Toxicology in Vitro 26 (2012) 1161–1169

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Antiproliferative effects of lectins from *Canavalia ensiformis* and *Canavalia brasiliensis* in human leukemia cell lines

Glaucia V. Faheina-Martins [a], Alethéia Lacerda da Silveira [a], Bruno C. Cavalcanti [b], Márcio V. Ramos [c], Manoel O. Moraes [b], Cláudia Pessoa [b], Demetrius A.M. Araújo [a,*]

[a] Departamento de Biotecnologia, Centro de Biotecnologia, Universidade Federal da Paraíba, João Pessoa, PB, Brazil
[b] Laboratório Nacional de Oncologia Experimental, Departamento de Fisiologia e Farmacologia, Universidade Federal do Ceará, Fortaleza, CE, Brazil
[c] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Fortaleza, CE, Brazil

ARTICLE INFO

Article history:
Received 15 March 2012
Accepted 29 June 2012
Available online 7 July 2012

Keywords:
Apoptosis
ConA
ConBr
Cytotoxicity
Flow cytometry
Leukemic cells

ABSTRACT

The antiproliferative activity of lectins *Canavalia ensiformis* (ConA) and *Canavalia brasiliensis* (ConBr) were studied using human leukemia MOLT-4 and HL-60 cell lines. It was revealed that both ConA and ConBr were markedly cytotoxic to cells using MTT and NAC assays. The IC_{50} values were approximately 3 and 20 μg/mL for ConA and ConBr, respectively, for both MOLT-4 and HL-60 cells. However, in normal human peripheral blood lymphocytes, the lectins were not cytotoxic, even when tested at concentrations as high as 200 μg/ml. Using comet assay, the lectins produced a rate of DNA damage exceeding 80% in MOLT-4 and HL-60 cells. Fluorescence analysis revealed the morphology characteristic of apoptosis, with low concentrations of apoptotic bodies and fragmented DNA (5 μg/ml). Flow cytometric analysis demonstrated an accumulation of cells in the sub-G1 cell cycle that is characteristic of DNA fragmentation, and a decrease in membrane integrity at high concentrations. Lastly, we evaluated the alterations in mitochondrial potential that reduced after treatment with lectins. Our results indicate that ConA and ConBr inhibited cell proliferation selectively in tumor cells and that apoptosis was the main death mechanism. Therefore, lectins can be considered a class of molecules with a high antitumor activity potential.

1. Introduction

Lectins are proteins of non-immune origin that either bind to carbohydrates and sugar-containing substances in a specific and reversible manner or precipitate glycoconjugates (Goldstein et al., 1980). They are widely distributed in nature and can be found in almost all living organisms, including plants, algae, fungi, animals (vertebrates and invertebrates), microorganisms, and viruses (Peumans and Van Damme, 1996). Additionally, it has been shown that lectins can be used to differentiate malignant tumors from benign cells and their degree of glycosylation, which is associated with metastasis. It has also been demonstrated that lectins inhibit cell proliferation and have cytotoxic effects on human tumor cells (De Mejía and Prisecaru, 2005). Furthermore, lectins exert an immunostimulatory effect at low amounts and a cytotoxic effect at higher concentrations.

In recent years, a great number of lectins with *in vivo* and *in vitro* antiproliferative properties against cancer cells have been isolated and characterized (Dhuna et al., 2005; Liu et al., 2009a; Zhang et al., 2010). Among the seven major lectin families, legume lectins have received more attention from cancer biologists due to their remarkable anti-tumor properties compared to the other lectin families. In their review, Li et al. (2011) focused on analyzing the anti-tumor activities of Concanavalin A (ConA), the first and most typical representative of the legume lectin family, and its related mechanisms of cell death implicated in apoptosis and autophagy. Induction of *in vitro* and *in vivo* cell death (apoptosis and autophagy) in cancer cells by ConA has been reported (Kulkarni et al., 1998; Suen et al., 2000; Chang et al., 2007; Liu et al., 2009a–c).

Of note is the fact that the development of cancer can be associated with programmed cell death (PCD), which is an evolutionary conserved process that plays a crucial role in metazoan development (Bortner and Cidlowski, 2007). Apoptosis, type I of PCD, is characterized by the condensation of the cytoplasm and nucleus, DNA fragmentation, chromatin merging in the nuclear periphery, cell contraction, dynamic membrane blebbing, and cell phagocytosis. Several antitumor drugs are now known to induce apoptosis in cancerous cells. Cell apoptosis is considered to be one of the most

* Corresponding author. Address: Universidade Federal da Paraíba, Centro de Biotecnologia, Departamento de Biotecnologia. Campus I, Cidade Universitária, CEP: 58051-900 João Pessoa, Paraíba, Brazil. Tel.: +55 83 32167173; fax: +55 83 32167502.
E-mail addresses: demetrius.araujo@pq.cnpq.br, dam@ltf.ufpb.br, demetrius@cbiotec.ufpb.br (D.A.M. Araújo).

0887-2333
http://dx.doi.org/10.1016/j.tiv.2012.06.017

important mechanisms regulated by numerous cellular signaling pathways for tumor cell suicide (Andrew, 2008).

It has been shown that the mitochondrial membrane permeabilization can be sensitive to the redox state and reactive oxygen species (ROS) can also enable such membrane permeabilization both *in vitro* and *in vivo* approaches (Kroemer and Reed, 2000). Although free radicals are essential for normal cells, they can cause cell damage or act directly as intermediate signaling molecules, leading to oxidative stress as well as a variety of biological effects, including apoptosis (Nakano et al., 2006). These results on ROS signaling have been employed for the improvement of novel therapeutic applications in human diseases (Trachootham et al., 2009).

Our recent studies have shown that lectins ConA, ConBr, and CFL are all structurally related and induce apoptosis in the MCF-7 cell line (Faheina-Martins et al., 2011). Therefore, this study explores the antileukemic and DNA-damaging activities of ConA and ConBr in terms of two human leukemia cell lines (HL-60 and MOLT-4). Furthermore, we investigate the mechanism of death induction caused by these lectins.

2. Materials and methods

2.1. Chemicals

Fetal bovine serum (FBS), phytohaemagglutinin, and trypsin–EDTA were purchased from Cutilab (Campinas, SP, Brazil). RPMI 1640 medium was purchased from GIBCO (Invitrogen, Carlsbad, CA, USA). ConA, Rhodamine 123 (Rho-123), etoposide, penicillin, streptomycin, and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Normal melting point agarose (NMPA) and low melting point agarose (LMPA) were obtained from Invitrogen (Carlsbad, CA, USA). Doxorubicin (Doxolem®) was purchased from Zodiac Produtos Farmacêuticos S. A. (São Paulo, SP, Brazil). All other chemicals and reagents used were of analytical grade. ConA was obtained from SIGMA (São Paulo, Brazil) and ConBr was purified from the crude saline extract of seed flour through affinity chromatography on Sephadex G-50 fast flow (SIGMA) according to Cavada et al. (1998).

2.2. Cell line and cell culture

The human promyelocytic leukemia (HL-60) and acute lymphoblastic cell (MOLT-4) lines were acquired from Rio de Janeiro Cell Bank (Federal University of Rio de Janeiro, RJ, Brazil). Leukemia cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO_2. For experiments, the concentration of FBS was reduced to 1% so that the lectins would display their effects (Faheina-Martins et al., 2011).

