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Can Ginger (*Zingiber officinale*) Aqueous Crude Extract Induce Apoptotic Pathways in Drug-Resistance Acute Myeloid Leukemia: *In Vitro* Study?

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Abstract

While chemotherapy remains to be one of the main using approaches in the clinical treatment of acute myeloid leukemia (AML), multidrug resistance (MDR) is considered the major obstacle that limits the therapeutic efficacy. Nowadays, Herbal therapy as an adjuvant therapy has been used for many health problems. Ginger (*Zingiber officinale*) is considering one of the promising herbal spices showing high therapeutic and preventive effects against many disorders specially cancer. In the current work, we focused on the role of ginger crude extract in fighting drug resistant AML. MTT assay showed a significant decrease in cell viability and clear cytotoxic effect on HL60/ADR and HL60 cell under the high concentrations (100 and 1000 µg/Ml) of ginger extract. The flow cytometry results showed a significant apoptotic cell death by ginger in HL60 and ADR/Hl60 and also confirmed by immunostaining of nucleus by DAPI which showed apoptotic nuclei. Our data clearly declared that the high concentration of ginger extract is promising anticancer drug by induction of apoptotic cell death in HL60/ADR cells especially in drug resistant AML.

Keywords

Acute Myeloid Leukemia, Drug Resistance, Ginger

1. Introduction

Acute myeloid leukemia (AML) is one of the most complex diseases associated with abnormal differentiation and high cellular proliferative rate of hematopoietic stem cells [1]. Many of current clinical treatment approaches as chemothera-

peutic drugs, immune-based therapeutics, radiation and stem cell transplantation are associated with incomplete recovery and many side effects [2]. In AML patients, developing multiple drug resistance (MDR) is the major challenge changes in successful treatment of AML patients with chemotherapy [1] [3]. Many of experimental cell lines treated with cytotoxic drugs such as colchicine, Actinomycin and Adriamycin are normally developed resistance to the selecting agent [4] [5]. The developing of the multidrug resistance occurs because of decline of the intracellular accumulation of the drugs in these cancer cells after a period of drug administration, which subsequently related to an enhanced drug efflux system [6]. ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp) and MRP1 mediate the active drug efflux system [7] [8].

Recently, completely food-based therapeutics as using herbal food is categorized as Natural Medicines (NMs) in prevention and treatment of chronic diseases like cancer. Herbal medicine is one of the most commonly used complementary and alternative therapies by cancer people [9] [10]. Ginger, a common natural herb, widely consumed all over the world as a spice. Ginger has a high medical value due to the presence of phenolic compounds as shogaols, gingerols, paradols, gingerdiones and many other bioactive compounds [11] [12]. Many published data proved that all of the phenolic gradients of ginger as 6-gingerol (6 G), 8-gingerol (8 G), 10-gingerol (10 G) and 6-shogaol (6 S) are the most abundant bioactive constituents of the crude ginger extract that exert a variety of therapeutic and preventive effects' properties as anti-proliferative, anti-inflammatory, anti-oxidative and anti-tumor effects' properties [11]-[19]. The present work focused on the effect of the crude water extract of ginger on drug resistant acute myeloid leukemia cells and the way by which ginger can induce leukemic cells' death.

2. Materials and Methods

2.1. Chemicals

RPMI growth medium, fetal bovine serum (FBS) and antibiotic mix (Penicillin/Streptomycin) were bought from Gibco (Invitrogen, CA, USA). MTT (3-[4, 5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H tetrazolium bromide), trypane blue, Annexin-V-FLUOS Staining Kitand7-Aminoactinomycin D Ready Made Solution (7AAD) were purchased from Sigma (St. Louis, MO, USA).

