



## *In vitro* Study of Anti-Leukemic Potential of Ursolic Acid in Jurkat Cell Line

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### Abstract

This study aims to assess the possible antitumor effects of the Ursolic Acid through cell death studies. Hence, Jurkat cell lines related to leukemia were subjected to treatment with different concentrations of the Ursolic Acid in order to identify their likely mechanism of death. After completion of cell viability tests, we suggest that this acid used in this study have antiproliferative/cytotoxic activity in a dose-dependent manner. The results obtained indicate that the Ursolic Acid assessed in this study have cytotoxic activity with IC50% value of 10  $\mu$ M. In addition, we observed that the IC50% value tested on the same cell line revealed a significant percentage of stagnant cells in cell cycle sub-G1 phase, a finding that allows us to infer the cytotoxic ability of the acid assessed.

### Keywords

Ursolic acid; Cytotoxicity; Jurkat cell line

### Introduction

Acute myeloid leukemia (AML) is characterized by the rapid proliferation of myeloblasts, precursor cells of the myeloid lineage, that do not differ and thus does not perform its physiological functions and accumulate in the bone marrow. It is the most common type of leukemia; accounting for about 90% of all acute leukemia's in adults and is rare in children and slightly more common in males. Although AML is a relatively rare disease, accounting for about 1.2% of deaths caused by oncological diseases in the United States, it is expected to increase its incidence due to increased survival of the population. One of the remarkable features of this disease is its rapid progression, and may be fatal in a short time when receiving no treatment or with inadequate treatment. The chemotherapy of choice for this disease includes cytosine arabinoside associated with an anthracycline (idarubicin or daunorubicin). These drugs are used both in remission induction therapy and consolidation therapy. In more specific cases, such as disease recurrence, only high doses of Ara-C are used. Even with the therapy described above, most patients relapse in a short period of time (12-18 months), because many factors contribute to treatment failure and low percentage of cure of AML, among these are the heterogeneity of the disease, the involvement of chromosome rearrangements, the resistance to Ara-C and anthracyclines, and the peak onset in adulthood. According to Appelbaum, white blood

cell count above 20,000 is a major contributor to the non-sustained remission of AML. Moreover, it is noteworthy that the systemic toxic effects of drug therapy usually result in myelosuppression with increased risk of infection, thus contributing to the low percentage (about 20-30%) of AML patients who respond well to induction therapy, achieving a disease-free survival for more than five years. The ursolic acid is a pentacyclic triterpene acid present in several fruits, especially in the peel of pears, plums, apples and pomegranate, being also found in medicinal plants like *Jacaranda decurrens* [1], *Eugenia brasiliensis* [2] and *Eriobotrya japonica* [3]. Previous studies show that ursolic acid has several pharmacological properties, such as antiviral, antioxidant, hepatoprotective, anti-tumor, anti-angiogenesis and anti-metastatic activity [6]. Studies have correlated the presence of ursolic acid to the purifying and healing activity of some medicinal plants such as *Jacaranda decurrens* Cham [4]. Studies also show that ursolic acid has regenerative, antiaging and anti-inflammatory properties, which makes it effective in cosmetic applications [5-11]. In addition to stimulating the formation of collagen in fibroblasts, it increases the production of ceramides in human skin by keratinocytes stimuli, and thus has anti-invasive and anti-metastatic activity [12-14]. Other studies show that the ursolic acid is capable of inducing apoptosis in several types of tumor cells, such as colon cancer [15], breast cancer [16], leukemia [17], lung cancer [18], melanoma and prostate cancer cells [19]. However, the pathway responsible for ursolic acid mediated apoptosis needs to be better understood. Furthermore, it is suggested that ursolic acid may be a non-toxic chemopreventive agent in clinical practice with few side effects [20-22]. Other studies show that the ursolic acid is capable of inducing apoptosis in several types of tumor cells, such as colon cancer [15], breast cancer [16], leukemia [17], lung cancer [18], melanoma and prostate cancer cells [19]. However, the pathway responsible for ursolic acid mediated apoptosis needs to be better understood. Furthermore, it is suggested that ursolic acid may be a non-toxic chemo preventive agent in clinical practice with few side effects [20-22].

### Objectives

#### General objective

To assess the possible anti-tumor effects of ursolic acid through cytotoxicity studies and to evaluate cell death pathways and modalities using the cell line Jurkat.

#### Specific objectives

To assess the ursolic acid cytotoxicity through the exclusion techniques by Trypan Blue and reduction of MTT to formazan crystals.

To quantify the fraction of cells population in cell cycle sub-G1 phase by incorporation of propidium iodide technique and analyzed by flow cytometry.

