

## Grandisin induces apoptosis in leukemic K562 cells

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In this study, the potential antileukemic activity of grandisin, a lignan extracted from *Piper solmsianum*, was evaluated against the leukemic line K562. The cytotoxicity of grandisin (0.018 to 2.365  $\mu$ M) was evaluated in K562 and normal peripheral blood lymphocytes by Trypan Blue Exclusion and MTT methods after 48h exposure to the drug. In both methods, cellular viability was concentration-dependent and the IC<sub>50</sub> values were lower than 0.85 $\mu$ M. Analysis of K562 cells after treatment with grandisin showed that the cell cycle was arrested in the G1 phase with a 12.31% increase, while both S and G2 phases decreased. Morphological studies conducted after the exposure of K562 to grandisin revealed changes consistent with the apoptosis process, which was confirmed by annexin V stain and caspase activation. Thus, lignan grandisin showed antileukemic activities against the K562 cell line and the cell death process occurred via apoptosis.

**Uniterms:** Grandisin/antileukemic activities. Leukemic. K562. Apoptosis.

### INTRODUCTION

Chronic myeloid leukemia (CML) is a clonal myeloproliferative syndrome characterized by the presence of the Philadelphia chromosome - Ph (Faderl *et al.*, 1999). The Ph chromosome contains the chimeric gene *BCR-ABL*, which produces a tyrosine kinase. Uncontrolled BCR-ABL tyrosine kinase activity is a *conditio sine qua non* for the protein's skill to transform cells (Eiring *et al.*, 2011). The expression of BCR-ABL also contributes to apoptosis resistance after the withdrawal of growth factors, oxidative stress and DNA damage. The ability to escape normal cell death programs provides a remarkable proliferative advantage for tumoral cells, especially during the accelerated or blast phase of the disease and hinders successful treatment since it is well-known that CML cells are highly resistant to apoptosis induced by chemotherapeutic drugs (Ren, 2005; Fernandez-Luna, 2000; O'Hare *et al.*, 2012; Jabbour, Kantarjian, 2014).

Apoptosis, a type of programmed cell death which occurs in physiological and pathological conditions, is a prime target in designing therapeutic strategies to induce cancer cell death. This process shows typical cellular morphologic changes such as rounding-up of the cell, chromatin condensation, nuclear fragmentation, plasma membrane blebbing and engulfment by resident phagocytes (*in vivo*) (Ziegler *et al.*, 2004; Kroemer *et al.*, 2009). Moreover, many coordinated pathways with a wide range of proteins and enzymes are involved in this process. Caspase activation and translocation of phosphatidylserine from the inner leaflet of the cytoplasmic membrane to the outer leaflet are common events which occur in apoptotic cells. In addition, the Bcl-2 protein family plays a pivotal role in the regulation of apoptosis (Wong, 2011; Fleischer *et al.*, 2006).

Currently, the most promising treatment for CML is TKI (tyrosine kinase inhibitors)-based therapy (O'Hare *et al.*, 2012). The gold standard of care in CML is imatinib mesylate which inhibits BCR-ABL activities and induces apoptosis in tumoral cells. In recent years, however, a second and third generation of TKIs have been introduced in therapeutics especially due to imatinib drug resistance

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and toxicity. Despite the benefits of TKI therapy, the persistence of minimal residual disease (MRD) and the presence of active disease even with BCR-ABL1 inhibition, the development of new target drugs against CML is necessary (O'Hare *et al.*, 2012; Piccaluga *et al.*, 2012). In this context, natural compounds have been investigated as potential sources for new chemical entities and in order to develop drugs against various pharmacological targets, including leukemias (De Martino *et al.*, 2011).