2.2. Preparation of Ginger Crude Extract

Collection of Plant Part

Ginger plant part was purchased from the local market. Rhizome was first washed under running tap water then by ultrapure water, air-dried and then powdered with the help of sterilized pestle and mortar. Ginger powder was stored in airtight bottles and subjected to the aqueous extraction procedures [20]. Aqueous decoction of air-dried powder of ginger rhizome was prepared by weighting (10 g) and boiled in 400 ml ultrapure water till one-fourth of the ex-

tract initially taken was left behind after evaporation. The remaining solution was then filtered using muslin cloth and the filtrate subjected to centrifugation at 5000 rpm for 15 min. The supernatant was again filtered with Whatman Filter No. 1 under strict aseptic conditions and the filtrate was collected in fresh sterilized bottles, prepares our concentrations, and stored at 4˚C until use.

2.3. Cell Lines

Acute human myeloid leukemia (AML) HL60 and HL60/ADR cell lines were obtained from ATCC (American Tissue Culture Collection). HL60 and HL60/ADR cells were maintained in RPMI cell culturemedium containing 10% inactivated FBS (fetal bovine serum) and provided with 1% penicilin/streptomycin and incubated in 37˚C and 5% CO_2 in humid conditions for the following experiments.

2.4. Cell Proliferation Assay

To study the cell growth rate in HL60 and HL60/ADR cells, both HL60 and HL60/ADR cells were cultured in RPMI provided with 10% FBS and 1% penicilin/streptomycin at initial cell number (2×10^5 cell/Ml) and incubated for (24, 48 and 72 Hrs.). Viable cells were detected by trypane blue dye exclusion method.

2.5. MTT Cell Viability Assay

The cytotoxic effect of Adriamycin and ginger crude extract on the HL60 and HL60/ADR cells viability and toxicity were investigated using MTT cell viability assay as previously described [21]. In brief, a number of 2×10^4 cells/well were cultured in 96-well plates, then the drugs (Adriamycin and ginger extract) were added to the cells at the indicated concentrations, negative control was included, and then the cells were incubated for 12 and 24 hrs. Next, fifty µl of MTT solution (2 mg/ml of MTT in PBS) were added to each well and left for 3 - 4 hrs and then the supernatants were removed carefully and 150 µl DMSO were added to each well. Then, 96-well plates was shaken for 10 minutes and then were read at A570 with reference filter at A650 using Elisa plate reader.

2.6. Apoptosis and Necrosis Using FACS Analysis

The apoptotic and necrotic cell death by Ginger extract and Adriamycin were studied using Annexin V and 7AAD. In brief, HL60 and HL60/ADR cells were cultured in 10 tissue culture dish with initial number 4×10^5 cell/Ml in RPMI growth media and treated with ginger extract at concentration (100 and 1000 µg/Ml) and incubated in CO_2 incubator for 24 Hrs. Briefly, the cells washed once with PBS and suspended in 100 µL 1X Annexin v binding buffer and add 5 µL FITC Annexin v and 5 µL of 7AAD to each tube and incubate for 15 minutes at RT. Finally, add 400 µL of 1X Annexinv binding buffer to each tube and analyze by flow cytometer.

2.7. Immunocytochemistry

HL60/ADR/HL60 cells were cultured in RPMI growth medium containing 1

mg/ml of ginger crude extract for 24 Hrs. Cells were attached on the glass slides using thecytospin and fixed with 2% PFA for 10 minutes at 4˚C. Then, cells were permeabilized with 50 µg/mL digitonin for 10 minutes at room temperature (RT). After washing twice with PBS, nuclei were stained with DAPI for 20 minutes at RT. Images were obtained with confocal microscopy.

2.8. Western Blot Analysis

The effect of ginger crude extract treatment with the indicated concentration on the apoptotic cell death was investigated in HL60/ADR cells using western blot analysis. The cells were treated with (0.00, 50, 100 and 1000 µg/Ml) of coumarinand combined with doxorubicin (100 ng/Ml) and incubated for 24 hrs. After treatment, Cells were harvested in a proper amount of RIPA buffer and collected in eppendorf tubes and left for 10 min. on ice with regular vortexing. Then the tubed were centrifuged at 12,000 rpm for 20 minutes at 4˚C. The supernatants were transferred to another eppendorf and kept at −80 to be used for protein electrophoresis using 30 µg protein from each sample. Protein samples were loaded in SDS PAGE and the proteins were transferred to nitrocellulose membranes. Membranes were processed for blocking and incubated overnight in 4 C° with anti Caspase-3, PARP, and actin (cell signal 1:1000) primary antibody and then incubated with the proper secondary antibody for 1 hr at RT. Finally, the proteins were visualized by enhanced chemiluminescence luminal (ECL) solution. The membranes were then scanned on C-Digits scanner using Image Studio Digits version 3.1.

