Pharmacogn. Res., 2023; 15(4):771-775.
https://www.phcogres.com

Original Article

Acacia nilotica Pod Extract has an Anti-cancer Effect on the U937 Cell Line

Halla Falih Bakheit[1,2,*], Sebastien Taurin[1], Elwaleed Mohamed Elamin[2], Moiz Bakhiet[1]

[1]Department of Molecular Medicine and Al-Jawhara Centre for Molecular Medicine, Genetics, and Inherited Disorders, College of Medicine and Medical Sciences, Arabian Gulf University, Manama, KINGDOM OF BAHRAIN.
[2]Department of Histopathology and Cytology, College of Medical Laboratory Sciences, Elzaiem Alazhari University, Khartoum North, SUDAN.

ABSTRACT

Background: *Acacia nilotica* (*A. nilotica*), a tree that thrives in tropical regions, has been reported to have various medicinal uses and various pharmacological properties. Potential anti-cancer effects are one of the medical properties of parts of this tree. **Objectives:** To explore and evaluate the cytotoxic effect of *A. nilotica*, we used *Acacia nilotica's* pod extract on U937 cell lines as an acute myeloid leukemia model. **Materials and Methods:** Microscopic analysis, flow cytometry, and WST-1 tests were performed on the U937 cell line after 72 hr of treatment with different concentrations of ethanol pod extract from *Acacia nilotica* (*A. nilotica*) to test for DNA cell cycle, cell viability and apoptosis, and to determine the IC_{50} of the drug. **Results:** Treatment with *A. nilotica* pod extract significantly induced cell death in U937-treated cells compared with untreated control cells. Microscopic analysis revealed morphological changes in dead and fragmented cells associated with a reduction in cell number. A reduction in cell viability with an increase of cells in the late apoptosis stage by flow cytometry indicated the cytotoxic effect of *A. nilotica* and accumulation of cells in the sub-G1 phase of the DNA cell cycle analysis. The IC_{50} was identified by the WST-1 assay. **Conclusion:** The findings of the present study suggest that *A. nilotica* may be employed as a natural anti-cancer agent in research on acute myeloid leukemia in humans.

Keywords: Flow cytometry, Cytotoxicity, Natural drugs, Apoptosis.

Correspondence:
Dr. Halla Bakheit
Department of Molecular Medicine and Al-Jawhara Centre for Molecular Medicine, Genetics, and Inherited Disorders, College of Medicine and Medical Sciences, Arabian Gulf University, Salmaniya Avenue, Building 293, Road 2904, Block 329. Manama, KINGDOM OF BAHRAIN.
Email: hallafb@agu.edu.bh.

Received: 13-04-2023;
Revised: 20-06-2023;
Accepted: 31-07-2023.

INTRODUCTION

***Acacia nilotica*:** *A. nilotica*, a tree that grows in tropical countries, belongs to the family Fabaceae of the genus *Acacia*. *A. nilotica* is a tree with diverse potential anti-cancer and anti-toxic abilities[1] and has been reported to have different medicinal uses and various pharmacological properties.[2-5] Ranging from anti-bacterial,[6,7] anti-viral, anti-oxidant,[8] anti-diabetic, anti-mutagenic, and cytotoxic activities.[9] Different chemical components were found in *A. nilotica* from different parts with potential for different anti-cancer and anti-toxic actions.[1] *A. nilotica* has a diverse variety of biological activities, making it one of the best candidates for developing novel, secure, environmentally friendly, and renewable pharmacological foundations with a broad range of therapeutic applications. Acute myeloid leukemia, or AML, is the uncontrollable growth of non-properly developed immature blood cells and is one of the most common cancer diseases that is aggressive and rabidly progressing, being one of the leading causes of human death in the world. The U937 cell line is one of the human AML cell lines. In biomedical research, the U937 cell line was isolated from histiocytic lymphoma in a 37-year-old male patient.[10] Here, we are exploring the cytotoxicity of the *Acacia nilotica* pod ethanol extract on the U937 cell line as a model for AML. Microscopic analysis defines the presence of dead cells by the reduction in cell numbers of *A. nilotica* pod extract-treated cells compared to control, untreated U937 cells. In addition, a viability and apoptosis flow cytometry experiment showed a high increase in apoptotic cells upon *A. nilotica* pod extract treatment. The WST-1 assay in the other had defined the IC_{50} as 0.23% concertation of *A. nilotica* pod extract that was required to kill 50% of U937 cells. Moreover, DNA cell cycle flow cytometry analysis showed an increase in cells in the sub-G1 zone and a decrease in cells in the G1, S, and G2/M phases. Our research established the *Acacia nilotica* pod extract's cytotoxicity as an agent with an anti-cancer effect that may aid research for the treatment of AML.