### Materials and Methods

#### Chemicals and ursolic acid

These were RPMI-1640, minimum essential medium (MEM), fetal calf serum, trypsin, trypan blue, ethanol, penicillin, streptomycin,

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gentamycin, phosphate buffer saline (MERCK, Germany); distilled water, sodium hydroxide. All other chemicals used in this study were of analytical grade and were purchased locally. The Ursolic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Jurkat cell culture

Jurkat cell line was generously donated by Dr. Claudia Bincoletto Trindade, from the Instituto Nacional de Farmacologia e Biologia Molecular (INFAR-EPM-UNIFESP), The cells were cultured in RPMI medium (Gibco®, USA) supplemented with 10% foetal calf serum, 100 UI/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere at 37°C in 5% CO<sub>2</sub>.

### Cytotoxicity evaluation by MTT and Trypan Blue exclusion assays

To cell viability assessments, control and treated (24h) jurkat cells were resuspended in equal volumes of medium and trypan blue (0.05% solution) and were counted using a haemocytometer chamber. Cell viability was also measured using a standard methylthiazol-tetrazolium (MTT) assay, as previously described by Mossmann, 1983. Briefly, 5×10<sup>4</sup> viable cells were seeded into 96-well flat plates (Corning, USA) in RPMI medium supplemented with 10% foetal calf serum and incubated with different concentrations of the extracts for 24 h. Then, 10 µl of MTT (5 mg/mL/well) was added and incubated for 4 h. After that, 100 µl of sodium dodecyl sulphate (SDS) 10% solution in MiliQ H<sub>2</sub>O was added to each well to an overnight solubilisation of the formazan crystals. Absorbance was measured at 560 nm in FlexStation® 3 Multi-Mode Benchtop Reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

### Cell cycle evaluation by propidium iodide (pi) staining

For cell cycle analysis, 1×10<sup>6</sup> cells were harvested for each sample. Jurkat cells were washed in PBS, adjusted to 1×10<sup>6</sup> cells/mL and fixed in ethanol 50 % solution for 30 minutes. Fixed cells were resuspended in PBS that contained propidium iodide (PI) 50 µg/mL and RNase 100 µg/mL for 30 minutes and then analysed by flow cytometry. Data were collected (10<sup>5</sup> events) in a FACS Calibur (Becton-Dickinson, Mountain View, CA, U.S.A.) and were acquired with Cell Quest software (Becton-Dickinson). Acquired data was analysed using Flow Jo V<sub>x</sub> 0.7 (Flow Jo enterprise).

### Statistical analysis

The results were expressed as the mean ± SEM of at least three independent experiments, unless stated otherwise. Paired data were evaluated by Student's t-test. A one-way ANOVA was used for multiple comparisons. A value of p<0.05 was considered significant.

### Results

After conducting the initial tests (cell viability by trypan and MTT), we determined the concentration that inhibits proliferation of 50% of the viable cells in Jurkat cell cultures, thus suggesting that ursolic acid present antiproliferative/cytotoxic activity in a dose-dependent manner. The dose-response curve was determined using different concentrations of ursolic acid and the inhibitory concentrations of 50% (IC<sub>50</sub>) for each test are shown in Figures 1 and 2. The value of 10 µM was considered the concentration corresponding to IC<sub>50</sub>. Finally, with the help of flow cytometry, we quantified the fraction of cells in cell cycle sub-G1 phase, indicative of a group of cells in death pathway. After completion of MTT and trypan blue tests, the

IC<sub>50</sub> was determined as being 10 µM of ursolic acid, and then the propidium iodide incorporation technique was performed. Thus, Figure 3 shows the percentage of cells in cell cycle sub-G1 phase.

### Discussion

In recent years, the use of natural products has become widely accepted as a realistic option for the treatment of malignant tumors. In this context, new candidates for herbal antitumor compounds (Figure 4), represent attractive alternatives for the development of new drugs. The ursolic acid, an active pentacyclic triterpene acid, was isolated from several medicinal plants, such as *Eriobotrya japonica*, *Rosmarinus officinalis*, and *Glechoma hederaceae* [3]. Irregularities in the process of cell death is a common feature for many human diseases including cancer, stroke, and neurodegeneration, situations

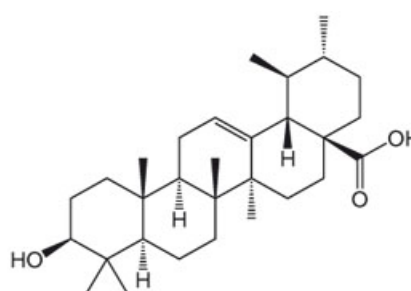


Figure 1: Chemical structure of ursolic acid.