2.9. Statistical Analysis

The quantitative data were expressed as averages ± standard deviation. Data were then graphed and statistically analyzed. Statistical differences were investigated using One-way analysis of variance (ANOVA) followed by Student's t-test.

3. Results

1) HL60/ADR show higher proliferative rate than HL60 cells.

HL60/ADR show high proliferative rate than HL60 cells after 24, 48 and 72 Hrs in the normal growth conditions as shown in Figure 1.

2) HL60/ADR show high resistance to chemotherapy than HL60 cells.

Cell viability was investigated in HL60 and ADR under different concentrations of Adriamycin (0, 0.1, 0.5, 1, 5 and 10 µg/Ml) using MTT assay. Quantitatively, it was shown that HL60 cells exert significant cell death than ADR cells with IC50 (3 and 17 µg/Ml) as shown in Figure 2(A) & Figure 2(B). Cell death by FACS analysis using Annexin V and 7AAd was confirmed the cell viability results and prove the high resistant of ADR cell to chemotherapy induced cell death (Figure 2(C) & Figure 2(D)).

3) Ginger crude extract shows clear cytotoxic effect on HL60/ADR cell at high concentrations.

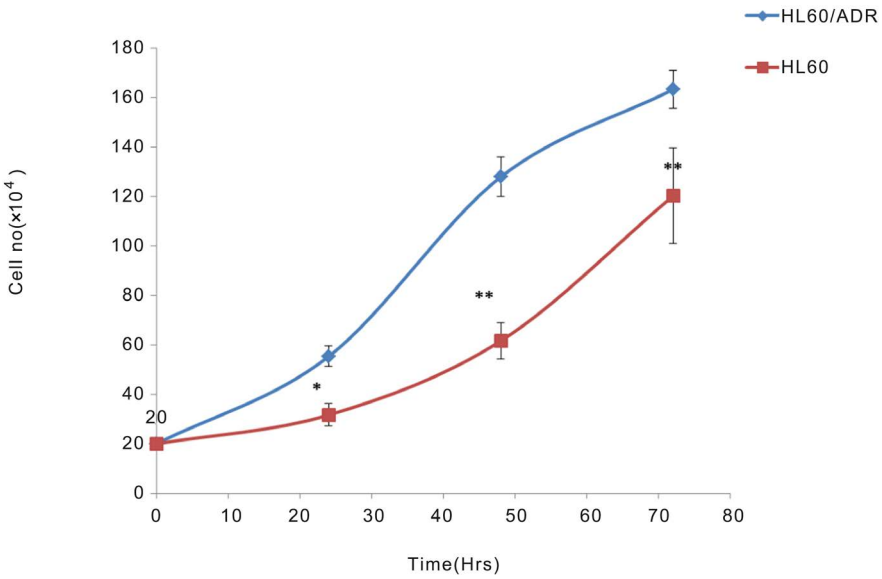
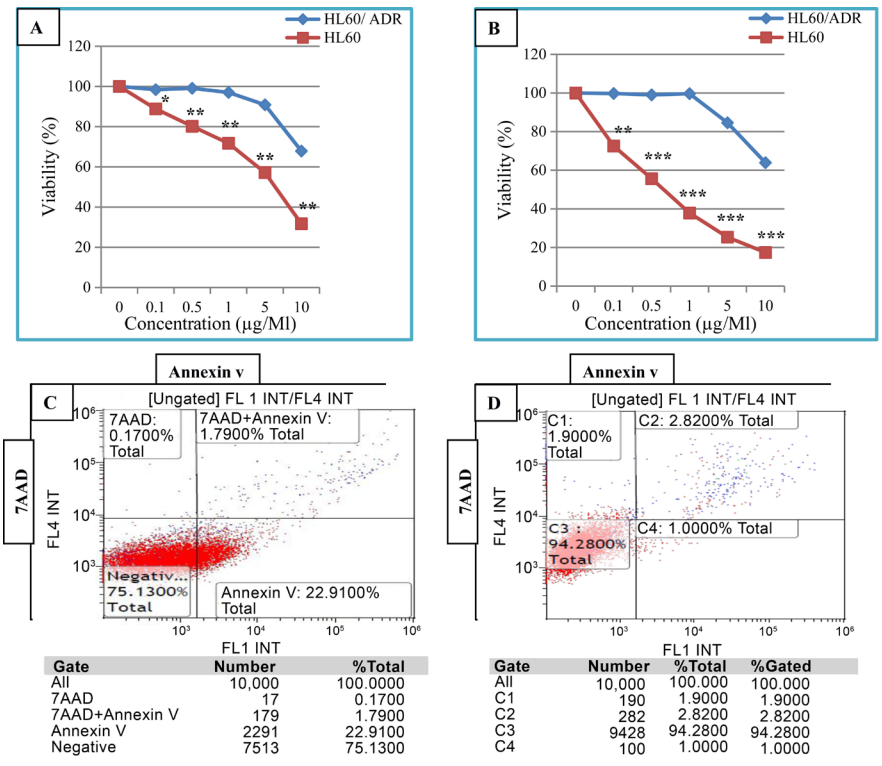


