

Research Article



Apoptosis Activity of Fruit Extract of *Rhamnus nepalensis* against Myeloid Leukemia Cells and Cell Cycle Arrest

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ABSTRACT

The present study investigated the apoptosis activity of fruit extract of *Rhamnus nepalensis* (FERN) on leukemia cell lines. The FERN significantly inhibited cell viability in a time and concentration dependent manner in both the cells. Morphological study showed several signs of apoptosis like chromatin condensation, nuclear fragmentation and formation of apoptotic bodies and degraded DNA bands in carcinoma cells than control. Cell cycle phase arrest was observed in the G0/G1 phase in the FERN treated cells. These findings suggest that extract possess cytotoxic and apoptosis activity against both the leukemia cells. Further mechanistic studies are under process to determine the different pathways attributing to apoptosis.

Keywords: *Rhamnus nepalensis*, Fruit, Leukemia, myeloid, Apoptosis, Cell cycle.

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INTRODUCTION

Medicinal plants are rich sources of biologically active compounds, containing immune modulatory and antioxidant properties, making them potential candidates as anticancer drugs. Secondary metabolites from plants are an excellent source of anticancer agents.¹ Different natural phytochemicals used for the treatment of various diseases including cancer are becoming important for drug discovery. These phytochemicals are found to be effective against various types of cancer. They show such effectiveness by alternating the cancer initiation, development and progression as well as interrupting several mechanisms like differentiation, cellular proliferation, angiogenesis, apoptosis and metastasis. They not only cause cytotoxicity but also manages the cancer microenvironment.² Leukemia was not only reported as the 14th most common cancer but also the 11th leading cause of mortality worldwide. Also, it was ranked 8th and 9th in men and women respectively in terms of mortality. Overall, this disease accounts for 2.5% of the total cancer cases in people below the age of 75 years.³ Leukemia is the most common paediatric malignancy and a major cause of morbidity and mortality in children. In a patient with leukemia, many of the white blood cells produced in the bone marrow do not mature normally. These

abnormal cells, called leukemic cells, are unable to fight infection the way healthy white cells can. As they grow in number, the leukemic cells also interfere with the production of other blood cells.⁴ It is also characterized by the aggressive nature of the disease and poor response to therapy.⁵ Acute myeloid leukemia (AML) is the most common acute leukemia in adults.⁶ Sequencing of acute myeloid leukemia (AML) genomes revealed a predominance of DNA mutations occurring in genes related to transcription, chromatin regulation, and DNA methylation.⁷ Chemotherapy and synthetic drugs lack specificity and targeted efficacy leading to drastic side effects. For thousands of years, humans have been using plants to treat various diseases.⁸ The plant *Rhamnus nepalensis*, a deciduous and hermaphrodite shrub found at elevation of 900-1800 metres have been used in treatment of Herpes and has also shown cytotoxicity on KB (HeLa) cells. *Rhamnus* is a genus of about 110 accepted species of shrubs or small trees, commonly known as buckthorns, in the family Rhamnaceae. The leaves are simple, 3 to 15 centimeters long, and arranged alternately, in opposite pairs, or almost paired (sub opposite). The plant bears fruits which are black or red berry-like drupes. The fruits of *Rhamnus nepalensis* when pounded and macerated in vinegar, can be used in treatment of Herpes. In the fruits of *Rhamnus nepalensis*, Emodin (3-methyl-1,6,8-trihydroxy anthraquinone) present in it, provides excellent basis for development of novel chemopreventive agents against cancer. *Rhamnus* species is found to contain anthraquinones such as emodin or chrysophanol, their reduced forms such as emodin anthrone and chrysophanol anthrone, dimers such as chrysophanol dianthrones and emodin dianthrones and their glycosides such as prinoidin and some may contain flavonoids. Some of these anthraquinone have been found



to possess anti-leukemic, cytotoxic, laxative, photosensitizing and vaso-relaxant properties. The plant under observation is *Rhamnus nepalensis*, a shrub of *Rhamnus* genus. The Fruit extract had shown cytotoxicity to KB cells (HeLa cells).⁹ The main aim of this study is to find out the anticancer activity of fruits of *Rhamnus nepalensis* on acute myeloid leukemic cell line Therefore we studied the methanolic extract of *Rhamnus nepalensis* fruits (FERN) was used for the cytotoxic activity on human acute myeloid leukemia cell lines, U937 and K562.

MATERIALS AND METHODS

Chemical and Reagents used

RPMI1640 medium with L-glutamine (Gibco), Fetal calf serum (FCS), Penicillin-Streptomycin, Gentamycin, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], Dimethyl sulphoxide (DMSO) Acridine Orange, Ethidium Bromide, Proteinase K, Agarose, Rnase (Sigma) Methanol, Ethanol, Petroleum ether etc.

Cell Culture

Human leukemic cell lines U937 & K562 were purchased from National Facility for animal tissue and cell culture, Pune, India. All the cells were sub cultured weekly at an initial concentration of 1×10^6 cells/ml and maintained in sterile RPMI 1640 medium supplemented with 10% heat inactivated FCS. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂ in air. In all the experiments untreated leukemic cells were termed as control group.

Collection and Identification of Plant Specimen

The specimen *Rhamnus nepalensis* fruits were collected from Siang District of Arunachal Pradesh, India in March 2015 and identified by Botanical Survey of India, Shibpur, Howrah- 711109.

