration of 5×10^4 cells per well for 30 min at room temperature and added in the wells. The incubation (BfL, cells and coats) was performed at 37 °C under an atmosphere of 5% (v/v) CO_2 for 3 h. Subsequently, non-adherent cells were removed by washing with PBS (100 μl/well), and the adhered cells were fixed with 100% (v/v) methanol (100 μl/well) for 5 min. The methanol was removed, and the cells were washed three times with PBS (100 μl/well) and stained with 1% (w/v) toluidine blue (100 μl/well) in 1% (w/v) sodium tetraborate for 5 min. The cells were washed four times with PBS (200 μl/well), and the pigment retained by the cells was released by a solution containing 1% (w/v) SDS for 20 min at 37 °C. The absorbance was read at 540 nm in a microplate reader (Spectra Count™). The inhibition of adhesion of the MCF7 cells to the coats was calculated using the following equation: $(\%) = (a - b) \times 100\% / a$, where a is the A_{540} of the cellular homogenate without lectin (control) and b is the A_{540} of the cellular homogenate treated with BfL.

2.6. Cell death assay

Cell death was studied using annexin V-FITC/propidium iodide (PI) double staining and analysis by flow cytometry [35]. MCF7 cells (1×10^5) were seeded in 6-well plates in DMEM containing 10% (v/v) FBS for 24 h. After incubation, the supernatant was removed, the medium replaced with DMEM supplemented with 0.2% FBS and the cells were incubated for 24 h at 37 °C under an atmosphere of 5% (v/v) CO_2 for cell cycle synchronization. The adhered cells were incubated with BfL (5 μM) or Con A (10 μM) in DMEM containing 2% FBS in a final volume of 1.2 ml for 24 and 48 h. After these periods, the cells were removed with 200 μl of 2.5% (v/v) trypsin, followed by the addition of 1 ml of DMEM containing 10% (v/v) FBS and centrifuged at 311 ×*g* for 4 min. The supernatant was removed, and the cells were incubated with 500 μl of binding buffer (0.01 M Hepes/NaOH, pH 7.4, containing 0.14 M NaCl and 2.5 mM $CaCl_2$). The suspensions were transferred to tubes and centrifuged at 311 ×*g* for 6 min. The cells were resuspended with 50 μl of binding buffer with 3 μl of annexin V/FITC and 5 μl of PI (50 μg/ml). The cells were incubated at room temperature for 30 min with the addition of 300 μl of binding buffer and analyzed in a FACSCalibur flow cytometer. Control cells were treated with medium containing 7 mM Hepes, pH 7.4 (BfL vehicle).

2.7. Cell cycle distribution analysis

MCF7 cells (1×10^5) were seeded in 6-well plates in DMEM containing 10% (v/v) FBS for 24 h. After incubation, the supernatant was removed, the medium was replaced with DMEM supplemented with 0.2% FBS and the cells were incubated for 24 h at 37 °C under an atmosphere of 5% (v/v) CO_2 for cell cycle synchronization. The supernatant was removed from the adhered cells, which were incubated with BfL (5 μM) or Con A (10 μM), in a final volume of 1.2 ml with DMEM containing 2% FBS for 24 and 48 h. After these periods, the cells were removed with 200 μl of 2.5% (v/v) trypsin, followed by the addition of 1 ml of DMEM containing 10% (v/v) FBS and centrifuged at 311 ×*g* for 4 min. The supernatant was removed, the cells were resuspended with 200 μl of 2% (v/v) paraformaldehyde in PBS, transferred into cytometer tubes and incubated for 30 min at room temperature to fix the cells. The suspension was centrifuged at 311 ×*g* for 5 min, the supernatant was discarded and 100 μl of PBS containing 0.01% (w/v) saponin and 10 μl of RNase A (4 mg/ml) were added to the cells. The incubation occurred for 30 min at 37 °C. After, 5 μl of PI (1 mg/ml) and 300 μl of PBS were added to each tube and analyzed by flow cytometry (FACSCalibur, Becton Dickinson). The results were analyzed using the ModFit LT 3.2 program.

2.8. DNA fragmentation: Apo-BrdU™ TUNEL assay

MCF7 cells (5×10^5) were seeded in 6-well plates in DMEM containing 10% (v/v) FBS for 24 h. After the supernatant was removed, the medium was replaced with DMEM supplemented with 0.2% FBS, and the cells were incubated for 24 h at 37 °C under an atmosphere of 5% (v/v) CO_2 for cell cycle synchronization. Subsequently, the supernatant was removed, and the cells were incubated with BfL (5 μM) in a final volume of 1.2 ml with DMEM containing 2% FBS for 48 h. After this period, the cells were removed with 200 μl of 2.5% (v/v) trypsin, followed by the addition of 1 ml of DMEM containing 10% (v/v) FBS and centrifuged at 311 ×*g* for 4 min. The supernatant was removed, and the cells were resuspended with 500 μl of PBS. In the cell suspension, 5 ml of 1% (v/v) paraformaldehyde in PBS was added and placed on ice for 15 min. The cells were centrifuged for 5 min at 300 ×*g*, the supernatant was discarded and the cells were washed with 5 ml of PBS. The cells were centrifuged for 5 min at 300 ×*g*, resuspended with 500 μl of PBS and 5 ml of 70% (v/v) ethanol and stored at −20 °C. One-milliliter aliquots of the positive and negative control cell suspensions (1×10^6 cells/ml fixed human lymphoma cell line) and treated cells with BfL were placed in flow cytometry tubes and centrifuged at 300 ×*g* for 5 min, and the ethanol was removed by aspiration. The cells from each tube were resuspended with 1 ml of wash buffer and centrifuged for 5 min at 300 ×*g*, the supernatants removed and the cell pellets were resuspended in 50 μl of DNA-labeling solution (10 μl of reaction buffer, 0.75 μl of TdT enzyme, 8.0 μl of BrdUTP and 31.25 μl of water). The incubation was performed at room temperature for 16 h. At the end of the incubation period, 1 ml of rinse buffer was added to each tube and centrifuged at 300 ×*g* for 5 min. The supernatants were removed, and the cells were resuspended with 100 μl of antibody staining solution (5 μl of the Alexa Fluor 488 dye-labeled anti-BrdU antibody with 95 μl of rinse buffer). The cells were incubated for 30 min at room temperature. After, 500 μl of PI/RNase A staining buffer was added to each sample, the cells were incubated for 30 min at room temperature and analyzed by flow cytometry (FACSCalibur, Becton Dickinson).