Heparinized blood (from healthy, non-smoking donors who had not taken any drugs for at least 15 days prior to sampling) was collected from donor blood at the blood bank of the João Pessoa, Paraíba, Brazil. From these blood samples, we isolated the peripheral blood mononuclear cells (PBMC). The study was approved by the Institutional Ethical Committee of Lauro Wanderley Hospital/Federal University of Paraíba. PBMC were isolated by a standard method of density-gradient centrifugation over Histopaque-1077 (GE Healthcare, USA). PBMC were washed and resuspended in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO_2. Phytohemagglutinin (2%) was added at the beginning of the culture. After 24 h of culture, cells were treated with the test lectins.

2.3. MTT assay

The cytotoxicity of ConA and ConBr to leukemic cells was evaluated using the original enzymatic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to produce formazan crystals (Mosmann, 1983). Cells were seeded at 5×10^4 cells/well in 96-well tissue culture plates. Cells were exposed to different concentrations of ConA or ConBr lectins (1–200 μg/mL) dissolved in the RPMI medium (three wells per concentration) with 1% FBS. After 72 h of incubation, plates were centrifuged (500*g*, 5 min) and the supernatant was removed, followed by the addition of MTT solution (0.5 mg/mL in PBS) and incubation for 4 h at 37 °C. After 4 h, the MTT formazan product was dissolved in SDS/HCl 0.01 N and absorbance was measured at 570 nm in reader plate ELISA (Biotek ELx800). Etoposide (1 μg/mL) was used as a positive control.

2.4. Nucleic acid content (NAC)

The number of cells in both the control and treated cell samples were estimated based on their total nucleic acid content, as described by Cingi et al. (1991). Cells were seeded at 5×10^4 cells/well in 96-well tissue culture plates and exposed to different concentrations of ConA or ConBr lectins (1–200 μg/ml) dissolved in the RPMI medium (with 1% FBS). After 72 h of incubation, cells were fixed (5% trichloroacetic acid), washed twice with ice-cold PBS, and a soluble nucleotide pool was extracted with cold ethanol. The cell pellet was dissolved in 0.5 M NaOH at 37 °C overnight. Following this, the absorbance at 260 nm of the NaOH fraction was used as an index of the cell number (Bianchi and Fortunati, 1990). The results are expressed as mean percentages of absorbance at 260 nm in treated cells compared to the controls. Etoposide (1 μg/ml) was used as a positive control.

In MTT and NAC assays the concentration that inhibits 50% of cell proliferation (IC_{50}) was determined from plots of cell viability.

2.5. BrdU incorporation assay

Proliferating cells can be identified using DNA labeling with nucleotide analogs such as bromodeoxyuridine (BrdU). Leukemic cells were plated in 24-well tissue culture plates (0.3×10^6 cells/mL) and treated with lectins at different concentrations dissolved in RPMI medium (with 1% FBS). After 21 h of exposure, 20 μl of BrdU (10 mM) was added to each well and incubated for 3 h at 37 °C. To determine the amount of BrdU incorporated into DNA (Pera et al., 1977), cells were harvested and then transferred to cytospin slides and allowed to dry for 2 h at room temperature. Cells that had incorporated BrdU were labeled by direct peroxidase immunocytochemistry using the chromogen diaminobenzidine (DAB). Slides were counterstained with hematoxylin, mounted, and coverslipped. Determination of BrdU positivity was performed by light microscopy (Olympus, Tokyo, Japan). Two hundred cells were counted per sample to determine the percentage of BrdU-positive cells. Etoposide (1 μg/ml) was used as a positive control.

2.6. DNA strand breaks

The comet assay, which is used to detect DNA strand breaks, was conducted under alkaline conditions as described by Singh et al. (1988) with minor modifications (Klaude et al., 1996) following the recommendations of the International Workshop on Genotoxicity Test Procedures (Tice et al., 2000). HL-60 and MOLT-4 (0.3×10^6 cells/ml) cells were incubated for 24 h with lectins at 5, 25, and 50 μg/ml. After this, the cells were centrifugated and resuspended in the medium. Subsequently, 20 μl of the cells in suspension ($\sim 10^6$ cells/ml) were dissolved in 0.75% low melting point

agarose and immediately spread onto a glass microscope slide precoated with a layer of 1% normal melting point agarose. The agarose was allowed to set at 4 °C for 5 min. The slides were incubated in an ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100 and 10% dimethylsufoxide, DMSO, pH 10) at 4 °C for at least 1 h to remove cellular proteins, leaving the DNA as 'nucleoids.' After the lysis procedure, the slides were placed on a horizontal electrophoresis apparatus, which was filled with fresh buffer (300 mM NaOH and 1 mM EDTA, pH > 13) to cover the slides, for 20 min at 4 °C to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted for 20 min at 25 V (300 mA). All of the above steps were conducted either under a yellow light or in the dark to prevent additional DNA damage. The slides were then neutralized (0.4 M Tris, pH 7.5), dried with 100% ethanol, stained with ethidium bromide (20 μg/mL), and analyzed using a fluorescence microscope. Two hundred randomly selected cells (100 cells from each of the two replicate slides) were analyzed for each concentration of the test substance (Faheina-Martins et al., 2011). Cells were grouped visually according to tail length into the following five classes: (1) class 0—undamaged, without a tail; (2) class 1—with a tail shorter than the diameter of the head (nucleus); (3) class 2—with a tail length of 1–2× the diameter of the head; (4) class 3—with a tail longer than 2× the diameter of the head; (5) class 4—comets with no heads. A value (damage index, DI) was assigned to each comet according to its class using the equation below:

$$DI = (0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)$$

where n = the number of cells in each class that were analyzed. The damage index thus ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4), and damage frequency (%) was calculated based on the number of cells with a tail versus the number of those without (Cavalcanti et al., 2009). Etoposide (1 μg/mL) was used as a positive control.

2.7. Determination of the proportion of apoptotic and necrotic cells

Staining of cells with acridine orange/ethidium bromide (AO/EB) was performed (McGahon et al., 1995) to observe the cell death pattern induced by increasing concentrations of compounds after 24 h of incubation. HL-60 and MOLT-4 (0.3×10^6 cells/ml) cells were incubated for 24 h with lectins at 5, 25, and 50 μg/ml. After incubation, each sample (25 μl) was mixed with 1 μl of AO/EB solution (1 part of 100 μg/ml of AO in PBS; 1 part of 100 μg/ml EB in PBS) just prior to microscopic examination and quantification. At least 300 cells were examined under a fluorescence microscope using a fluorescein filter and 40X objective lens. The cells were then classified as either apoptotic or necrotic. The percentage of apoptotic and necrotic cells was then calculated. Experiments were performed in duplicate in three independent experiments. Etoposide (1 μg/ml) was also used as a positive control.

2.8. Determination of internucleosomal DNA fragmentation and mitochondrial transmembrane potential analysis

For internucleosomal DNA fragmentation, after 24 h of exposure with lectins, cells were incubated at 37 °C for 30 min in the dark in a lysis solution containing 0.1% citrate, 0.1% Triton X-100, and 50 μg/ml PI. The percentage of degraded DNA was determined by the number of cells displaying subdiploid (sub-G_o/G_1) DNA divided by the total number of cells examined.