The tetrahydrofuran lignan grandisin, isolated from *Virola* and *Piper* species, presents antimalarial and trypanocidal activities, as well as having antitumoral properties. Furthermore, grandisin CYP450-mediated biotransformation obeys Michaelis–Menten kinetics and its metabolism results in the formation of four demethylated metabolites (Zhang *et al.*, 2001; Valadares *et al.*, 2009; Barth *et al.*, 2015). Grandisin antitumor properties were investigated by our group both in *in vitro* and *in vivo* assays using the Ehrlich Ascites Tumoral (EAT) model. The treatment of EAT-bearing mice with grandisin (2.5, 5.0 or 10.0 mg/kg) for 10 days, significantly increased the survival of the animals, in a dose-dependent manner. At the same time, a 66% reduction in intraperitoneum tumor cell burden in the animals treated with 10mg/kg of grandisin was observed. Furthermore, the marked increase in vascular endothelial growth factor (VEGF) levels induced by EAT development in these animals was also significantly reduced after treatment with grandisin, and resulted in a 32% reduction of VEGF levels, when compared to the control. Finally, caspase activation (caspase-3, -6, -8 and -9) was detected after EAT cells were exposed to grandisin (Valadares *et al.*, 2009). On-going studies performed by our group demonstrated that this lignan has a protective effect against cyclophosphamide-induced mutagenicity and this effect could be associated to grandisin bioactivation (Valadares *et al.*, 2011).

In the light of this, the present study was designed to explore the potential antileukemic properties of grandisin on K562 cells (a human erythroleukemia cell line) and normal lymphocytes. Furthermore, the effects of this potential drug on the cell cycle and death mechanism were also investigated by flow cytometry with caspase activation.

## MATERIAL AND METHODS

### Grandisin

Grandisin was isolated from the extract obtained from the leaves of *Piper solmsianum* previously described (Martins *et al.*, 2003).

### Grandisin nanoemulsion

Grandisin was prepared as a nanoemulsion disperse system in order to obtain a formulation with an aqueous external phase as previously described (Valadares *et al.*, 2009).

Briefly, the dispersion was prepared as follows: first, a mixture of 50 µg of soy phosphatidylcholine (PC), sunflower oil (4:1 mol/mol PC), and grandisin (10 mg/mL) were dissolved in 1.0 mL of chloroform. The mixture was dried under a nitrogen atmosphere and kept under vacuum overnight to ensure the complete removal of chloroform. Then 1 mL of water was added to the dried lipid film to promote the hydration of the lipid-drug mixture, which after a 2 min vortex mixing, resulted in a coarse O/W emulsion. After 1 hour, the mixture was sonicated for 10 min in a Titanium probe sonicator to obtain a homogeneous submicrometric emulsion. For the control group, the nanoemulsion was prepared without adding grandisin to the oil phase.

### Cell lines and culture

K562 erythroleukemia cell line, obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA), was cultured in suspensions in RPMI 1640 medium (Sigma Chemical Co.) supplemented with 10% fetal calf serum (FCS, Sigma Chemical Co.), 100 U/mL of penicillin, and 100 µg/mL of streptomycin in a humidified atmosphere at 37 °C in 5% CO<sub>2</sub>. Cells were seeded (1 x 10<sup>6</sup> cells/mL) in 96-well microtiter plates and incubated with different concentrations of grandisin for 48 hours (Queiroz *et al.*, 2009). The human blood used in the assays was collected from healthy volunteers who had signed a consent form.

### Lymphocyte culture

Peripheral human blood, obtained by venipuncture from healthy adult donors, was diluted with an equal volume of RPMI 1640 medium, then layered over Ficoll-Hypaque density gradient separation solution (1.077g/mL), and centrifuged at 400g for 20 min at room temperature. The mononuclear cell layer was removed, washed twice in RPMI 1640 medium and resuspended in RPMI 1640 medium supplemented with 2 mM glutamine (Sigma Chemical Co.), antibiotics and 10% FCS. The cells were then cultured with 10 µg/mL of phytohemagglutinin. Lymphocytes at a density of 1 x 10<sup>6</sup> cells/mL were seeded in 96-well microtiter plates, in the presence and absence of grandisin, for 48 hours in a humidified atmosphere at 37 °C in 5% CO<sub>2</sub> (Queiroz *et al.*, 2009).