2.9. Analysis of cell protein expression

2.9.1. Cell protein extraction

MCF7 cells (5×10^5) were seeded in 6-well plates in DMEM containing 10% (v/v) FBS for 24 h. After the supernatant was removed, the medium was replaced with DMEM supplemented with 0.2% FBS, and the cells were incubated for 24 h at 37 °C under an atmosphere of 5% (v/v) CO_2 for cell cycle synchronization. Subsequently, the supernatant was removed, and the cells were incubated with BfL (5 μM) for 48 h or Con A (10 μM) for 24 h in a final volume of 1.2 ml with DMEM containing 2% FBS. The cells were washed with 500 μl of PBS containing 0.25 mM sodium orthovanadate. The cells were collected by scraping in 500 μl of PBS containing 0.25 mM sodium orthovanadate and centrifuged at 460 ×*g* for 20 min at 4 °C. The supernatant was discarded, and the cells were lysed by homogenization in the vortex and ten freeze/thaw cycles in 30 μl of lysis buffer [0.05 M Tris–HCl, pH 7.4 containing 1% (v/v) Tween 20, 0.25% (w/v) sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM NaF and protease (1:100) and phosphatase (1:100) inhibitors], and incubated for 2 h on ice. The cell proteins were collected by centrifugation (22,400 ×*g* for 10 min at 4 °C), and the protein concentration was determined using the MicroBCA assay with a bovine serum albumin (BSA) standard curve over the range of 5–40 μg/ml.

2.9.2. Determination by fluorescence of caspase-9 activation

Thirty micrograms of total proteins of the cell lysate without treatment and with treatment (BfL or Con A) was incubated in reaction buffer 25 mM Hepes, pH 7.4, containing 10% (w/v) sucrose, 0.1% (v/v) Chaps and 1 mM DTT, with 20 μM of the Ac-Leu-Glu-His-Asp-AFC substrate for caspase-9 at 37 °C, and readings were performed after 4 h, 7 h 30′ and 20 h 30′ in a fluorometer (Spectra Max M2^e, Molecular Devices), using $\lambda_{exc} = 400$ nm and $\lambda_{em} = 505$ nm.

2.9.3. Western blotting

Total proteins of the cell lysate (30 μg) were separated by electrophoresis under denaturing and non-reducing conditions (SDS-PAGE), performed according to the procedure described by Laemmli [36], using 5% stacking and 7.5% (for integrins α_1, α_2, α_5, α_6 and β_1) or 12% (p21, pRb and β-actin) separating gels. After SDS-PAGE, protein bands were transferred to polyvinylidene fluoride (PVDF) membranes for 2 h and 40 min in 0.05 M Tris, 0.384 M glycine and 30% (v/v) methanol. Membranes were blocked with 5% (w/v) BSA in 0.025 M Tris, 0.192 M glycine and 0.1% (v/v) Tween 20 for 2 h. The membranes were washed three times with 0.025 M Tris, 0.192 M glycine and 0.1% (v/v) Tween 20 for 5 min and incubated overnight at 4 °C with the appropriate primary antibody at a 1:1000 dilution (α_5, α_6, β_1, p21, pRb and β-actin) or at a 1:100 dilution (α_1 and α_2) in 5% (w/v) BSA in 0.025 M Tris, 0.192 M glycine and 0.1% (v/v) Tween 20. After washing in 0.025 M Tris, 0.192 M glycine and 0.1% (v/v) Tween 20 (three times for 5 min each), the membranes were incubated with an anti-rabbit HRP-conjugated secondary antibody at a 1:2000 dilution or with an anti-mouse HRP-conjugated

secondary antibody at a 1:1000 dilution, in 1% (w/v) BSA in 0.025 M Tris, 0.192 M glycine and 0.1% (v/v) Tween 20 for 2 h at room temperature. After, the membranes were washed three times for 5 min each with 0.025 M Tris and 0.192 M glycine and added a solution containing substrate for peroxidase (Super signal® west pico chemiluminescent substrate) for 5 min at room temperature. Signal detection was performed using the enhanced chemiluminescence (Bio-Imaging Systems) GelCapture program, and densitometric analysis was performed using the Scion imaging software program (Scion Corporation) with β-actin as a control for each sample.

2.10. Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Statistical analyses of the data were performed by one-way ANOVA followed by Tukey's test. Values were considered to be significant when $p < 0.05$.