Transmembrane mitochondrial potential was evaluated by incorporating Rhodamine-123 (Rho-123), which is a cell-permeable, cationic, fluorescent dye that is readily sequestered by active mitochondria without inducing cytotoxic effects. Treated and untreated leukemic cells during 24 h were centrifuged at 500*g* for 5 min and the pellet was resuspended in 200 μl of a 1 μg/ml solution of Rho-123 for 15 min in the dark. After incubation, cells were centrifuged at 500*g* for 5 min. The resulting pellet was resuspended in 200 μl of phosphate-buffered saline (PBS) and incubated for 30 min in the dark. Fluorescence was measured and the mitochondrial depolarization percentage was determined.

For all cytometric experiments, cell fluorescence was determined by flow cytometry in a Guava EasyCyte Mini (Guava Technologies, Inc., Hayward, CA, USA) using Guava Express Plus software, as described by Lograd o et al. (2010). Five thousand events were evaluated per experiment.

2.9. Measurement of intracellular reactive oxygen species (ROS)

The intracellular ROS was estimated by fluorescent probe, 2′,7′-dichlorohydrofluorescein diacetate (H2-DCF-DA). This dye is deacetylated by intracellular esterase and converted to non fluorescent 2′,7′-dichlorohydrofluorescein (H2-CDF), which is rapidly oxidized to the highly fluorescent compound 2′,7′-dichlorohydrofluorescein (DCF) in the presence de ROS. The MOLT-4 and HL-60 cells (4×10^5 cells/well) were treated with lectins ConA or ConBr (5, 25 and 50 μg/ml). As a positive control H_2O_2 (50 μM) was used for 15 min. After 24 h of exposure, the samples were centrifuged (200*g*, 5 min), washed with PBS at 37 °C and labeled of H_2-DCFH-DA for 30 min, in the dark conditions, at 37 °C. The cells were then washed with PBS and analysed by flow cytometry in BD FACSCalibur, NJ, USA (Ravidran et al., 2011).

2.10. Statistical analysis

The data are expressed as mean ± SEM from three replicates per treatment. Results were analyzed by one-way ANOVA followed by Newman-Keuls post-test. The level of significance was set at $p < 0.05$. Data of all the results in this study were obtained from at least three independent experiments.

The correlation between numbers of late apoptosis cells, expressed as arbitrary unit of DNA damage, was performed using the least squares linear regression, $f = ax + b$, where a means the slope of the line and b determines the point at which the line crosses the y-axis. The Pearson's correlation coefficient (r), was considered to be significant when $p < 0.05$.

3. Results

3.1. Lectins reduce both proliferation and viability of leukemic cells

As displayed in Table 1, the MTT-based assay and total nucleic acid content (NAC) measurements show that ConA and ConBr lectins have cytotoxic effects in leukemic cells. The MOLT-4 cell line was more sensitive to exposure of ConA and ConBr than the HL-60 cells were after 72 h of treatment. Among the tested lectins, ConBr was much less active than ConA in the MTT assay. To further study the inhibition of proliferation, the incorporation of the nucleotide BrdU into DNA was evaluated by direct peroxidase immunocytochemistry in treated and untreated leukemic cells (Fig. 1). After 24 h, ConA at concentrations of 5, 25, and 50 μg/ml inhibited BrdU incorporation by 47.66 ± 2.79%, 72.45 ± 1.95%, and 87.58 ± 2.16%, respectively, in MOLT-4 cultures (Fig. 1A), and 39.12 ± 2.69%, 61.18 ± 3.68%, and 78.95 ± 2.66%, respectively, in HL-60 cultures (Fig. 1B). Leukemic cell cultures exposed to ConBr showed an inhibition of BrdU incorporation equal to 47.78 ± 4.52%, 69.31 ± 3.53%, and 86.60 ± 1.80% for MOLT-4 cells treated at 5, 25, and 50 μg/mL, respectively, and 28.65 ± 2.95%, 58.10 ± 3.01%, and 66.81 ± 3.49% for HL-60 cells treated at 5, 25,

Table 1
Cytotoxic activity of ConA and ConBr lectins on HL-60 and MOLT-4 cell lines.

Substances	HL-60		MOLT-4	
	MTT	NAC	MTT	NAC
Etoposide	2.6 (1.7–4.0)	10.0 (6.0–15)	0.1 (0.04–0.18)	0.1 (0.07–0.15)
ConA	14.0 (7.0–27.0)	10.0 (6.6–15.0)	3.6 (2.8–4.5)	3.0 (2.8–3.6)
ConBr	90.0 (75.0–109.0)	12.0 (5.0–28.0)	21.0 (20.0–23.0)	7.0 (6.2–7.8)

Results were expressed as CI_{50} in μg/ml and statistical confidence interval of 95% (CI 95%), which are shown by numbers in parentheses, was obtained using non-linear regression of three independent experiments in triplicate. Etoposide was applied as a positive control.

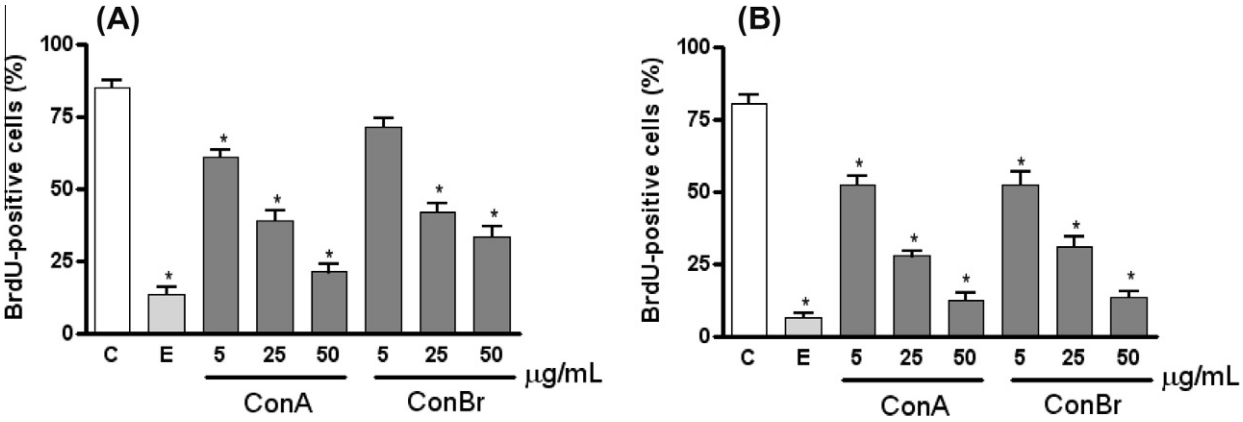

Fig. 1. Effect of ConA and ConBr on the BrdU incorporation by MOLT-4 (A) and HL-60 (B) cells. Negative control (C): cells not treated. Etoposide (5 μM) was used as a positive control (E). $^{*}p < 0.001$ compared with control by ANOVA, followed by Newman–Keuls post-test. Data are presented as mean values ± SEM from three independent experiments of percentage of BrdU positivity.

and 50 μg/mL, respectively. The positive control, etoposide, strongly inhibited the BrdU incorporation, as expected.