Figure 1. Growth curve of HL60/ ADR and HL60 in RPMI complete growth medium for 24, 48 and 72 Hrs. Viable cells were detected by trypane blue dye exclusion method P < (0.05 and 0.01) (n = 6).



Gate	Number	%Total
All	10,000	100.0000
7AAD	17	0.1700
7AAD+Annexin V	179	1.7900
Annexin V	2291	22.9100
Negative	7513	75.1300

Gate	Number	%Total	%Gated
All	10,000	100.000	100.000
C1	190	1.9000	1.9000
C2	282	2.8200	2.8200
C3	9428	94.2800	94.2800
C4	100	1.0000	1.0000

Figure 2. A and B show the cytotoxic effect of different concentrations of Adriamycin on HL60/ADR and HL60 cell after 12 and 24 Hrs. respectively, by MTT assay, (C) show theHL60 apoptotic cell death and (D) show HL60/ADR resistant to apoptotic and necrotic cell death by Adriamycin (5 µg/Ml) using FACS analysis P < (0.05 and 0.01) (n = 3).

MTT assay results explain the significant decreasing in cell viability of HL60/ADR and HL60 cell under the high concentrations (100 and 1000 µg/Ml)

of ginger extract as shown in Figure 3(A) & Figure 3(B) after 12 and 24 Hrs. respectively.

4) Ginger crude extract induces apoptotic cell death in HL60/ADR and HL60 cells at high concentrations.

Consistently with the flow cytometry analysis with annexin v and 7AAD, immunocytochemistry by using DAPI show lobed nuclei which clearly prove that the high concentrations (100 and 1000 µg/Ml) of ginger induce apoptotic cell death and in parallel to that, western blot data show the increased level of cleaved caspase-3 and PARP proteins under the indicated doses of ginger as shown in Figure 4 and Figure 5.