3. Results and discussion

3.1. Effect of BfL on cell viability

In the early phase of proliferation (48 h), BfL leads to a significant inhibition of approximately 38% of MCF7 cell viability at a concentration

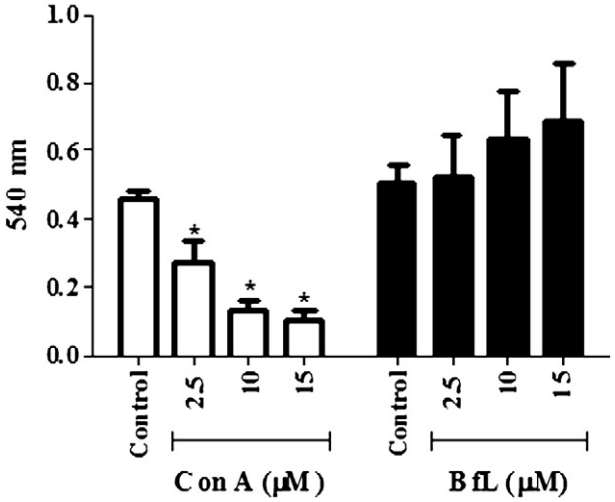

Fig. 2. Cytotoxicity of BfL and Con A in MCF 10A human breast cells. MCF 10A cells (5×10^3) were treated with different concentrations of BfL or Con A (2.5–15 μM) for 48 h in 96-well microtiter plates. Cell viability was assessed by the MTT reduction test. The statistical significance was evaluated using one-way ANOVA followed by Tukey's test. A p-value <0.05 was considered to indicate significance (*). Data represent means ± SD of two independent experiments in triplicate.

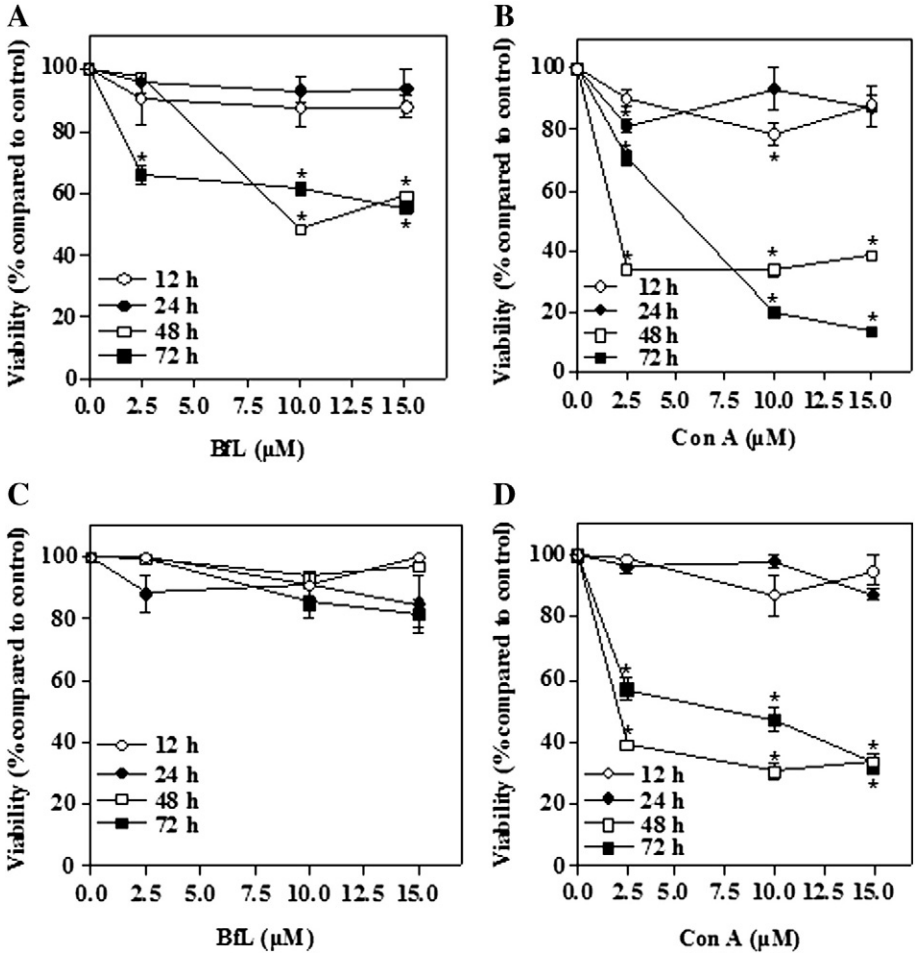

Fig. 1. Cytotoxicity of BfL in MCF7 human breast cancer cells (A) and its effect on the MDA-MB-231 cell line (C). The MCF7 or MDA-MB-231 cells (2×10^3) were treated with different concentrations of BfL (2.5–15 μM) for 12, 24, 48 and 72 h in 96-well microtiter plates. Cell viability was assessed by the MTT reduction test. In the absence of the proteins, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction was considered to be 100%. In B (MCF7) and D (MDA-MB-231), the positive control is shown with Con A (2.5–15 μM). The statistical significance was evaluated using one-way ANOVA followed by Tukey's test. A p-value <0.05 was considered to indicate significance (*). Data represent means ± SD of three independent experiments. Each experimental condition was performed in triplicate.