Etoposide exhibited potent cytotoxicity against HL-60 and MOLT-4 cell lines, as expected. The results presented in Fig. 2 demonstrate that ConA and ConBr are not cytotoxic for normal cells (PBMC) at 200 μg/ml using MTT assay.

3.2. Lectins induce DNA strand breaks followed by cell death (apoptosis and necrosis) in leukemic cells

Fig. 3A and B show the effects of ConA and ConBr on DNA damage index and frequency (tailed cells) as measured by DNA damage in leukemic cells according to the alkaline version of the comet assay. In both cell line cultures exposed to ConA and ConBr, the treated cells clearly show a significant increase in the means of DNA damage index ($p < 0.001$) and tailed cells ($p < 0.001$) at all evaluated concentrations. Etoposide, used as the positive control, induced a significant increase in DNA damage and frequency when compared to the negative control, or vehicle (data not shown). While attempting to determine the mechanism responsible for their antiproliferative effects, both the induction of apoptosis or necrosis and the DNA integrity of cells that were treated with lectins were assayed. After 24 h, more than 90% of the counted HL-60 and MOLT-4 cells in the control groups were uniformly green, viable, and had normal morphology (Fig. 4). As shown in Fig. 4A and B, both lectins reduced the number of viable cells in a concentration-

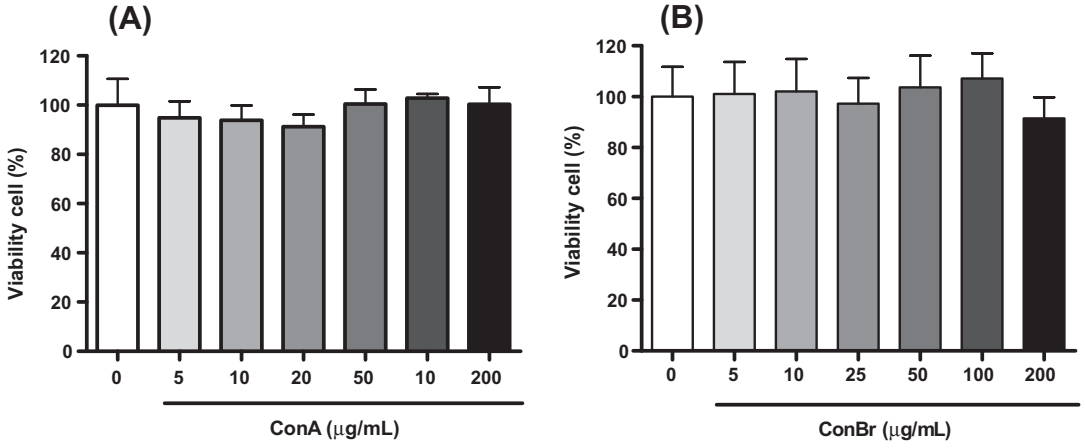

Fig. 2. Effect of ConA (A) and ConBr (B) after 24 h of treatment on PBMC viability using MTT assay. All data showed $p > 0.05$ compared with control by ANOVA, followed by Newman–Keuls post-test. Bars represent the mean ± SEM of three independent experiments in triplicate.

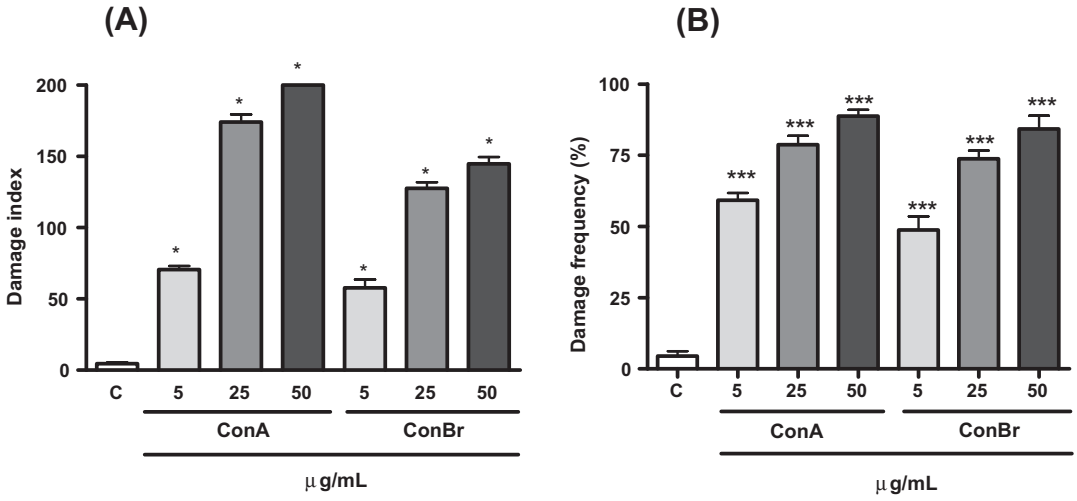

Fig. 3. Effect of lectins ConA and ConBr after 24 h of treatment on DNA damage index using MOLT-4 (A) and HL-60 cells (B) determined by comet assay. Control (untreated) or treated cells with lectins at concentrations 5, 25, and 50 μg/ml were used. Data are mean ± SEM from three experiments in duplicate and were analyzed by ANOVA followed by Newman–Keuls post-test. $^{*}p < 0.001$ compared to control.

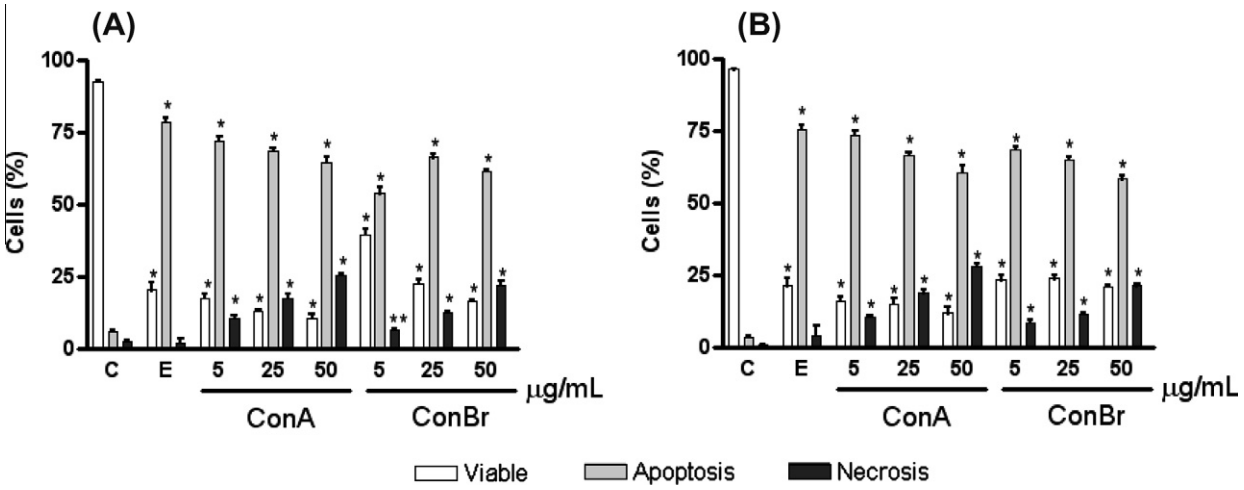

Fig. 4. Effect of lectins ConA and ConBr on MOLT-4 (A) and HL-60 (B) viability using acridine orange and ethidium bromide staining determined by fluorescence microscopy after 24 h of incubation. Negative control (C): cells were treated with medium alone. Bars represent the mean ± SEM of three independent experiments in triplicate. $^{*}p < 0.001$ and $^{**}p < 0.01$ compared with control by ANOVA followed by Newman–Keuls post-test.