4. Discussion

Acute myeloid leukemia as a clonal disorder is characterized by the highly increasing of an immature, abnormally differentiated, myeloid cell population in the bone marrow and peripheral blood. AML is the most common acute leukemia in adults aged above 45 years [22]. About one-third of the AML patients

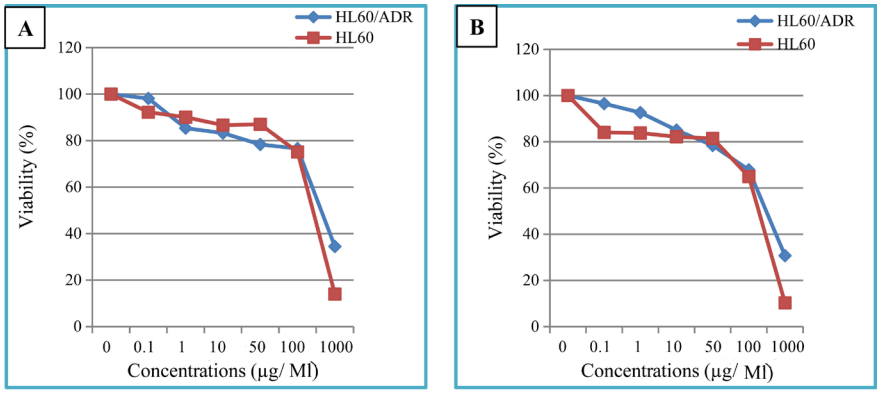


Figure 3. A and B show the cytotoxic effect of different concentrations of ginger crude extract on HL60/ADR and HL60 cell after 12 and 24 Hrs. respectively, by MTT assay. P < (0.05 and 0.01) (n = 6).

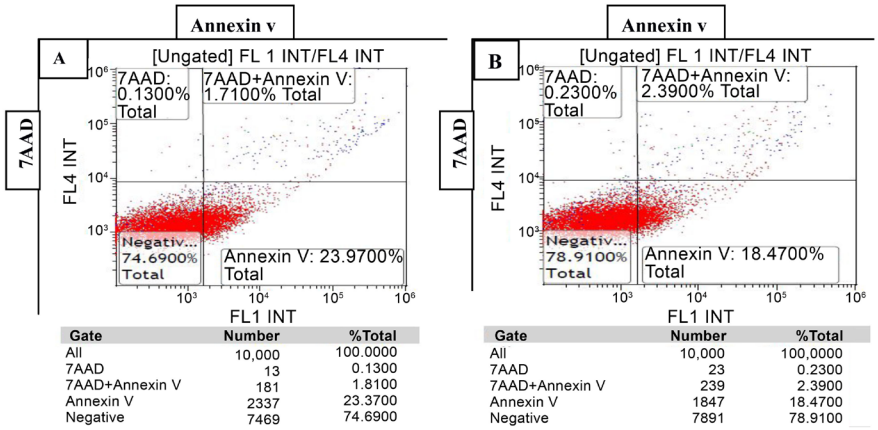


Figure 4. (A) and (B) show FACS analysis of apoptotic and necrotic cell death after 24 Hrs of (1000 µg/Ml) ginger extract treatment in HL60 and ADR respectively.