of 10 μM; however, after 72 h at a concentration of 2.5 μM, the cell viability was impaired approximately 35% (Fig. 1A). In the MDA-MB-231 cell line, BfL was not effective at these concentrations (Fig. 1C). The lectin Con A (*Canavalia ensiformis*) was used as a positive control in MCF7 (Fig. 1B) and MDA-MB-231 cells (Fig. 1D) for cell viability inhibition. In recent years, there has been an increasing interest in lectins because of their role in the agglutination, toxicity and antiproliferation of different cell lines [37–39]. Antiproliferative properties of lectins have been demonstrated in vitro and in vivo [39], in addition to the inhibition of cell adhesion [40]. It has been reported that these proteins interact better with tumor cells compared to normal cells because of the large number of altered glycoprotein receptors on the cell surface or because of the distribution of these receptors in tumor cells [40–42]. These differences may explain the high cytotoxic effect of lectins on tumor cells [40], which indicates that these proteins can contribute to the understanding of the multifactorial events involved in cancer [43]. The action of BfL and Con A on the viability of MCF 10A non-malignant cells was analyzed (Fig. 2). BfL did not inhibit the viability of these cells after 48 h of incubation, differently of the treatment with Con A, which indicates the selectivity of BfL for tumor cells. This effect is important, since cytotoxic drugs generally do not differentiate normal from metastatic tissues [44]. The effect of BfL on cell viability, using the human breast cancer cell lines MCF7 and MDA-MB-231, shows an important action of this protein, which is the inhibition of viability of the MCF7 cell line at low concentrations, and indicates its specific action. MDA-MB-231 is a triple-negative cell line that does not express hormone receptors (estrogen and progesterone) neither overexpress the HER2 receptor, therefore the treatment for this line is mostly based on chemotherapy [45,46], differently of MCF7 cells, which present intermediate levels of the receptor HER2. The activation of this receptor is blocked by the monoclonal antibody trastuzumab [47]. Differences in the specificity of plant lectins to the tumor cell lines have been reported [48–50], and the differences in specificity to carbohydrates distinguish their antiproliferative activities [39,49]. For example, the purified lectin from *Phaseolus coccineus* L. has specificity to sialic acid and shows antiproliferative properties, and the mechanism is related to the induction of caspase-dependent apoptosis in murine fibrosarcoma L929 cells. The antiproliferative activity decreases when the specific activity to sialic acid is completely inhibited, which indicates that carbohydrate binding specificity may be the main reason for the impairment in the cell viability of this line [7]. Lectins from *Polygonatum cyrtonema* (PCL), *Ophiopogon japonicus* (OJL) and *Liparis noversa* (LNL) inhibit the cell viability of MCF7 cells, and all these proteins present the conserved mannose-binding domain 'QXDXNXVXY', although the number of

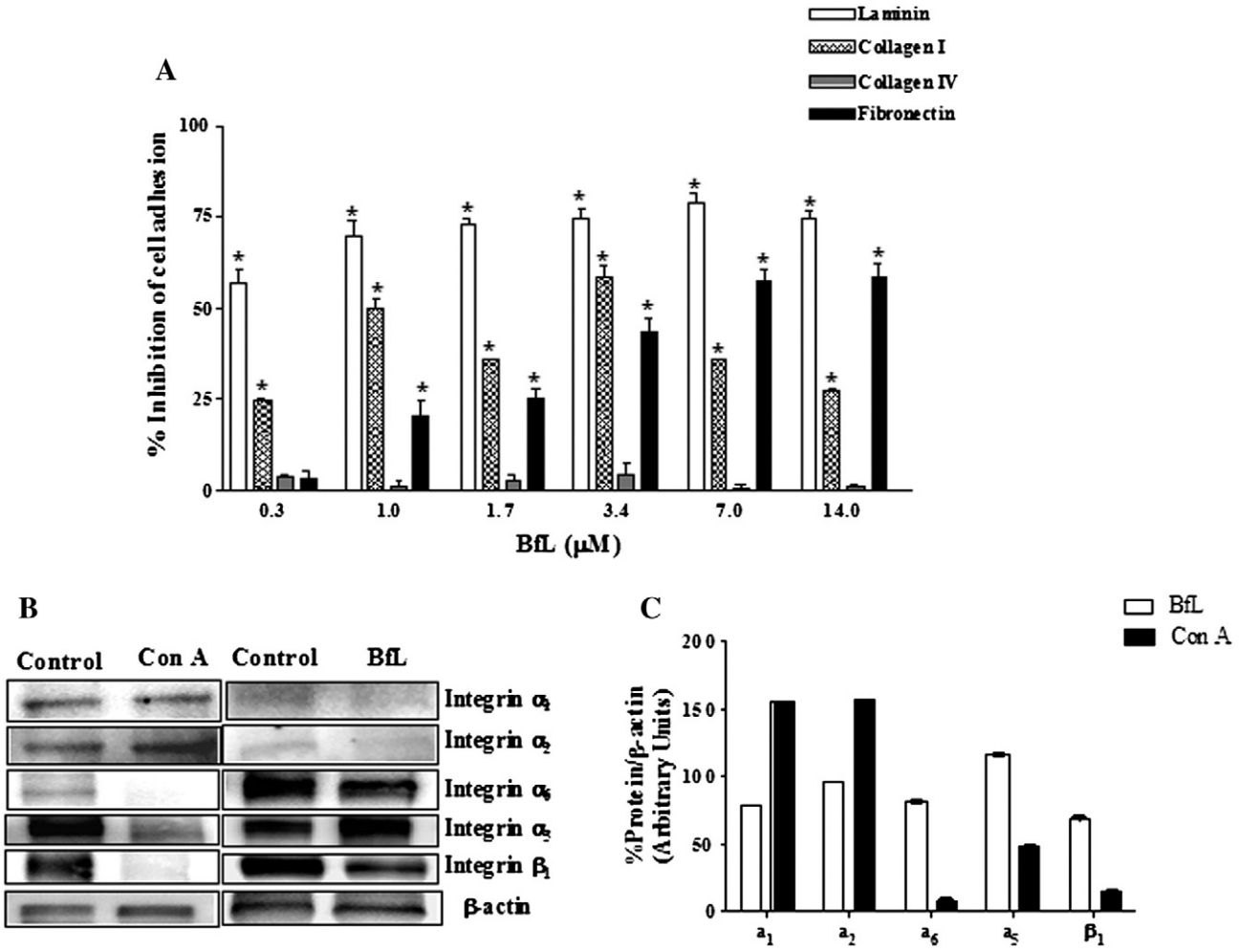

Fig. 3. Anti-adhesive effect of MCF7 cells (5×10^4) on laminin, fibronectin and collagen I by BfL (A). BfL (0.3–14 μM) was incubated with MCF7 cells for 30 min at room temperature and added in the wells with coats. The incubation was performed at 37 °C for 3 h. The statistical significance was evaluated using one-way ANOVA followed by Tukey's test. Data represent the means ± SD of two independent experiments performed in triplicate, $^*p < 0.05$. (B) Effect of BfL (5 μM) or Con A (10 μM) on α_1, α_2, α_5, α_6 and β_1 integrin subunit expression in MCF7 cells (5×10^5) for 48 h or 24 h, respectively. Lysate proteins were separated by 7.5% SDS-PAGE and transferred to a PVDF membrane. Membranes were incubated with anti-integrin α_1, anti-integrin α_2, anti-integrin α_5, anti-integrin α_6, anti-integrin β_1 and anti-β-actin (control) antibodies and the antibody binding was visualized by chemiluminescence. (C) The relative levels of the proteins were determined by densitometric analysis with the Scion imaging software using β-actin as a control for each sample. The experiment is representative of two independent experiments. The values are expressed as the means ± standard deviation.