## Cell proliferation and viability assays

Cell proliferation was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co.) reduction test, performed as previously described by Mosmann (1983). Briefly,  $1 \times 10^6$  cells/mL in RPMI 1640 medium supplemented with 10% FBS were seeded in a 96-well, flat-bottom plate (TPP, Trasadingin, Swiss) and treated with different concentrations of grandisin (0.018 – 2.365  $\mu$ M) for 48 hours. After incubation, 10  $\mu$ L/well MTT (5mg/mL) were added and the plate was incubated again for 4 hours. It was then centrifuged at 800 rpm for 10 min and the supernatant removed. A total of 100  $\mu$ L of dimethylsulphoxide (DMSO) was added to each well to solubilize the formazan crystals. Absorbance was measured at 545nm using a spectrophotometer (Stat Fax 2100, Awareness Technology, Dusseldorf, Germany).

Trypan blue dye exclusion assay was used to assess cell viability as previously described (Bromberg *et al.*, 2005). The cells were seeded in a 96-well, flat-bottom plate (TPP, Trasadingin, Swiss), at  $1 \times 10^6$  cells/mL in RPMI 1640 medium supplemented with 10% FBS and treated with different concentrations of grandisin (0.018 – 2.365  $\mu$ M) for 48 hours. An aliquot of the cell suspension was then mixed with trypan blue solution (0.2% in phosphate-buffered saline-PBS) (1:10) and the viability of the cells was estimated using a hemocytometer (Reichert, USA). Cells which incorporated the dye and turned blue were reported as dead. Each concentration was tested in three independent experiments run in four replicates. Proliferation and viability of cells exposed to grandisin were expressed as a percentage of the cell proliferation or viability of untreated control cells (100%).

## Methods for detection of cell death by apoptosis

### Apoptotic index

K562 cells were incubated in a humid chamber at 37 °C, 5% CO<sub>2</sub>, with grandisin 0.036  $\mu$ M for 24 h. After this period, cells were stained with hematoxylin and analysed at 40X magnification. The number of apoptotic cells was expressed as a percentage of the total number of cells. The Apoptotic index was determined after a minimum of 100 cells were counted. Cells showing morphological characteristics of apoptosis such as marginalization or chromatin condensation, cellular or nuclear fragmentation, and cellular structures fragmented into vesicles (apoptotic bodies) were considered positive.

## Annexin V-FITC/PI double-staining and analysis by flow cytometry

After incubation with grandisin as described above, K562 cells were harvested, washed with cold PBS and resuspended in binding buffer at a concentration of  $1 \times 10^6$  cells/mL, according to the manufacturer's instructions (eBioscience, USA). The suspensions were transferred to 5 mL tubes, and 5  $\mu$ L Annexin V-FITC and 5  $\mu$ g/mL PI were added. The cells were incubated at room temperature for 20 min, after which 300  $\mu$ L binding buffer were added and analysis was performed in a FACSCanto II flow cytometer using the CellQuest software (10.000 events were collected per sample). Control cells were treated with medium only.

## Cell cycle

For analysis of the cell cycle,  $5 \times 10^5$  cells were incubated with or without grandisin 0.036  $\mu$ M for 24 h in RPMI 1640 medium and 10% fetal calf serum, in an incubator with 5% CO<sub>2</sub> at 37 °C. After that, the cells were washed twice with 1 mL of cold PBS, and centrifuged at 1500 rpm for 10 min. The cell button was resuspended with 1 mL of fixative solution (previously prepared 70% ethanol) and incubated at 4 °C overnight. Cells were then washed in cold PBS and resuspended in 1 mL of 200  $\mu$ g/mL RNase A and 50  $\mu$ g/mL propidium iodide, and maintained at 4 °C, protected from the light, for 2 h. Samples were analyzed by flow cytometry (FACSCanto II, Becton Dickinson). Results were presented in graphs representing the number of cells versus DNA content indicated by fluorescence intensity.