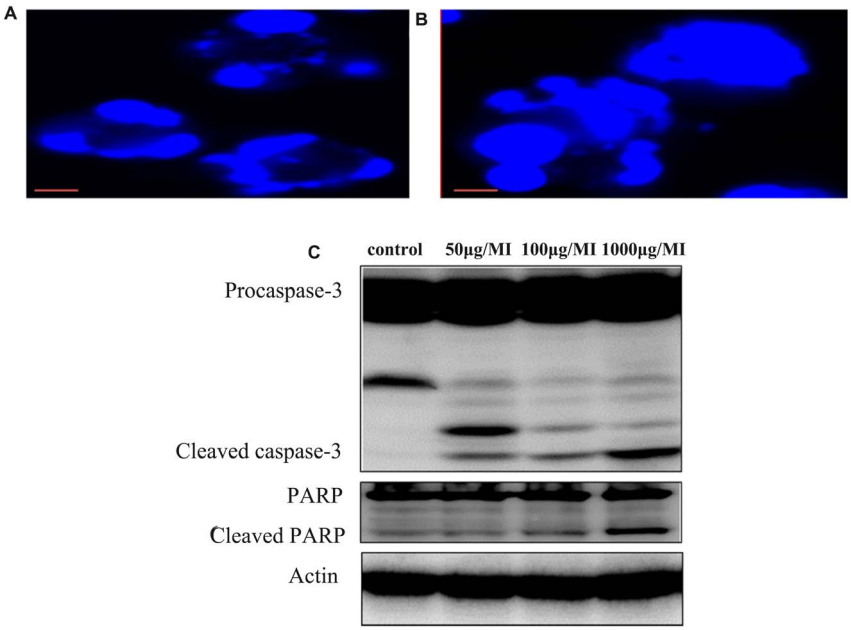


Figure 5. (A) and (B) show nuclei staining with DAPI after 24 Hrs of ginger extract (1000 μg/Ml) treatment in HL60 and ADR. Images were obtained with confocal microscopy. Bars, 10 μM. C after 24 Hrs of treatment with (50, 100 and 1000 μg/Ml) of ginger crude extract on HL60/ADR, cells were harvested and used for western blot analysis of Caspase-3, PARP, and Act in show western blotting results of AD.

do not attain a complete remission with conventional chemotherapy treatment developed drug resistant AML which considered a highly heterogeneous disease showing aggressive behavior toward all of normal strategies of treatment therapy [22] [23]. Therefore, our research focused on alternative natural dietary methods as ginger. In agreement with many previous published data that clearly state that the cell proliferation differs between leukemia subgroups and increased proliferation, is associated with developing drug resistant *in vitro* to several anticancer agents [24]. Our results show ADR/HL60 cells have aggressive proliferative rate compared with that of normal leukemic HL60 cells in the normal conditions. Ample published data reveals that the leukemic cells gradually activate their drug efflux system via change altering their membrane P-glycoprotein (P-gp) and MRP1 expression [6] [7] [8]. Adriamycin chemo-resistance is considerably clinical obstacles in chemotherapy for patients with AML. We found that Adriamycin showed no significant effects on cell viability at the indicated doses in ADR/HL60 cells with IC50 (17 μg/Ml) by MTT assay and confirmed by the FACS analysis for apoptosis and necrosis. However, it was clear that Adriamycin exerts high cytotoxic effect on HL60 cells at very low doses with IC50 (3 μg/Ml) as shown with cell viability and FACS analysis.

In recent years, ample data showed that the use of natural dietary agents has become widely accepted as a realistic option for the cure of malignant cancers because of their safety margin and there antioxidant and bioactive gradients [9] [10]. In the current work, we study the effect of ginger crude extract on the

ADR/HL60 cells by studying its cytotoxic effect and the cell death mechanisms. Recently, Mukkavilli *et al.* [25] explained the Pharmacokinetic-pharmacodynamics correlations in the development of ginger extract as an anticancer agent. Evidence has revealed that 6S, an active constituent of ginger, could induce cell death/apoptosis in many types of cancer cells including, colorectal carcinoma, ovarian cancer, hepatocarcinoma, human lung cancer, breast cancer cells and oral cancer [26] [27] [28] [29]. Inconsistent with Hang *et al.* [29] who explained that 6-shogaol inhibits breast cancer cell invasion by down-regulated matrix metalloproteinase-9 (MM-9) expression through blockade of nuclear factor-kappa-B (NFK-B) activation, Ling *et al.* [30] and Martin *et al.* [31] showed that 10 G induces apoptosis and inhibits metastasis in triple negative breast cancer *in vivo*, Liu *et al.* [32] demonstrated that 6-shogaol clearly induces apoptotic cell death in transformed and primary human leukemia cells, as well as in leukemia xenografts. This highlighted induction occurs in association with the cleavage of eIF2α that is dependent on caspase activation [32].

In consistent to these data, our results reveal the cytotoxic effect of ginger crude extract on HL60 and ADR/Hl60 cells as shown by cell viability assay in dose and time dependent manner. The flow cytometry results showed a significant apoptotic cell death by ginger in HL60 and ADR/Hl60 and also confirmed by immunostaining of nucleus by DAPI which showed apoptotic nuclei. From the above data, it could be concluded that Ginger as normal dietary spices is promising anticancer drug by induction apoptotic cell death especially in drug resistant AML.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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