dependent manner after 24 h of exposure at each evaluated concentration ($p < 0.001$) in leukemic cell cultures (MOLT-4 and HL-60). However, the effect on cell viability was more pronounced in cultures treated with ConA. The mechanism of induction of cell death in leukemic cells appears to be the same among the tested lectins. The antiproliferative capacity of both lectins seems to be predominately related to the apoptosis activation rather than necrosis. At the highest tested concentration, MOLT-4 and HL-60 cells exposed to ConA and ConBr showed that more than 60% of analyzed cells shared apoptotic features. These features include condensed or fragmented chromatin, blebs, and apoptotic bodies. The increase in the population of necrotic cells was smaller, achieving 25.33 ± 0.59% and 21.99 ± 1.14% when MOLT-4-treated with 50 μg/mL of ConA and ConBr, respectively. For HL-60 cultures exposed to ConA and ConBr at the highest concentration, the percentages of necrotic cells were 28.6 ± 2% and 21.3 ± 2%, respectively.

The number of late apoptosis cells induced by ConA and ConBr was compared with arbitrary unit of DNA damage induced by treatments. In MOLT-4 cultures, the increased induction of DNA damage correlated to the augmented late apoptosis cells induced by ConA ($a = 3.01$, $r = 0.958$, $p < 0.05$) and ConBr ($a = 2.24$, $r = 0,904$, $p < 0.05$) treatments. Also a correlation between arbitrary unit of DNA damage and late apoptosis cell number was observed for HL-60 treated cells with ConA ($a = 2.5$, $r = 0.976$, $p < 0.05$) and ConBr ($a = 2.57$, $r = 0.922$, $p < 0.05$). These correlations mean that an increase in DNA damage enhances the possibility of irreversible cell death, which can be late apoptosis in this case.

3.3. Lectins induce internucleosomal DNA fragmentation and alter mitochondrial transmembrane potential in leukemic cells

Both lectins induced mitochondrial depolarization in MOLT-4 and HL-60 cells, as measured by incorporation of Rho 123 after 24 h of exposure at all evaluated concentrations (Fig. 5A and B). This data suggests that ConA and ConBr induce apoptosis in leukemic cells by triggering an intrinsic mitochondrial pathway. At all tested concentrations, lectins caused cell shrinkage and nuclear condensation as evidenced by a decrease in forward light scattering and a transient increase in side scattering, respectively. The sub-diploid-sized DNA (sub-G_0/G_1) was considered to be due to

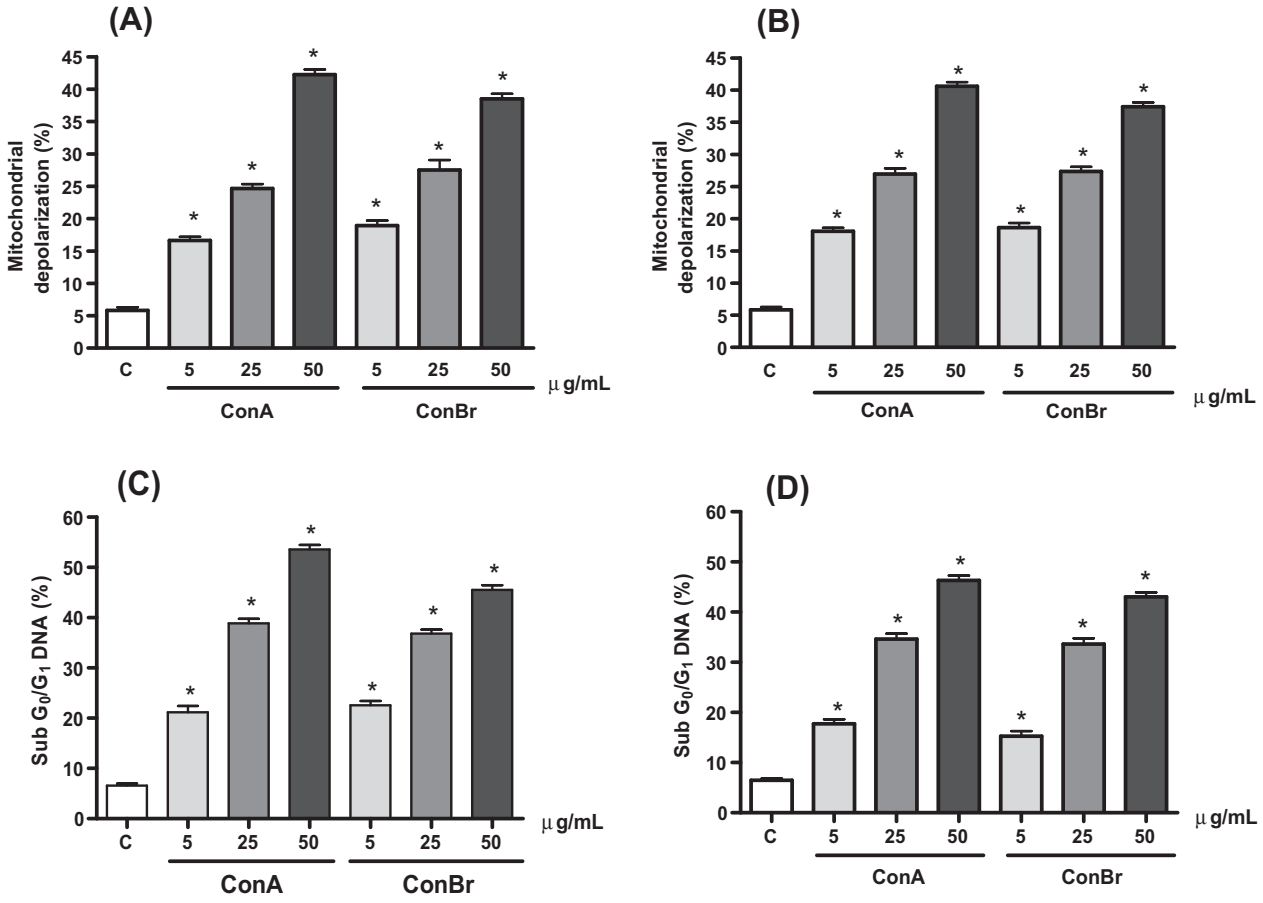

Fig. 5. Alteration of mitochondrial transmembrane potential ($\Delta\Psi m$) in MOLT-4 (A) and HL-60 lineages (B). Cells were treated with ConA and ConBr (5, 25 and 50 μg/ml) for 24 h. Those cells were stained with RHO 123 and the percentage of depolarized cells was quantified by flow cytometry. Analysis of DNA fragmentation in MOLT-4 (C) and HL-60 cells (D). Cells were treated with lectins ConA and ConBr (5, 25 and 50 μg/ml) for 24 h. After treatment, cells were stained with PI and analyzed by flow cytometry. Data are expressed as mean ± SEM of three independent experiments. $^{*}p < 0.01$ compared to control by ANOVA, followed by the Newman–Keuls post-test.

internucleosomal DNA fragmentation. Increased lectin-induced apoptotic sub-G_0/G_1 peaks mainly represent apoptotic cells having fractional DNA content and were observed at all concentrations 24 h after treatment (Fig. 5C and D).