MATERIALS AND METHODS

Cells

From ATCC, U937 was obtained (No. CRL-1593.2, American Type Culture Collection, Manassas, Virginia, USA). Cells were grown in RPMI-1640 medium with 10% heat-inactivated

DOI: 10.5530/pres.15.4.081

fetal bovine serum FBS (No. 26140087, Gibco by Life Technologies Corporation, Grand Island, NY, USA) and 1% of Pencillin-streptomycin (No. 15140122, Gibco by life technologies corporation, Grand island, NY, USA), and then incubated at 37°C, 5% CO_2 incubator, and 95% humidity.

Plant extract Preparation

The *A. nilotica* pod was bought from the Khartoum market in Khartoum, Sudan. 5 g of grounded *A. nilotica* pod (dry) were added to 15 mL of 70% ethanol in DI water. Plant: solvent ratio is 1:3, then soaked for 18 hr in the shaker at 4°C. Centrifugation at 1000 rpm for 5 min and transfer of the supernatant to a new tube were done, followed by filtration through a 0.45 mm filter (100% stock solution). The diluted extract (1:10) was then filtered through a 0.22 µm filter and stored at -20°C to be used for experiments.

Reagents

The Annexin V-FITC Apoptosis Detection Kit was purchased from BD Biosciences, Franklin Lakes, NJ, USA. WST-1, 2-(4-Iodopheny)-3-(4-nitrophenyl1)-5-(2,4-disulfophenyl)-2 H-tetrazolium, monosodium salt, from BioVision company, Waltham, MA, USA.

WST-1 Assay

This method was used to screen for cytotoxicity of cells and define the IC_{50} of *A. nilotica* pod extract. U937 cells were plated in a 96-well plate at a cell density of 6000 cells/ 100 µL well. Cells were incubated for 24 hr at 37°C with 5% CO_2 and 95% humidity, then treated with a different concentration of *Acacia nilotica* pod extract [0%, 0.025%. 0.05%, 0.075%, 0.1%, 0.25%, 0.5%, 1%]. Control cells had no treatment added. Following incubation for 72 hr, the WST-1 test was performed. It is based on the reduction of the tetrazolium salt to formazan by cellular dehydrogenase. 10 µL of WST-1 assay was added, following incubation for 1 hr the test was measured at 450nm and the color produced was correlated to the cell number.

Annexin V Apoptosis detection assay

U937 cells, $5x10^5$ cells/mL per each well, were cultivated 18 hr in RPMI media without FBS, media was changed with new RPMI-1640 complete and then activated with 1.5% of *A. nilotica* pod extract. Untreated cells were used as a control. Incubation was continued for 72 hr. Cell cycle analysis, cell viability, and apoptosis tests were executed by flow cytometry. Flow cytometry annexin V apoptosis detection test kit (No. I, 556547. BD Biosciences, Franklin Lakes, NJ, USA) was used following the manufacturer's instructions. Cells were harvested after 72 hr of incubation, washed twice with cold 1X PBS, and re-suspended in 1X binding buffer (1 x 10^6 cells/mL). Cells (1 x 10^5cells) were stained with FITC Annexin V and PI stains followed by flow cytometry analysis.