regions varies among the three lectins (3, 2 and 1 to LNL, OJL and PCL, respectively). The inhibitory ability of these lectins is directly proportional to the number of conserved domains of lectins [7]. Interestingly, as BfL does not contain this domain and is not specific to mannose, it likely interacts with another oligosaccharide. Lin and Ng [50] described the action of a single isolated lectin from *Bauhinia* (*variegata* species), which inhibits the viability of HepG2 and MCF7 cells, with IC_{50} values of 1.4 μM and 0.18 μM, respectively, but the mechanism of action is not elucidated. Whelan et al. [14] mapped the glycoproteins present in the membranes of the breast cancer lines MCF7, MDA-MB-453 and MDA-MB-468. In the MCF7 cell line, 14 glycoproteins with the consensus sequence NXS/T, which comprises the glycosylation site, were found, and these include galectin 3 binding protein, cathepsin D preproprotein, thrombospondin 1, leucine-rich α_2 glycoprotein 1 and α_2 integrin. Thus, there are various possibilities of BfL interaction that may lead to an induction or blockade of cellular events. Therefore, further studies are necessary to elucidate the mechanism involved in this process.

3.2. BfL affects the adhesion of MCF7 cells and alters the expression of integrins

The metastatic potential of cancer cells is largely attributed to their adhesive properties, which are affected by aberrant glycosylated structures different from those found on the surface of normal cells [20], promoting the interaction of the cell with extracellular matrix molecules. In an attempt to relate the action of BfL to its binding to the receptor involved in MCF7 cells, adhesion assays of these cells to four molecules of the extracellular matrix, collagens I and IV, laminin and fibronectin, were performed. Fig. 3A shows the significant inhibition of the adhesion of MCF7 cells to laminin, collagen type I and fibronectin by BfL. The cell adhesion was inhibited 70% and 50% to laminin and collagen type I, respectively, in the concentration of 1.0 μM of BfL. In the presence of fibronectin, the effect of BfL was more subtle, although in high doses of this lectin did exhibit significant concentration-dependent anti-adhesion effects (Fig. 3A). The adhesion molecules bind to receptors on the cell, called integrins, which are heterodimeric glycoproteins [51,52]. The common subunit to the adhesion molecules tested is the β_1 subunit, and the results obtained by western blotting (Fig. 3B) confirmed that the potent effect of BfL in inhibiting MCF7 cell adhesion to laminin, fibronectin and collagen type I (at a concentration of 1.0 μM) is mediated by the β_1 subunit because the results showed a reduction of 34% in the expression of this integrin. A reduction in the expression of the α_1 (20%), α_2 (3.2%) and α_6 (19%) subunits was observed. A 15% increase in the expression of the α_5 subunit was also observed. Con A decreased the expression of the subunits α_6 (91%), β_1 (86%) and α_5 (52%), and increased the expression of the subunits α_1 (55%) and α_2 (58%) (Fig. 3C). Based on these results, we suggest that the potent inhibitory effect of BfL on the cell adhesion to laminin may be related with a decrease of $\alpha_6\beta_1$ integrin expression. Moreover, the need for a higher concentration of BfL that effectively blocks the adhesion of MCF7 cells to fibronectin may be understood if we consider that although a decrease in β_1 expression was observed, an increase in α_5 expression occurs, which impairs the effect of this protein. In addition, an inhibition of 50% of cell adhesion to collagen may be due to the decreased expression of the β_1 and α_1 integrin subunits but not that of α_2. In this article, we focus on the expression of key molecules involved in the adhesion process, integrins, however, the extracellular matrix–integrin binding evokes molecular signaling cascades [44,53], such as FAK and Src [53], and this pathway will be further investigated.

3.3. Cell death induced by BfL in the MCF7 cell line

Fig. 4A shows the results obtained by flow cytometry using the annexin and PI assay. Annexin-V conjugated to the fluorochrome FITC