3.4. Lectins increase ROS in leukemic cells

It has been described that ROS can play an important role in inducing apoptosis in various cell types; therefore we measured the intracellular ROS level using the fluorescence dye, DCF-DA. In this case, MOLT-4 cells incubated with ConA and ConBr produced high levels of ROS. The rate of DCF-positive cells increased significantly from 0.97 ± 0.13% to 45.07 ± 14.5% and 60.33 ± 24.48% after treatment with ConA and ConBr, respectively, for 24 h of incubation (Fig. 6A). In HL-60 cell line an increase in ROS production was also demonstrated, when these lectins (50 μg/mL) were incubated separately. However, these results showed that levels of ROS produced did not exceed 10% when compared to control, even in presence of H_2O_2 (Fig. 6B).

4. Discussion

It was reported that anticancer agents have been derived from a form or other natural sources, including plants, marine organisms, and microorganisms (Cragg and Newman, 2005). In recent years, plant lectins, obtained mainly from seeds, have gained much attention from the scientific community due to their extreme usefulness in the identification of cancer and degrees of metastasis (De Mejía and Prisecaru, 2005; Liu et al., 2010). Literature has shown the effects of induction of cytotoxicity, apoptosis, and necrosis of certain lectins against tumor cells (Kim et al., 1993; Kim et al., 2000; Suen et al., 2000; Seifert et al., 2008; Liu et al., 2009a–c.

ConA and ConBr possess identical three-dimensional structures and carbohydrate-binding specificity (Sanz-Aparicio et al., 1997). However, these lectins commonly exhibit distinct affinity for the same ligand (Ramos et al., 2002). As a result, they frequently differ in terms of dose–response activity (Barbosa et al., 2001). Despite the numerous uses of lectins as tools, there are few studies that refer to their antitumor activity or report the underlying mechanisms involved in lectin cytotoxicity. One aspect of this study shows lectins ConA and ConBr to be cytotoxic against both MOLT-4 and HL-60 cells, with IC_{50} values being approximately 3 μg/ml (ConA) and 20 μg/mL (ConBr) after 72 h of incubation (Table 1). For PBMC, ConA and ConBr lectins were not cytotoxic at high concentrations (200 μg/ml), demonstrating selectivity for tumor cells (Fig. 2).

Several studies have revealed data that corroborates our findings, demonstrating the cytotoxicity of ConA lectin in tumor cells. ConA was shown to be more toxic because it becomes completely tetrameric at physiological pH, exposing its catalytic site better than ConBr. Hence, it is able to exert more pronounced activity than ConBr, which presents as a mixture of dimers and tetramers at physiological pH (Sanz-Aparicio et al., 1997; Calvete et al., 1999). In order to identify the mechanism of action related to the antiproliferative effect of lectins, genetic toxicity, morphological changes, and experiments of cell death using flow cytometry were conducted.

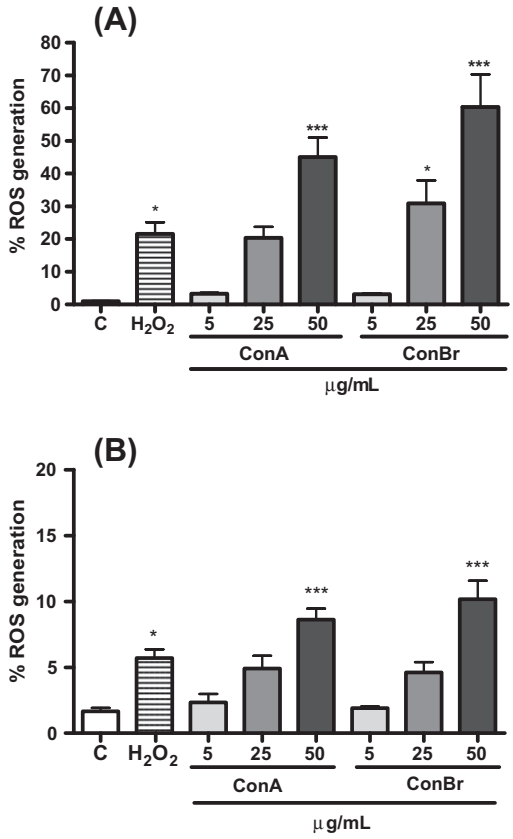

Fig. 6. Induction of ROS generation in MOLT-4 (A) and HL-60 (B) cell lines. Cells were treated with lectins ConA and ConBr (5, 25 and 50 μg/mL) for 24 h and H_2O_2 (50 μM) was used as positive control. These cell lines were then labeled with H_2-DCFH-DA and ROS production was quantified by flow cytometry in FL-1 band. Data are expressed as mean ± SEM from three independent experiments in duplicate. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared with the control by ANOVA, followed by the Newman–Keuls post-test.

Comet assay has shown that lectins ConA and ConBr promoted a significant increase in DNA strand breaks in MOLT-4 and HL-60 cells. Since the DNA lesions may disturb the maintenance of genomic integrity, the use of molecules that cause extensive damage to the DNA of these cells can induce programmed cell death and block tumor development (Hanahan and Weinberg, 2000; Hoang et al., 2007; Leonetti and Zupi, 2007).

The morphological analysis by differential staining with EB/AO demonstrated that cells treated with the lectins (ConA and ConBr) predominantly showed specific apoptosis features, as opposed to necrosis. Once again, our findings (Fig. 3) are supported by previous studies on the apoptotic effects of lectins (Barbosa et al., 2001; Gastman et al., 2004; Kulkarni et al., 1998; Liu et al., 2009a–c).

An important marker of cell death by apoptosis is the internucleosomal cleavage of chromatin and the fragmentation of DNA by DNases, such as the Apoptosis Induction Factor (AIF), endonuclease G, and caspase-activated DNase (CAD). These DNases are released from the mitochondria during apoptosis and are then translocated to the nucleus to promote DNA fragmentation (Elmore, 2007). The apoptotic nuclei can be distinguished by their hypodiploid DNA content, compared with the diploid DNA content of normal cells (Cury-Boaventura et al., 2003).

The lectins ConA and ConBr induced DNA fragmentation with an increase of fragmentation levels in a concentration-dependent manner. This coincides with the results obtained in our laboratory for DNA extraction and analysis using agarose gel electrophoresis, where the MOLT-4 and HL-60 line cells treated with lectins ConA and ConBr showed the pattern of a DNA "ladder" characteristic of apoptosis (data not shown). The literature has shown that the lectin ConA induces apoptosis of mice macrophages PU5–1.8, DNA fragmentation by agarose gel electrophoresis at 25 μg/ml, and liberation of cytochrome-*c* (Suen et al., 2000). A375 human melanoma cells treated with ConA at 25 μg/ml showed a considerable increase in sub-G1 cells with hypodiploid content, which is characteristic of apoptotic cell death (Liu et al., 2009c).