DNA cell cycle Test

U937 Cells were collected, fixed, and permeabilized by cold ethanol at 70% after being activated with 1.5% of *A. nilotica* for 72 hr. Following 1X PBS washing, cells were stained with 2.5 µg/mL propidium iodide mixed with 0.5 mg/mL RNase A, and the data were collected using flow cytometry.

For cell imaging, an Olympic light microscope using DP2-BSW software using an X40 objective was used.

Statistical Analysis

All experiments were repeated at least three times. A Student *t* test was used for comparison between groups. Statistically significant data was expressed as $p \leq 0.05$.

RESULTS

Microscopic Analysis

Microscopic cell images of U937 cells after treatment with 0.25%, 0.5% and 1% of *Acacia nilotica* pod extract for cytotoxicity following 72 hr incubation featured a reduction in cell numbers associated with apoptotic and fragmented cells on *A. nilotica* extract treatment were revealed when compared with untreated cells Figure 1.

The WST-1 test for cytotoxicity

A. nilotica pod extract was used on the U937 cell line following treatment with different concentrations of *A. nilotica* pod extract: 0%, 0.025%, 0.05%, 0.075%, 0.1%, 0.25%, 0.5%, 1% or untreated cells for 72 hr. Following the manufacturer's instructions, WST-1 assay reagent was added, and measurement was done at 450 nm (Figure 2). Cell viability is reduced while increasing the concentration of *A. nilotica* pod extract compared with untreated cells. The cytotoxic activity is expressed as the IC_{50} of *A. nilotica* pod extract, which was found to be 0.23% to kill 50% of cancer cells and reflect the drug's effectiveness (OD of treated cells/OD of control) x 100 = cell viability%.

Acacia nilotica pod extract induces apoptosis in U937 cells

U937 cells were subjected to an Annexin V apoptosis flow cytometry detection test on the third day of incubation after treatment with a higher concentration of 1.5% of *Acacia nilotica* ethanol pod extract for cytotoxicity. The treatment with *Acacia nilotica* extract revealed that the majority of the cell population was in late apoptosis (Figure 3).

DNA cell cycle Analysis

Flow cytometry cell cycle analysis using the DNA stain propidium iodide to explore DNA cell cycle pattern changes after 1.5% *A. nilotica* pod extract treatment for 72 hr revealed reductions in cells in the G1, S, and G2/M phases were found to be associated