## Caspase activity

Direct measurements of caspase activity were performed using colorimetric protease kits (R&D Systems, USA), according to the manufacturer's recommendations, after the incubation of cells with grandisin (0.036  $\mu$ M/mL) for 24 h. The caspase activity assay is based on the spectrophotometric detection of the *p*-nitroanilide (pNA) chromophore after cleavage from the substrates X-*p*NA, where X stands for amino acid sequences recognized by specific caspase-6 (VEIDI-*p*NA), caspase-8 (IETD-*p*NA) and caspase-9 (LEHD-*p*NA). According to the procedure,  $2 \times 10^6$  K562 cells were pelleted by centrifugation and lysed on ice. The protein concentration in the lysate was measured using a Bio-Rad Protein assay (Bio-Rad Laboratories Inc., USA). This assay is based on the Bradford method in which 200  $\mu$ g of protein

were incubated with each X-*p*NA substrate (200  $\mu$ M final concentration) at 37 °C in a microtiter plate. The optical density of samples was measured at 405nm. After subtraction of the blank, the increase in caspase activity was determined by comparing these results with the levels of the control.

## Statistics

Cytotoxicity evaluation was performed in three independent experiments. Results were converted into percentage of control. IC<sub>50</sub> (concentration which produces an inhibitory effect of 50% of the evaluated parameter) was obtained graphically from the concentration-response curve. Results were expressed as mean  $\pm$  S.D. of four replicates. For the parameters ‘apoptotic index’ and ‘caspase activity’, statistical analysis was performed using the nonparametric Mann-Whitney test to compare the treated groups with controls and the Kruskal-Wallis test to compare the treated groups with each other. Statistical significance was considered when  $p < 0.05$ .

## RESULTS AND DISCUSSION

### Effects of grandisin on cell proliferation and viability of K562 cells and normal lymphocytes.

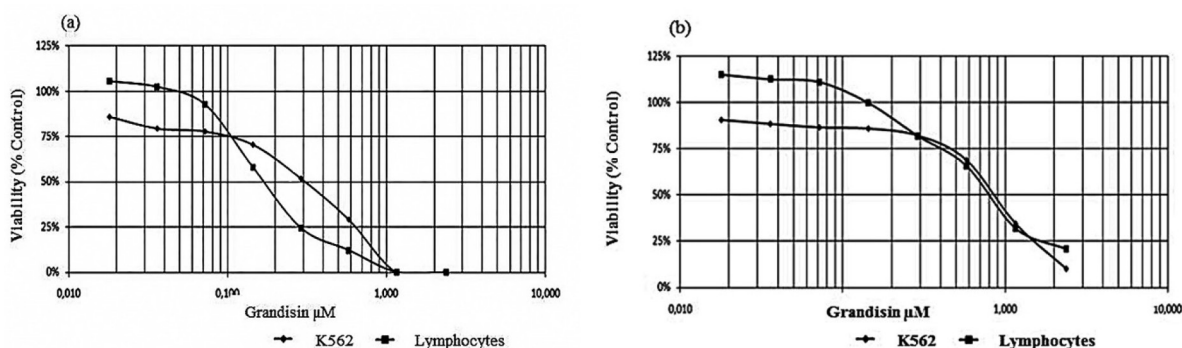
The effect of grandisin on the growth and viability of K562 cells and lymphocytes was examined, using the Trypan blue exclusion method, after 48 hours of exposure in culture. The proliferation of K562 cells and lymphocytes was inhibited in a concentration-dependent manner in response to increased concentrations of grandisin (0.018 to 2.365  $\mu$ M) (Figure 1a). The 50%

inhibition (IC<sub>50</sub>) was obtained with a concentration of 0.851  $\mu$ M for K562 and 0.685  $\mu$ M for lymphocytes after 48 hours of exposure (Figure 1a). On the other hand, results obtained by the MTT method showed that the 50% inhibition (IC<sub>50</sub>) values were 0.198  $\mu$ M for K562 and 0.200  $\mu$ M for lymphocytes (Figure 1b).