Our results indicate that lectins ConA and ConBr promoted mitochondrial depolarization in a concentration-dependent manner with MOLT-4 cells from 5 μg/ml ($p < 0.001$). The lectin ConA produced a greater depolarization than ConBr (Fig. 5A). These results are confirmed by data from Kulkarni et al. (1998), which shows that ConA (50 g/ml) induces apoptosis in FGH human fibroblast cells and is associated with a decrease in transmembrane potential, reduced intracellular calcium levels, and decreased expression of Bcl-2, a protein involved with cell death protection. Another report shows that treatment with ConA on A375 melanoma cells resulted in the induction of mitochondrial dysfunction due to an increased depolarization, release of cytochrome c, and increased activity of caspases -8, -9, and -3, which are all characteristic of apoptosis (Liu et al., 2009c).

The generation of ROS from mitochondria and other intracellular sources can cause serious damage to fundamental cellular molecules such as lipids, proteins and DNA. In addition, chemical agents that induce cytotoxicity have been implicated in the production of ROS. Indeed, ROS is known to be involved in the early stages of apoptosis and induces mitochondrial membrane depolarization (Ravidran et al., 2011). In this paper we demonstrated that ConBr and ConA lectins provoked apoptosis on MOLT-4 and HL-60 cells and this action also showed an increase of ROS levels. However the production of these radicals was significant only at the highest concentration tested (50 μg/mL, $p < 0.001$) suggesting that ROS production stimulated by lectins is not the initial factor inducing apoptosis, since the cellular damage and apoptotic events induced by ConA and ConBr might already be observed from the lowest concentration of lectins tested (5 μg/mL).

It is reported in the literature that apoptosis is the main mechanism of cell death caused by lectins (Oliveira et al., 2011; Li et al., 2011). The lectin from rice bran induced chromatin condensation, phosphatidylserine externalization, the formation of a DNA "ladder" in human monoblastic leukemia U937 cells, and apoptosis with cell cycle arrest. This mechanism is similar to that of wheat germ agglutinin (WGA), yet different from that of *Viscum album agglutinin* (Miyoshi et al., 2001). It is also reported that the mistletoe extract inhibited protein synthesis in malignant cells and the lectins isolated from this extract (ML-I, ML II, and ML-III) dissociated into catalytic subunits before they translocated across the membrane and entered the cytoplasm. The involvement of caspases cascade and their effects on U937 cells, by lectin ML-II, explains its cytotoxicity and apoptosis induction (Kim et al., 2000), as well as the lectin ML-I (Lyu et al., 2001). Along with confirming pre-existing data for ConA, the present results show that legume lectins ConA and ConBr promoted apoptosis (Figs. 4A,B and 5A–D). Even though these lectins have slight structural differences, they have specificity for the same type of carbohydrate (glucose/mannose) and show a similar effect on the tumor cell lines MOLT-4 and HL-60. Nonetheless, in each trial, it was noticeable that lectin ConA was more potent in its effects. This confirms the idea that the cytotoxicity exhibited by the lectins ConA and ConBr on tumor cells was caused mainly by induction of cell death via apoptosis, but also by necrosis when they are at higher concentrations. Thus, the cytotoxic agent may induce either apoptosis or necrosis depending on the concentrations and time of contact with the substance. Generally, apoptosis induction in tumor cells is a beneficial

effect for chemotherapy treatment of cancer. The lectins may promote apoptosis via two mechanisms. One possibility is by interacting with the cell surface, being endocytosed, and then reaching the mitochondria. This possibility would occur directly through the intrinsic pathway, as with ConA in some cell lines and other lectins such as WGA (Chang et al., 2007; Gastman et al., 2004; Suen et al., 2000). A second possibility is by binding to glycosylated portions of death receptors and then leading to its activation and apoptotic signal transduction through the extrinsic pathway.

It is expected that this cytotoxicity will be mediated by the carbohydrate-binding site of the lectins. These sites should specifically recognize membrane glycoreceptors on the cell surfaces of both HL-60 and MOLT-4 leukemic cells. Data from this study has shown the *in vitro* antitumor potential of legume lectins ConA and ConBr in breast tumor MCF-7 cells (Faheina-Martins et al., 2011). This is in agreement with existing literature on ConA and other lectins known for their cytotoxic potential, such as that obtained from mistletoe (Pryme et al., 2007).

In summary, ConA and ConBr lectins induce cell death in leukemic cells and promote apoptosis with DNA fragmentation, mitochondrial depolarization and increased production of ROS. Apoptosis plays a critical role in the molecular pathogenesis of cancer and can influence the outcome of chemotherapy and radiotherapy. Because of this, dietary compounds such as plant lectins should be considered promising for cancer treatment.

Acknowledgments

This research was supported by Grants from CNPq (Conselho Brasileiro de Desenvolvimento Científico e Tecnológico), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and RENORBIO (Rede Nordeste de Biotecnologia). The authors thank Silvana França dos Santos and Erivanda França Rios for their technical assistance. We also thank Dr Cosme R.M. Salinas, Department of Chemistry, Federal University of Paraíba, for his assistance in statistical analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2012.06.017.