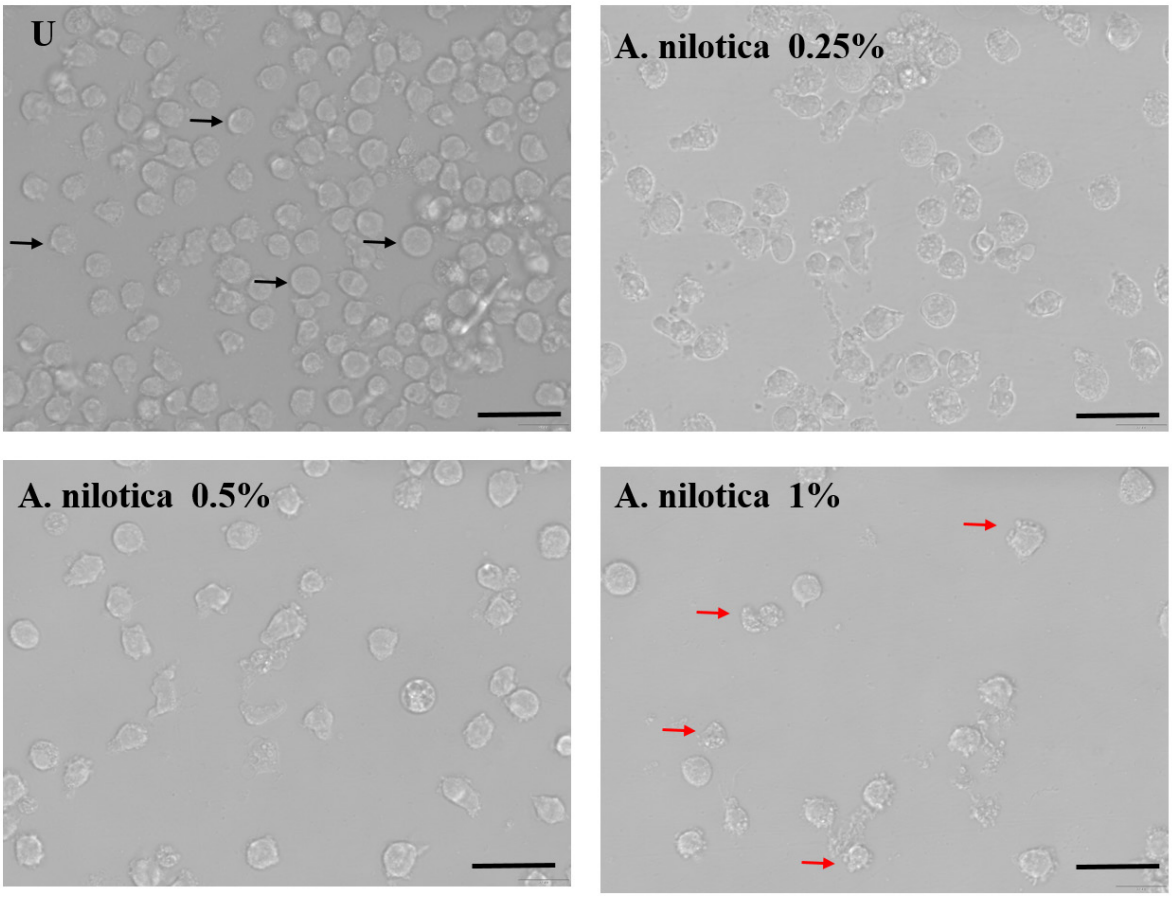

Figure 1: Microscopic Examination of U937 Cells. Morphological changes of U937 cells U: Untreated cells; *A. nilotica*: 0.25%, 0.5%, and 1% of *Acacia nilotica* pod extract treatments were done for 72 hr of incubation. Viable cells are indicated by black arrows while apoptotic cells are indicated by red arrows. Scale bar = 100 µM.

with an accumulation of cells in the sub-G1 zone upon *A. nilotica* treatment (Figure 4).

DISCUSSION

A tropical region tree, *A. nilotica*, has been found to have different medical uses, including potential anti-cancer capabilities.[11] The U937 cell line is one of the human AML cell line, acute myloid leukemia the aggressive, an uncontrollable growth of non-properly developed immature blood cell and one of the major cancer diseases in the world that requires a different approach for treatment, control, and prevention. Natural plant based medicine is an important component for treating different kinds of human diseases.[12] In this study, we tested the cytotoxicity of an *A. nilotica* pod extract on the U937 cell line as a model for AML. Diverse concertation of *A. nilotica* pod extract were tested on U937 cells line in order to define its cytotoxic effect, within 72 hr of cell treatment *A. nilotica* pod extract was found to be an effective cytotoxic agent with the indication of apoptotic cells in the microscopic examination, the identification of the IC_{50} by WST-1 as 0.23%, and accumulation of cells in late apoptosis on the PI- Annexin V Apoptosis test

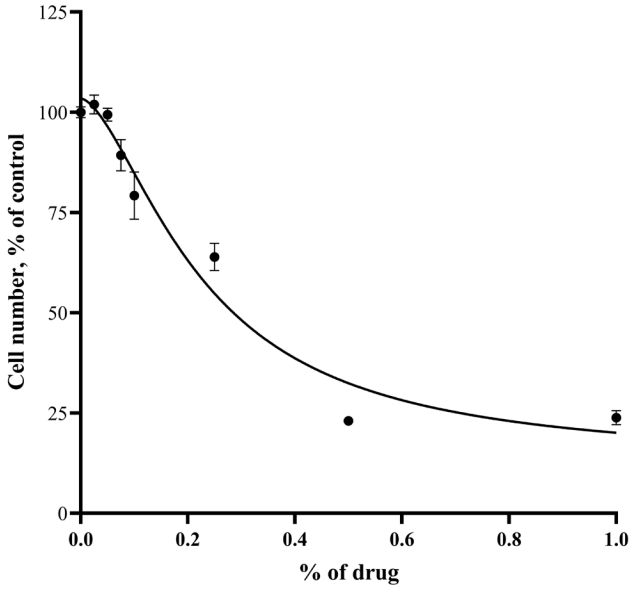

Figure 2: *A. nilotica* pod extract drug effectiveness on U937 cells by WST-1 for cytotoxicity. Illustrations of the cytotoxic activity of *A. nilotica* pod extract on U937 after 72 hr of *A. nilotica* pod extract treatments. Each point represents the average standard deviation of six replicates.