Thus, our data demonstrated that grandisin was more cytotoxic for lymphocytes than for K562 cells in the Trypan blue assay. However, in the MTT assay the cytotoxicity of grandisin was quite similar for both types of cells. Although grandisin proved to be cytotoxic to K562 cells, it was also toxic for lymphocytes in the same range of concentrations tested. These results are important because they demonstrate that even though grandisin exerts anti-leukemic activity, it may act similarly to many other chemotherapeutic agents exhibiting toxicity to normal cells and triggering undesirable side effects, which thus limits its application in the clinical field (Bhatt, Saleem, 2004). However, the lymphotoxicity of grandisin opens up new treatment fields, such as the immunosuppressive agent similar to PKC412 (CGP41251), a protein kinase inhibitor which has antitumoral and immunosuppressive activities (Ganeshaguru *et al.*, 2002; Miyatake *et al.*, 2007; Kawamoto *et al.*, 2008).

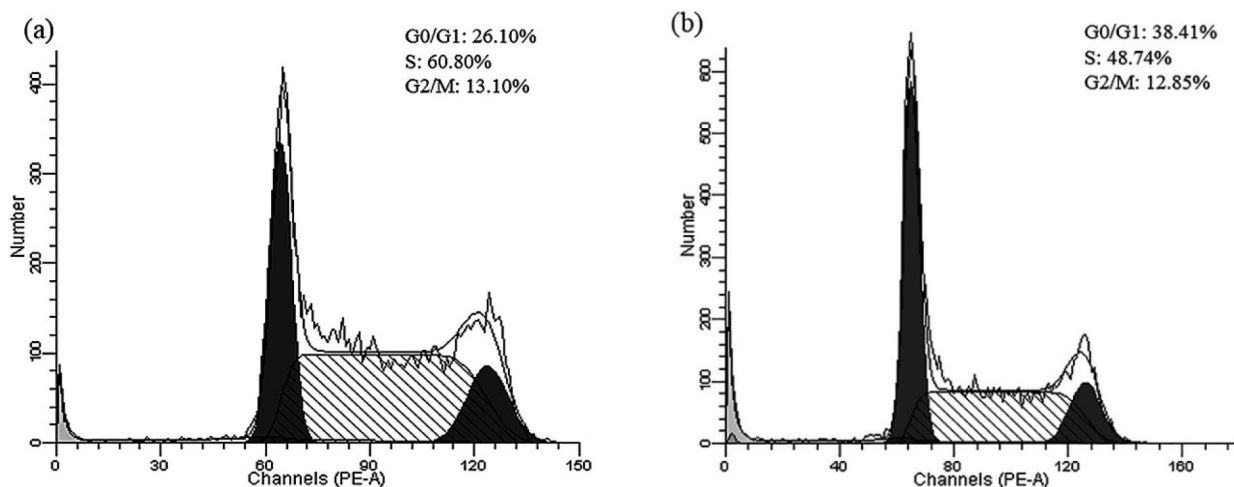
### Cell cycle analysis by flow cytometry

Results from the experiments designed to evaluate whether grandisin interferes with the cell cycle showed that it caused a significant G1 phase arrest with an increase of 12.31% in the population of cells and a decrease of 12.06% and 0.25% in the population of cells in the S and G2 phases, respectively, when compared with the control (Figures 2a and 2b).



**FIGURE 1** - Cytotoxicity in K562 cells and normal peripheral blood lymphocytes ( $1 \times 10^6$  cells/mL) treated with different concentrations of grandisin (0.018 – 2.365  $\mu$ M) for 48 hours. (a) Cell viability was determined by the Trypan blue exclusion method. (b) Cell proliferation was assessed by the MTT reduction test. In the absence of compound, the viability was considered as 100%. Results represent the mean  $\pm$  SD of three experiments run in four replicates ( $p < 0.05$  compared with control cells).