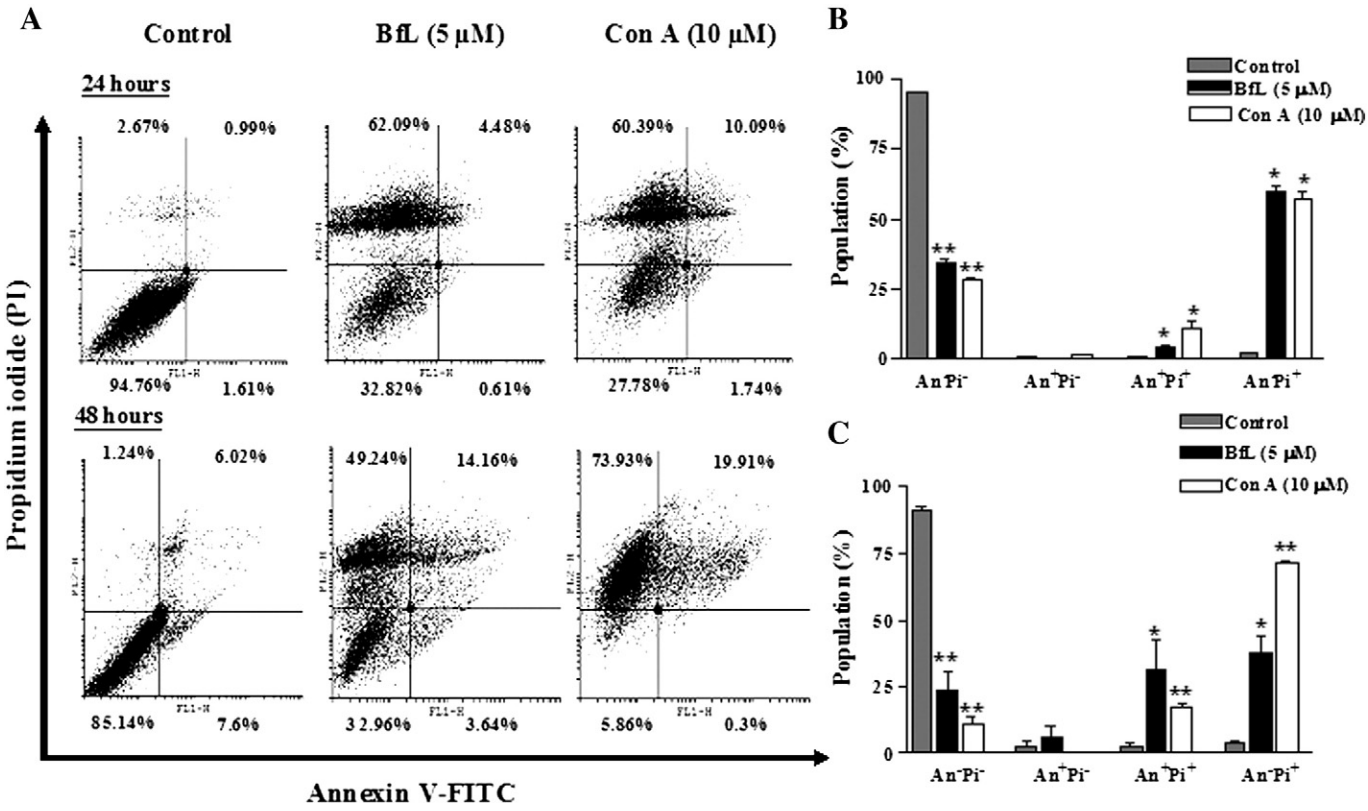

Fig. 4. Necrosis induction by BfL (5 μM) and Con A (10 μM) (positive control) in MCF7 cells (1×10^5) for 24 h (A, B) and 48 h (A, C), analyzed by flow cytometry using Annexin V-FITC/PI double-staining. The percentages of viable (An^-PI^-), apoptotic (An^+PI^-), secondary necrotic (An^+PI^+) and necrotic (An^-PI^+) cells are presented. The statistical significance was evaluated using one-way ANOVA followed by Tukey's test. *$p < 0.05$; **$p < 0.001$. Data represent means ± SD of two independent experiments performed in duplicate.

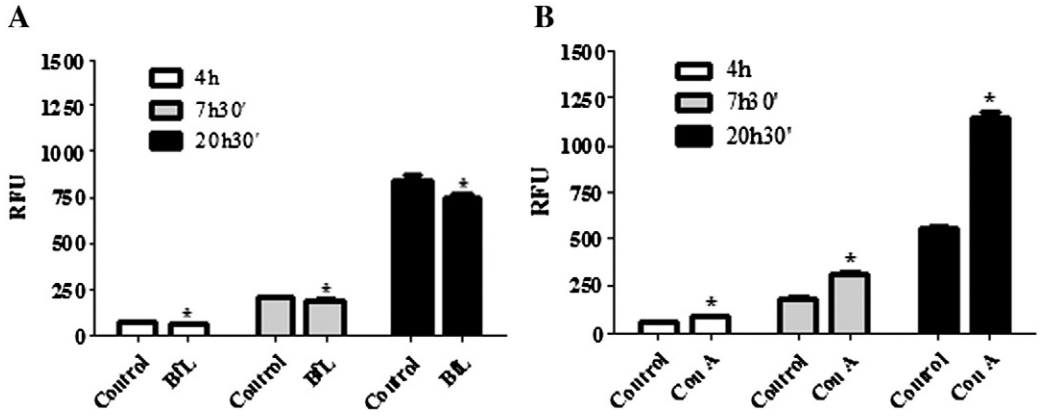

Fig. 5. Effect of BfL (A) or Con A (B) in the activation of caspase-9 in MCF7 cells (5×10^5) for 48 h or 24 h, respectively. The caspase-9 activation assay was performed by incubation of the substrate Ac-LEHD-AFC with the cellular lysate at 37 °C for 4 h, 7 h 30′ and 20 h 30′. The values are expressed as the means ± standard deviation of a representative experiment performed in triplicate. The statistical significance was evaluated using one-way ANOVA followed by Tukey's test, $^*p < 0.05$.