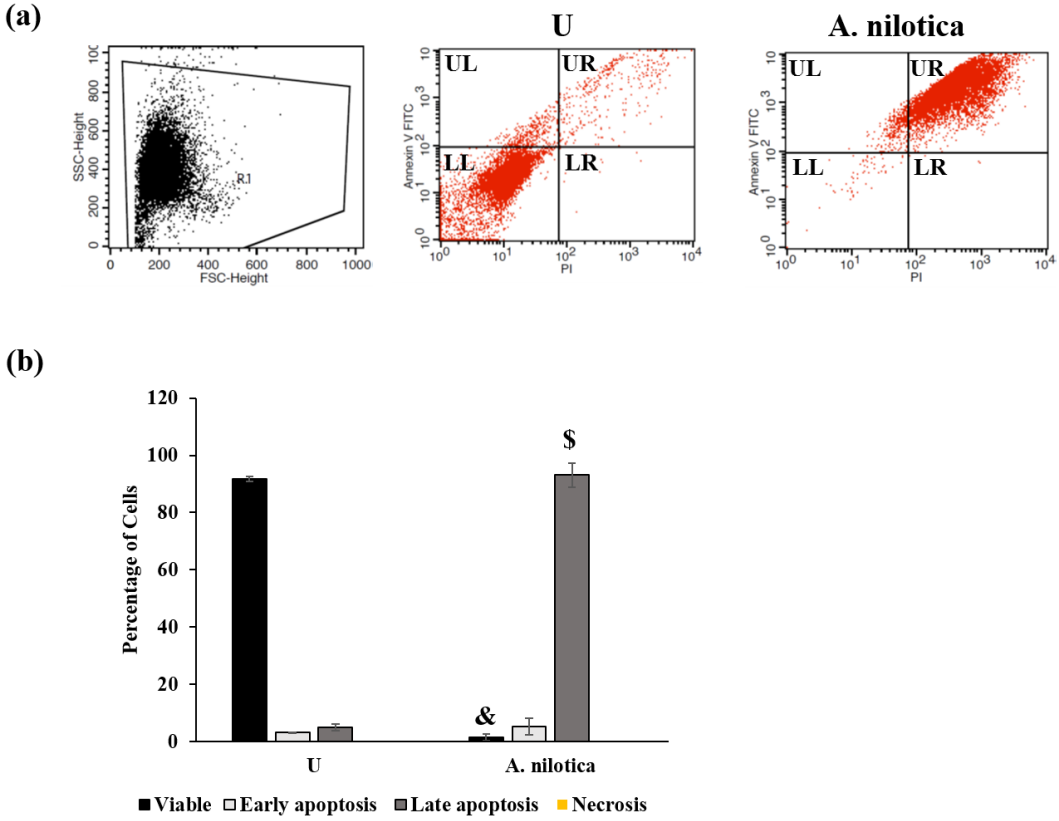

Figure 3: In U937 cells, apoptosis is induced by *Acacia nilotica* pod extract. U937 cells were subjected to an Annexin V Apoptosis flow cytometry detection test on the third day of incubation after treatment with higher concentration of 1.5% of *Acacia nilotica* pod extract for cytotoxicity. The treatment with *Acacia nilotica* extract revealed that the majority of the cell population was in late apoptosis. (a) Flow cytometry dot blot analysis. (b) The percentage quantifications of positive events of FITC Annexin V and PI on U937 cells are indicated. Average (SD) of the three experiments. $^{\&}p \leq 0.05$ comparing viable cells to untreated cells. $^{\$}p \leq 0.05$ comparing late apoptosis cells to untreated cells.