**FIGURE 2** - (a) Analysis of the cell cycle of K562 cells (control) by Flow Cytometry. (b) Analysis of the cell cycle of K562 cells after treatment with grandisin 0.036 μM for 24 hours.

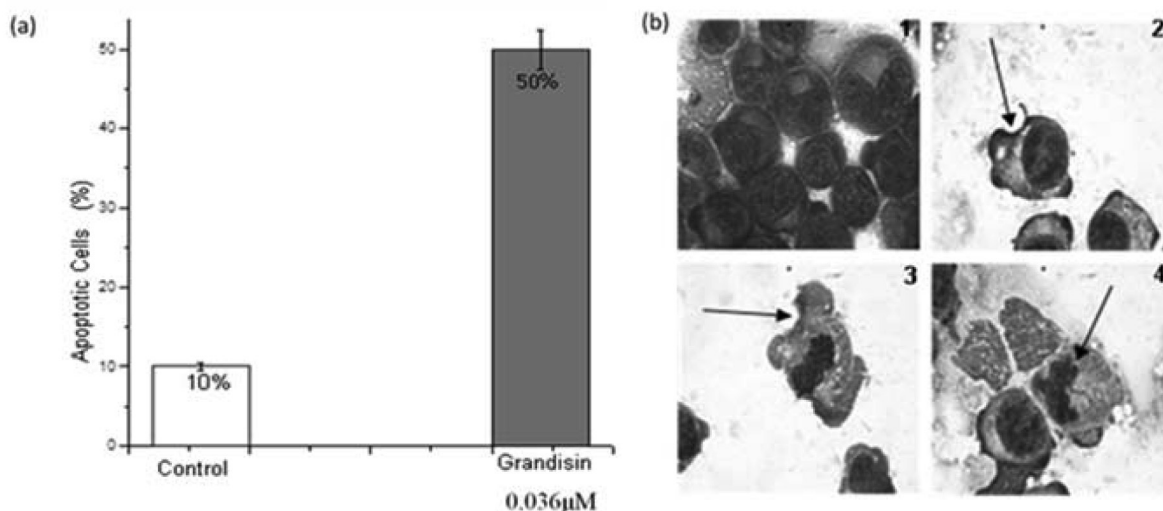
This analysis demonstrated that there was an increase in G1 population and a decline in cell population in the S and G2 phases which indicates that grandisin can interfere with cellular proliferation and with the dynamics of the cell cycle.

### Apoptosis induction by grandisin in K562 cells

#### Apoptosis index

Once the ability of grandisin to induce the death of leukemic cells was detected, we studied the sequence of mechanisms involved in cell death, especially the

apoptotic mechanisms. To understand the mechanism by which grandisin promotes loss of viability in K562 cells, a number of apoptosis related experiments were performed. The effects of grandisin on the morphology of K562 cells were examined after 24 hours of exposure in culture (Figures 3a and b). Results showed a 4-fold increase in the number of cells undergoing apoptosis when compared to the control. As indicated in the photomicrographs, the cells showed morphological characteristics of death by apoptosis such as condensed chromatin, vacuoles, and fragmentation of the DNA and of the cellular membrane. A 40% increase in the number



**FIGURE 3** - (a) Apoptosis rate of K562 cells exposed to grandisin 0.036 μM for 24 hours. The rate of apoptosis was determined by counting 100 cells, each sample was duplicated and the number of apoptotic cells was expressed as a percentage of the total number of cells. (b) (2, 3 and 4) Morphological characteristics of apoptosis induced K562 cells treated with grandisin 0.036 μM for 24 hours, determined by hematoxylin coloration. Arrows indicate vacuoles, membrane fragmentation and condensed chromatin, respectively. (b) (1) Morphological characteristics of untreated cells.

of cells undergoing apoptosis was also observed when compared with the control.