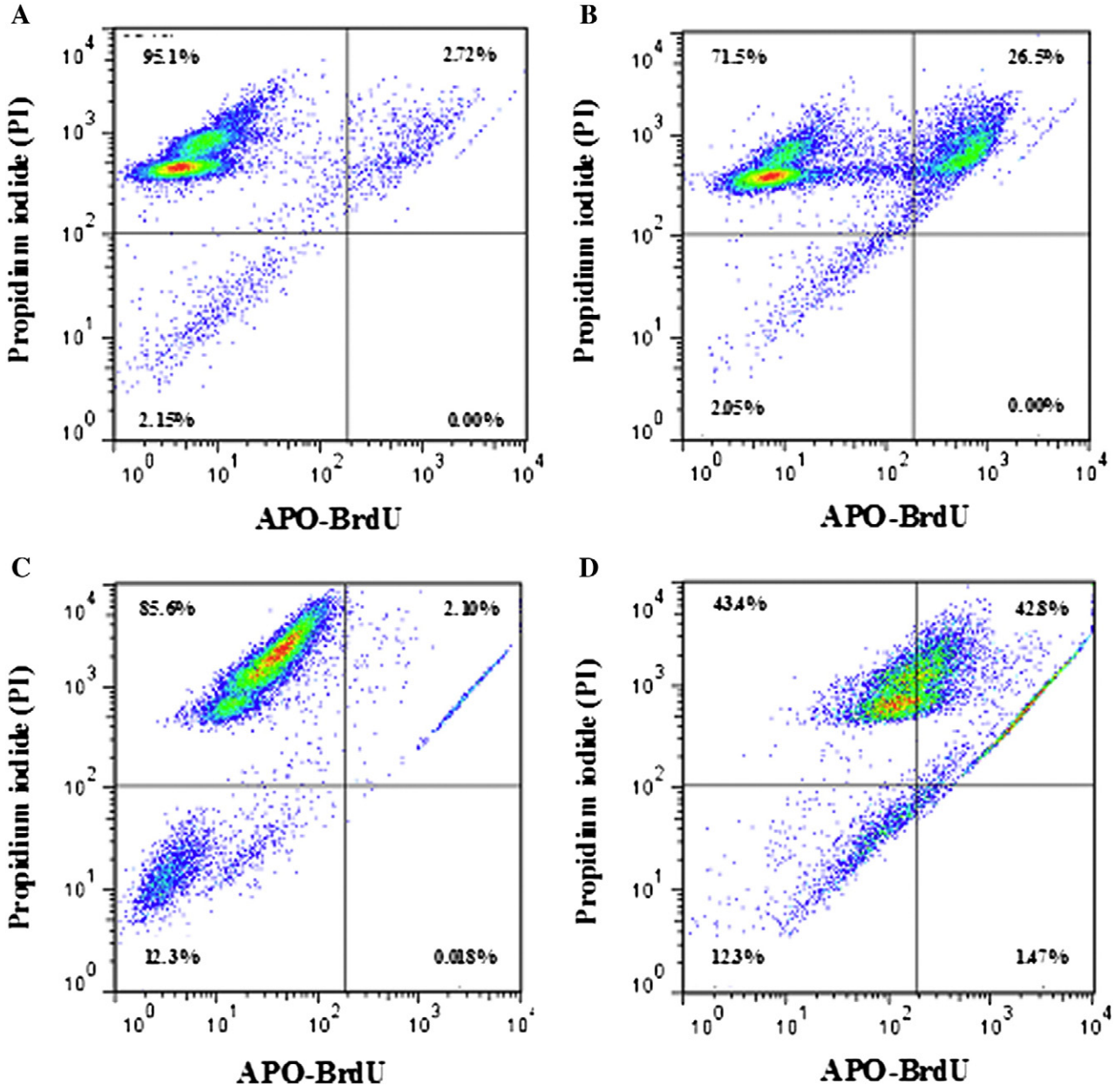

Fig. 6. Detection of DNA fragmentation by the TUNEL assay analyzed by flow cytometry using the anti-BrdU antibody conjugated to Alexa Fluor 488. In A, negative control cells, fixed human lymphoma cell line; in B, positive control cells, fixed human lymphoma cell line; in C, MCF7 cells treated with 7 mM HEPES, pH 7.4 (BfL vehicle) and in D, MCF7 cells (5×10^5) treated with 5 μM BfL for 48 h.

is a protein that binds to phosphatidylserine exposed on the extracellular membrane during apoptosis in the presence of calcium, and PI is a nuclear stain that cannot penetrate to viable cells [54]. Divided into four quadrants, the lower left quadrant is the negative staining for annexin V-FITC and PI (propidium iodide), which is representative of viable cells. The lower right quadrant shows positive staining for annexin V-FITC and negative PI, which occurs in early apoptotic cells. The upper right quadrant is representative of cells in secondary necrosis because of the double positive staining for annexin V-FITC and PI. The last quadrant (upper left) represents cells in necrosis with negative staining for annexin V-FITC and positive staining for PI [54]. Both lectins, Con A and BfL, at concentrations of 5 and 10 μM, respectively, were able to induce significant MCF7 cell death after 24 h (Fig. 4B) and 48 h (Fig. 4C). We observed differences in the mode of cell death depending on the time. After 24 h of treatment with Con A and BfL, approximately 33% and 28% of the cell population, respectively, were viable cells; however, the majority of the cells were in necrosis for both the lectins (Fig. 4B). At 48 h, the proportion of viable cells in the presence of BfL remained constant (33%), whereas with Con A, the proportion of viable cells significantly decreased (6%). For both treatments, the majority of the cell population was in necrosis, however, a significant number of cells were in secondary necrosis (Fig. 4C). Many lectins have the ability to induce cell death [55]. BfL triggers cell death in MCF7 cells in addition to inducing cell cycle arrest. Galluzzi et al. [56] reported that death receptor signaling, caspase inhibition and RIP1 and/or RIP3 activation can directly influence the cell death mode regulated necrosis. To corroborate whether cell death induced by BfL occurs by necrosis, caspase-9 activity analysis was performed in MCF7 cells. By fluorescence using the substrate Ac-LEHD-AFC, monitoring the cleavage of the AFC fluorophore, we observed an inhibition of caspase-9 activity in MCF7 cells after incubation for 48 h with BfL (Fig. 5A), but not with Con A (Fig. 5B), which showed a significant activation of caspase-9 in these cells, indicating that BfL triggers cell death with caspase inhibition (caspase-9).