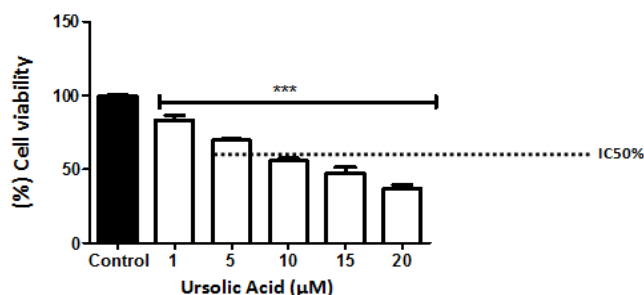


Figure 2: Cytotoxicity of ursolic acid in Jurkat leukemic cell line by trypan blue incorporation assay. Results obtained after 24 hours of treatment with ursolic acid. (\*) P<0.001 relative to the control group without treatment (ANOVA; a posteriori test of Tukey). All experiments were performed in triplicate and are represented by the mean and standard deviation.

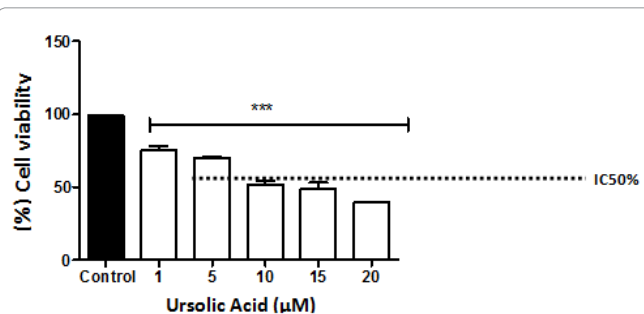
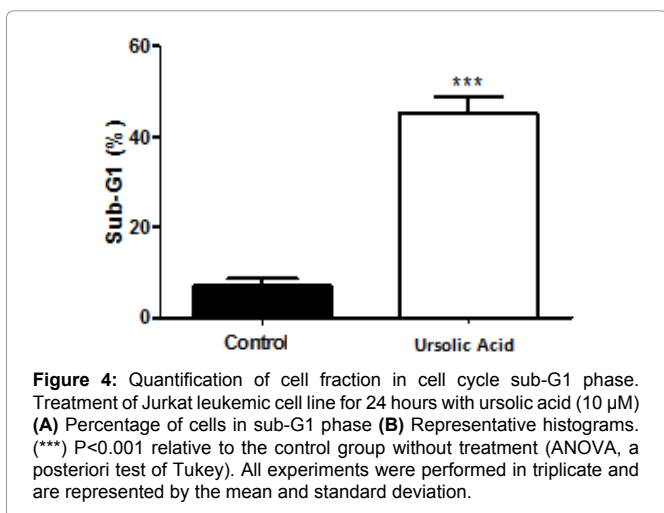


Figure 3: Cytotoxicity of ursolic acid in Jurkat leukemic cell line by MTT test. Results obtained after 24 hours of treatment with ursolic acid. (\*) P<0.001 relative to the control group without treatment (ANOVA; a posteriori test of Tukey). All experiments were performed in triplicate and are represented by the mean and standard deviation.



in which the modulation of such cell response proved to be an effective therapeutic strategy [23]. In this study, we demonstrated that ursolic acid significantly reduces the viability of Jurkat cells (lymphoid leukemia) with ursolic acid dose-response pattern, confirming other studies in the literature, which demonstrate the inhibition of human cancer cell lines proliferation such as colon cancer cells [15], breast cancer [16], leukemia [17], lung cancer [18], melanoma and prostate cancer cells [19]. Moreover, the study of the fraction in the cell cycle sub-G1 phase indicated that the concentration selected as the IC50% (10  $\mu$ M) was able to retain, in this stage, 45% of cells suggesting cell death since they are not able to start division. In summary, the results indicated that ursolic acid showed cytotoxic activity on Jurkat cell line, with concentration of 10 $\mu$ M being the one that best behaved for conducting *in vitro* studies in this work [24-26].

## Conclusion

The results indicate that ursolic acid showed cytotoxic activity with concentration of 10  $\mu$ M. We also observed that the concentration of 10  $\mu$ M was shown to be able to retain a significant percentage of cells in cell cycle sub-G1 phase, allowing us to suggest that this compound exhibits cytotoxic activity. Therefore, other tests should be performed to elucidate the mechanism of ursolic acid, and thus continue with the studies of this promising natural product in the development of new candidates for antitumor agents.

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