References

Andrew, T., 2008. Apoptosis and autophagy: regulatory connections between two supposedly different processes. Apoptosis 13, 1–9.
Barbosa, T., Arruda, S., Cavada, B., et al., 2001. In vivo lymphocyte activation and apoptosis by lectins of the diocleinae subtribe. Mem. Inst. Oswaldo Cruz 96, 673–678.
Bianchi, V., Fortunati, E., 1990. Cellular effects of an anionic surfactant detected in V79 fibroblasts by different cytotoxicity tests. Toxicol. in Vitro 4, 9–16.
Bortner, C.D., Cidlowski, J.A., 2007. Cell shrinkage and monovalent cation fluxes: role in apoptosis. Arch. Biochem. Biophys. 462, 176–188.
Calvete, J.J., Thole, H.H., Raida, M., et al., 1999. Molecular characterization and crystallization of Diocleinae lectins. Biochim. et Biophys. Acta 1430, 367–375.
Cavada, B.S., Santos, C.F., Grangeiro, T.B., et al., 1998. Purification and characterization of a lectin from seeds of Vatairea macrocarpa duke. Phytochemistry 49, 675–680.
Cavalcanti, B.C., Bezerra, D.P., Magalhães, H.I.F., et al., 2009. Kauren-19-oic acid induces DNA damage followed by apoptosis in human leukemia cells. J. Appl. Toxicol. 29, 560–568.
Chang, C.-P., Yang, M.C., Liu, H.S., et al., 2007. Concanavalin A induces autophagy in hepatoma cells and has a therapeutic effect in a murine *in situ* hepatoma model. Hepatology 45, 286–296.
Cingi, M.R., De Angelis, I., Fortunati, E., et al., 1991. Choice and standardization of test protocols in cytotoxicology: a multicentre approach. Toxicol. In Vitro 5, 19–25.
Cragg, G.M., Newman, D.J., 2005. Plants as a source of anti-cancer agents. J. Ethnopharmacol. 100, 72–79.
Cury-Boaventura, M.F., Pompéia, C., Curi, R., 2003. Comparative toxicity of oleic acid and linoleic acido n Jurkat cells. Clin. Nutr. 23, 21–732.
De Mejía, E.G., Prisecaru, V.I., 2005. Lectins as bioactive plant proteins: a potential in cancer treatment. Crit. Rev. Food Sci. 45, 425–445.
Dhuna, V., Bains, J.S., Kamboj, S.S., et al., 2005. Purification and characterization of a lectin from *Arisaema tortuosum* Schott having *in vitro* anticancer activity against human cancer cell lines. J. Biochem. Mol. Biol. 38, 526–532.
Faheina-Martins, G.V., Silveira, A.L., Marques-Santos, L.F., et al., 2011. Influence of fetal bovine serum on cytotoxic and genotoxic effects of lectins in MCF-7 cells. J. Biochem. Mol. Toxic. 25, 290–296.
Gastman, B., Wang, K., Han, J., et al., 2004. A novel apoptotic pathway as defined by lectin cellular initiation. Biochem. Biophys. Res. Commun. 316, 263–271.
Goldstein, I.J., Hughes, R.C., Monsigny, M., et al., 1980. What should be called a lectin? Nature 285, 66.
Hanahan, D., Weinberg, R.A., 2000. The hallmarks of cancer. Cell 100, 57–70.
Hoang, B.X., Levine, S.A., Pham, P., et al., 2007. Hypothesis of the cause e development of neoplasms. Eur. J. Cancer Prev. 16, 55–61.
Kim, M., Rao, M.V., Tweardy, D.J., et al., 1993. Lectin-induced apoptosis of tumour cells. Glycobiology 3, 447–453.
Kim, M.S., Lee, J., So, H.S., Lee, et al., 2000. Activation of caspases cascades in Korean mistetloe (Viscum album var coloratum) lectin-II-induced apoptosis of human myeloleukemic U937 cells. Gen. Pharmacol. 34, 349–355.
Klaude, M., Erikson, S., Nygren, J., et al., 1996. The comet assay: mechanisms and technical considerations. Mut. Res. 363, 89–96.
Kroemer, G., Reed, J.C., 2000. Mitochondrial control of cell death. Nat. Med. 6, 513–519.
Kulkarni, G.V., Lee, W., Seth, A., et al., 1998. Role of mitochondrial membrane potential in concanavalin a-induced apoptosis in human fibroblasts. Exp. Cell Res. 245, 170–178.
Leonetti, C., Zupi, G., 2007. Targeting different signaling pathways with antisense oligonucleotides combination for cancer therapy. Curr. Pharma. Design 13, 463–470.
Li, W.W., Yu, J.Y., Xu, H.L., et al., 2011. Concanavalin A: a potential anti-neoplastic agent targeting apoptosis, autophagy and anti-angiogenesis for cancer therapeutics. Biochem. Biophys. Res. Commun. 414, 282–286.
Liu, B., Zhang, B., Min, M.W., et al., 2009a. Induction of apoptosis by *Polygonatum odoratum* lectin and its molecular mechanisms in murine fibrosarcoma L929 cells. Biochim. Biophys. Acta 1790, 840–844.
Liu, B., Min, M.W., Bao, J.K., 2009b. Induction of apoptosis by Concanavalin A and its molecular mechanisms in cancer cells. Autophagy 5, 432–433.
Liu, B., Li, C.Y., Bian, H.J., et al., 2009c. Antiproliferative activity and apoptosis-inducing mechanism of Concanavalin A on human melanoma A375 cells. Arch. Biochem. Biophys. 482, 1–6.
Liu, B., Bian, H.J., Bao, J.K., 2010. Plant lectins: potential antineoplastic drugs from bench to clinic. Cancer Lett. 287, 1–12.
Lograd, L.P.L., Santos, C.O., Romeiro, L.A.S., et al., 2010. Synthesis and cytotoxicity screening of substituted isobenzofuranones designed from anacardic acids. Eur. J. Med. Chem. 45, 3480–3489.
Lyu, S.Y., Park, W.B., Choi, K.H., et al., 2001. Involvement of caspase-3 in apoptosis induced by Viscum album var coloratum agglutinin in HL-60 cells. Biosci. Biotech. Biochem. 65, 534–541.
McGahon, A.J., Martin, S.M., Bissonnette, R.P., et al., 1995. The end of (cell) line: methods for the study of apoptosis in vitro. Methods Cell Biol. 46, 153–185.
Miyoshi, N., Koyama, Y., Katsuno, Y., et al., 2001. Apoptosis induction associated with cell cycle dysregulation by rice bran agglutinin. J. Biochem. 130, 799–805.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Imunol. Methods 65, 55–63.
Nakano, H., Nakajima, A., Sakon-Komazawa, S., Piao, J.H., Xue, X., Okumura, K., 2006. Reactive oxygen species mediate crosstalk between NF-κB and JNK. Cell Death Differ. 13, 730–737.
Oliveira, C., Nicolau, A., Teixeira, J.A., et al., 2011. Cytotoxic effects of native and recombinant frutalin, a plant galactose-binding lectin, on Hela cervical cancer cells. J. Biomed. Biotechnol. 2011, 1–9.
Pera, F., Mattias, P., Detzer, K., 1977. Methods for determining the proliferation kinetics of cells by means of 5-bromodeoxyuridine. Cell Tissue Kinet. 10, 255–264.
Peumans, W.J., Van Damme, E.J., 1996. Prevalence, biological activity and genetic manipulation of lectins in foods. Trends Food Sci. Technol. 7, 132–138.
Pryme, I.F., Dale, T.M., Tilrem, P., 2007. Oral mistletoe lectins: a case for their use in cancer therapy. Cancer ther. 5, 287–300.
Ramos, M.V., Cavada, B.S., Mazard, A.M., et al., 2002. Interaction of Diocleinae lectins with glycoproteins based in surface plasmon resonance. Mem. Inst. Oswaldo Cruz 97, 275–279.
Ravidran, J., Gupta, N., Agrawal, M., Bhaskar, B., Rao, P.V.L., 2011. Modulation of ROS/MAPK signaling pathways by okadaic acid leads to cell death via, mitochondrial mediated caspase-dependent mechanism. Apoptosis 16, 145–161.
Sanz-Aparicio, J., Hermoso, J., Grangeiro, T.B., et al., 1997. The crystal structure of *Canavalia brasiliensis* lectin suggests a correlation between its quaternary conformation and its distinct biological properties from Concanavalin A. FEBS Lett. 405, 114–118.
Suen, Y.K., Fung, K.P., Choy, Y.M., et al., 2000. Concanavalin A induced apoptosis in murine macrophage PU5-1.8 cells through clustering of mitochondria and release of cytochrome c. Apoptosis 5, 369–377.
Seifert, G., Jesse, P., Laengler, A., et al., 2008. Molecular mechanisms of mistetloe plant extract-induced apoptosis in acute lymphoblastic leukemia in vivo and in vitro. Cancer Lett. 264, 218–228.

Singh, N.P., McCoy, M.T., Tice, R.R., et al., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191.
Tice, R.R., Agurell, E., Anderson, D., et al., 2000. Single cell gel/Comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Env. Mol. Mutagen. 35, 206–221.
Trachootham, D., Alexandre, J., Huang, P., 2009. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat. Rev. Drug Discov. 8, 579–591.
Zhang, G., Sun, J., Wang, H., Ng, T.B., 2010. First isolation and characterization of a novel lectin with potent antitumor activity from a Russula mushroom. Phytomedicine 17, 775–781.