### Annexin V-FITC/PI double-staining and analysis by flow cytometry

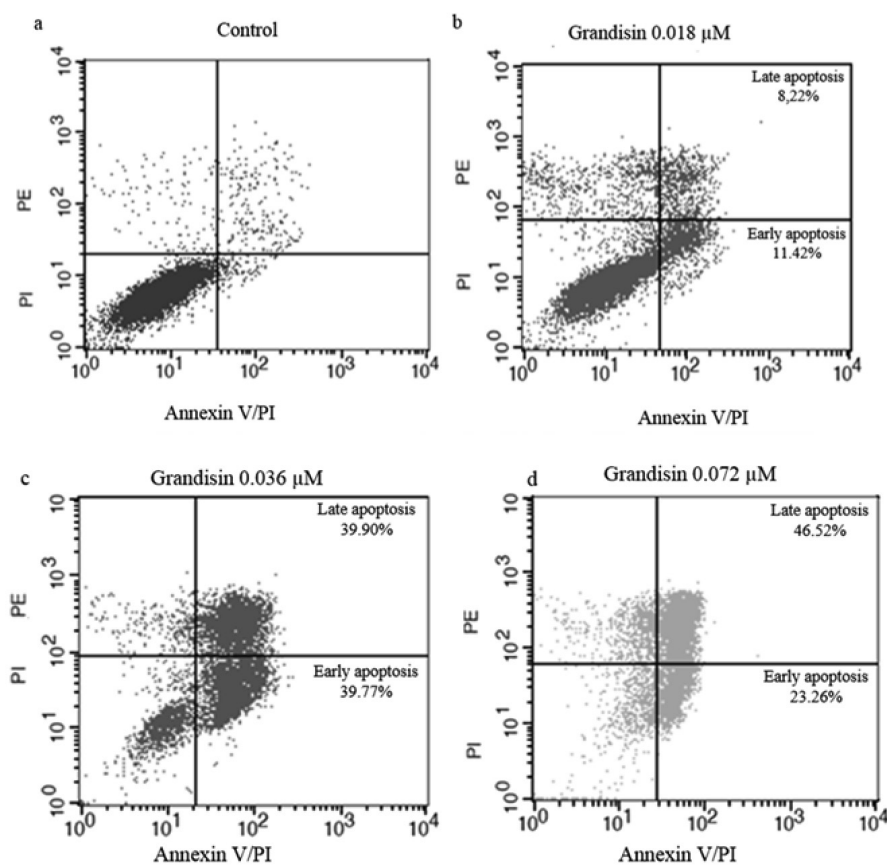
Once the antitumoral properties of grandisin were determined, we focused on the mechanisms involved in this process. Since morphological changes in K562 after treatment pointed to death by apoptosis, flow cytometry analysis with Annexin V/PI stain was conducted. The exposure to grandisin was performed in three concentrations (0.018, 0.036 and 0.072  $\mu\text{M}$ ) for 24 hours (Figure 4). In the concentration of 0.018  $\mu\text{M}$ , the increase in cells in early and late apoptosis was 11.42% and 8.22%, respectively. In the intermediate concentration (0.036  $\mu\text{M}$ ), the percentage of cells in early apoptosis was 39.77% and 39.90% in late apoptosis. Finally, in 0.072  $\mu\text{M}$  the increase in early and late apoptosis was 23.26% and 46.52%, respectively. The results confirmed that the death mechanism triggered by grandisin was apoptosis.