3.4. DNA fragmentation and cell cycle arrest in the presence of BfL

As BfL induces cell death, we verified whether BfL causes DNA fragmentation in MCF7 cells, which was directly measured using the terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) assay. The MCF7 cells showed approximately 43% of staining with the anti-BrdU antibody (Fig. 6D), which indicates that fragmentation of the nuclear DNA occurs in the presence of BfL, similarly to the effects of the lectins from *Sophora flavescens* and *Abrus* agglutinin in HeLa cells [55,57,58]. Galluzzi et al. [56] reported that regulated necrosis can also be induced by DNA damage, and this event may lead to the inhibition of caspase. Analysis by flow cytometry showed that MCF7 cells treated with 5 μM of BfL or 10 μM of Con A for 24 h (Fig. 7A) and 48 h (Fig. 7B) led to a cell cycle arrest in the G2/M phase. Cell cycle arrests are frequently observed in cells treated with lectins because of DNA damage [7,9,55,58]. BfL leads to cycle arrest in the G2/M phase with a decrease in the expression of pRb (86%), which is a critical regulator of DNA replication, and in p21 (96%), such as Con A (10% for pRb and 18% for p21) (Fig. 7C, D). The cell cycle process, particularly the transition from one phase to another, is regulated by the activities of different cyclin-dependent kinases (CdKs) and their specific regulatory subunits, which are called cyclins. Each complex CdK/cyclin phosphorylates proteins that are required for cell cycle progression. The catalytic activity of this complex is regulated by binding CdK inhibitors [59]. p21 is a CdK inhibitory protein that binds to the CdK-cyclin of the S phase (CdK2-cyclin E) and blocks the action of this complex. In other words, the progress of

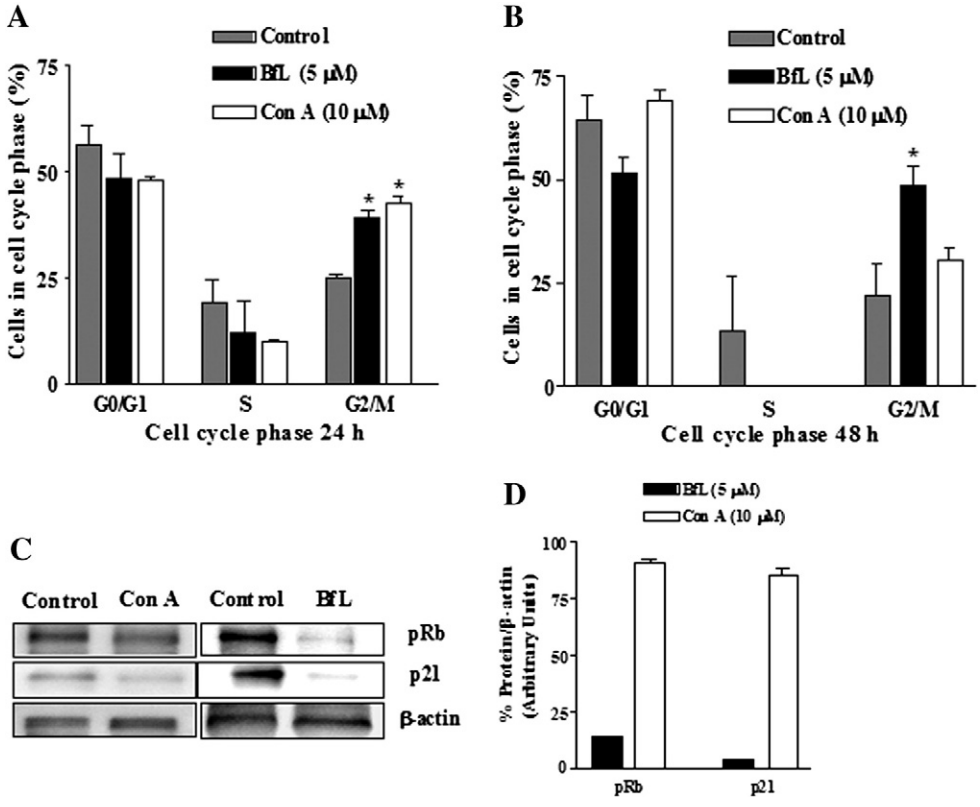

Fig. 7. Percentages of the cell population of MCF7 cells (1×10^5) in each phase of the cell cycle were evaluated by flow cytometry using propidium iodide (PI) staining after treatment with BfL (5 μM) or Con A (10 μM) for 24 h (A) and 48 h (B). The statistical significance was evaluated using one-way ANOVA followed by Tukey's test. $^*p < 0.05$. Data represent means ± SD of two independent experiments performed in duplicate. (C) Effect of BfL (5 μM) or Con A (10 μM) on the expression of cell cycle regulatory proteins p21 and pRb in MCF7 cells (5×10^5) for 48 h or 24 h, respectively. Lysate proteins were separated by 12% SDS-PAGE and transferred to a PVDF membrane. Membranes were incubated with anti-p21, anti-pRb and anti-β actin (control) antibodies, and the antibody binding was visualized by chemiluminescence. (D) The relative levels of the proteins were determined by densitometric analysis using the Scion imaging software with β-actin as a control for each sample. The experiment is representative of two independent experiments. The values are expressed as the means ± standard deviation.

the cell cycle to S phase [59], and to the Cdc2-cyclin B, which controls the progress of the cell cycle to the M phase. The decrease in the expression of p21 and pRb in MCF7 cells treated with BfL confirms its interference in the cell cycle, which led to cell cycle arrest in G2/M. These results were consistent with other studies that demonstrated cell cycle arrest in the G2/M phase by other lectins, such as Con A [9,55,60,61].

4. Conclusions

Based on these findings, we propose that BfL induces cell death with caspase-9 inhibition, DNA fragmentation, which results in cell cycle arrest in the G2/M phase in MCF7 cells, and anti-adhesion of these cells on laminin, fibronectin and collagen type I, with reduced α_1, α_6 and β_1 integrin subunit expression. In summary, our results provide a basis for using a plant lectin to investigate the participation of glycoproteins and glycoconjugates in signaling pathways in different cell lines, however the precise mechanism of BfL action needs to be clarified, whether it is a lectin-independent activity or by the property of the protein.

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