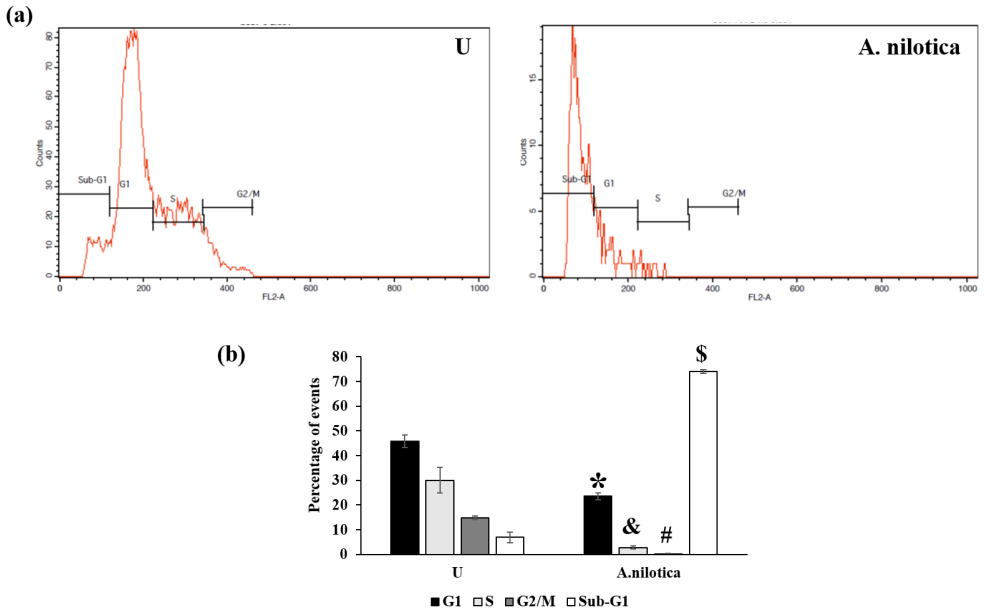

Figure 4: U937 cell accumulation in the sub-G1 zone upon *A. nilotica* treatment. U937 cells following a 72 hr treatment with 1.5% *A. nilotica* pod extract, DNA cell cycle pattern changes by flow cytometry using the DNA dye propidium iodide (FL-2A). (a) A histogram examination of U937 cells after being treated with *A. nilotica* pod extract shows that these cells accumulated in the sub-G1 zone as opposed to untreated, control cells and reductions in cells in the G1, S, and G2/M phases. *A. nilotica*: cells treated with *Acacia nilotica* pod extract, and U: Untreated cells (control). (b) The percentage calculation of PI-positive cells in the various cell cycle phases. The mean (SD) of the three studies was $p \leq 0.05$ when compared with untreated cells.

by flow cytometry (Figures 1, 2, and 3). This finding agrees with other studies that have shown the cytotoxicity of different cell lines. The anti-proliferative effect of *A. nilotica* leaf on the carcinoma cell line KB was studied by Thiagrajan.[13] Another anti-cancer effect study against a human cancer liver cell line was performed by nanoparticles synthesized from *A. nilotica* bark extract in 2022.[9] An animal study also indicated a decrease in tumor size after treatment with *A. nilotica* leaf extract.[14] In addition, our study also indicated an accumulation of U937 cells following treatment with *A. nilotica* pod extract in the sub-G1 zone in PI cell cycle analysis by flow cytometry (Figure 4), which reflects cell cycle arrest and cell death, which is consistent with other studies.[15]

CONCLUSION

Our research defines one of the different parts of *A. nilotica* tree that have great potential against various types of cancer cells, including AML.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Cite this article: Bakheit HF, Taurin S, Elamin EM, Bakhiet M. Acacia nilotica Pod Extract has an Anti-cancer Effect on the U937 Cell Line. Pharmacog Res. 2023;15(4):771-5.