### Caspase activity

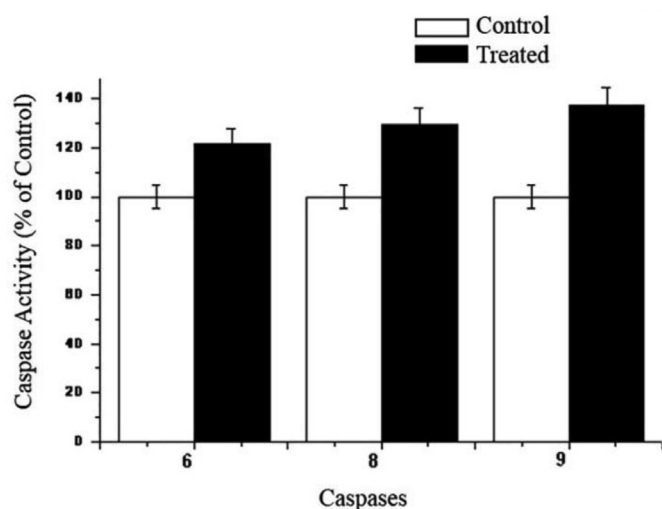
A molecular hallmark of apoptosis is the activation of caspases – specific proteases which bring about cell death through cleavage of multiple protein substrates such as nuclear and cytoskeletal proteins (Bao; Shi, 2007; Elmore, 2007). In this context, caspase-6, -8 and -9 activities were analyzed by colorimetric assays. All caspases analyzed showed increases in activities of 21.4%, 29% and 37% for caspases-6, -8 and -9, respectively (Figure 5).

Our results showed an increase in all caspase activities tested after exposure of K562 to grandisin which suggests that intrinsic and extrinsic cascades were activated. Similar results were observed with apigenin in human breast cancer cells and with emodin in HeLa cells (Choi, Kim, 2009; Yaoxian *et al.*, 2013).

Despite the many advances in cancer research, most antitumor compounds produce undesirable side effects. Thus, significant effort has been invested in finding new phytochemicals with antitumoral properties. Throughout history, natural compounds have been used for the treatment of cancer due to their safety, low toxicity,



**FIGURE 4** - Analysis of K562 cells treated with grandisin ( $1 \times 10^6$  cells/mL) for 24 hours. The cells were stained with Annexin V-FITC and propidium iodide. Dot plot of untreated (a) and treated (b, c and d). (b) K562 cells treated with 0.072  $\mu\text{M}$  of grandisin. (c) K562 cells treated with 0.036  $\mu\text{M}$  of grandisin. (d) K562 cells treated with 0.018  $\mu\text{M}$  of grandisin.



**FIGURE 5** - Caspase activities in K562 ( $2 \times 10^6$  cells/mL) cells after 24 hours of treatment with grandisin  $0.036 \mu\text{M}$ . Colorimetric assay was performed to determine caspase-6, -8 and -9 activities. The enzyme activities were calculated in  $\text{pmol min}^{-1} (\text{mg protein})^{-1}$  (extinction coefficient of pNA was  $10.000 \text{ M}^{-1} \text{ cm}^{-1}$ ) and expressed relative to controls. In the absence of grandisin the activity was considered to be 100%. Error bars represent standard deviation.

and availability from natural sources (Pratheeshkumar *et al.*, 2012). Grandisin is a lignan extracted from *Piper solmsianum* which presents antitumoral properties as well as antimalarial and trypanocidal activities (Martins *et al.*, 2003; Bernardes *et al.*, 2006; Valadares *et al.*, 2011). Despite its lymphocytic activities, grandisin did not exert mutagenic effects on the bone marrow cells of exposed mice. Moreover, grandisin proved to be a potent antiangiogenic compound due to the prolonged survival of mice bearing breast carcinoma (Valadares *et al.*, 2011). Other pharmacological properties, such as antinociceptive, anti-inflammatory and antioxidant activities, have also been attributed to grandisin, which thereby shows the therapeutic versatility of this compound (Carvalho *et al.*, 2010).

Thus, this study introduces a new agent capable of inducing apoptosis in a leukemic cell line which presents distinct features of resistance to many drugs. But it is important to highlight that further studies should be conducted to ensure the safety and efficacy of this substance and elucidate more clearly the mechanism which triggers apoptosis.

## CONCLUSIONS

The present study suggests that the lignin grandisin has antileukemic activities against the K562 cell line and

that the cell death process occurs via apoptosis. Grandisin also showed immunosuppressant activities indicating new potential uses for immunological pathologies.

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