Extraction of Test Sample

The fruit of the plant was separately cut into small pieces, shade dried and grinded into powder. 200gm of powder of fruits of *Rhamnus nepalensis* was soaked in 500ml of petroleum ether for three days at room temperature with intermittent shaking. The mixture was then subjected to filtration with the help of a filter paper and the filtrate was evaporated by Rotary vacuum evaporator to produce the petroleum ether extract. The residue obtained upon filtration was soaked in 500 ml of methanol for one week at room temperature with intermittent shaking and there after it was filtered. The filtrate obtained was subjected to evaporation by rotary vacuum evaporator to produce the methanol extract 20 gm of the methanolic extract was obtained. This was stored at 4° C in an airtight container and was designated as fruits extract of *Rhamnus nepalensis* (FERN) which was used for our study.

Cytotoxicity Study by MTT Assay

For cytotoxicity analysis, 1×10^5 concentration of human myeloid leukemic cell lines (U937 and K562) in log phase were used as stock suspension. 100 µl of this cell suspension were seeded in each well of 96 well tissue culture plates. They were treated separately with freshly prepared 1mg/ml stock solution of FERN, the doses of 25µg, 50µg, 100µg and 200µg for 24, 48, 72 hrs at 37° C in a humidified atmosphere containing 5% CO₂ in air. Untreated cells served as control. At the end of treatment, 20µl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] was added to each well and incubated for another 4 hours at 37-degree C in a CO₂ incubator. The MTT assay is a colorimetric assay for measuring the activity of enzymes that reduce MTT to formazan dyes, giving a purple colour. A solubilisation solution DMSO (Dimethyl sulphoxide) 100µl is added to dissolve the insoluble purple formazan product into a coloured solution. The absorbance was taken at 490 nm by micro plate manager (Reader Type: Model 680 XR Bio-Rad laboratories Inc.). From the absorbance values obtained at 490 nm the % inhibition was determined using the relation as stated below:

$$\text{Cell Inhibition: } 100 \times (\text{O. D of Control} - \text{O. D of Treated}) / \text{O. D of Control}^{10}$$

Cell Morphology by Fluorescence Microscope

Two DNA intercalates, acridine orange and ethidium bromide were used to visualise condensed chromatin of apoptotic dead cells. The differential uptake of these two dyes allows the identification of live and dead cells. The cationic dye acridine orange enters live cell containing normal nuclear chromatin and exhibits green colour, live cells exclude ethidium bromide and it stains fragmented nuclear chromatin in apoptotic cells in orange colour. 1ml cell suspension (1×10^6 cells) of U937 & K562 cells were treated with FERN at IC₅₀ concentration in a small petri plate and incubated for 24 hr at conditions mentioned above. Cell suspension incubated with medium was taken as control. The cells were then washed with cold PBS and treated with 10 µl of staining solution containing acridine orange (100µg/ml) and ethidium bromide (80 µg/ml) and immediately observed under fluorescence microscope (Leica Fluorescence microscope) at magnification of 60X, excitation range-blue, excitation filter-BP450-490, dichromatic mirror-510 and suppression filter- LP515.¹¹

Agarose Gel Electrophoresis Study

U937 cells were treated with 20µg/ml of FERN. After trypsinization cells were harvested and washed twice with PBS. The cells were resuspended in 500 µl of lysis buffer (50mM Tris- HCl, pH- 8.0, 10mM EDTA, 0.5% SDS), 100 µg/ml of Proteinase K was added and incubation was done at 50 °C for 1 hour and 37°C overnight respectively. DNA extraction was done by following the general phenol-chloroform extraction procedure and kept at -20 °C overnight. After centrifugation, DNA precipitates were washed with 70% ethanol, dried and evaporated at room



temperature and dissolved in TE buffer (pH 8.0) at 4°C overnight. To detect the DNA fragments, the isolated DNA samples were electrophoresed overnight at 20V in 1% agarose gel and stained with ethidium bromide. DNA fragmentation was observed in UV transilluminator.¹²

Cell Cycle Arrest Study

To assay the stage of cell cycle arrest in a flow cytometry, U937 and K562(1x10⁶) cells were treated with FERN (IC₅₀ dose) for 18 hrs. Cells were washed with PBS, fixed with cold methanol. They were then resuspended in cold PBS and kept at 4 °C for 90 min. Cells were pelleted down, dissolved in cold PBS, treated with RNase for 30 min at 37 °C and stained with Propidium iodide and kept in dark for 15 min. Cell cycle phase distribution of nuclear DNA was determined on FACS (Becton Dickinson FACS Fortessa 4 laser cytometer), fluorescence detector equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale) using BD FACS Diva software (Becton Dickinson).¹³

Statistical Analysis

This was done by Student's t-test P < 0.05 was considered as significant. The percentage cell inhibition was calculated by the following formula:

$$\% \text{ Cell Inhibition} = 100 \times (\text{O. D of Control} - \text{O. D of Treated}) / \text{O. D of Control}$$

Where O. D= Optical Density

The percentage cell viability was calculated by the formula:
% Viable Cells= (Total Number of Viable Cells per ml/ Total Number of Cells per ml) X 100.

RESULTS

Cytotoxicity Study by MTT Assay

FERN treated plant extract inhibited the growth and produced significant cytotoxicity of leukemic cell lines in a concentration-dependant manner. Reduction in the mean OD of cells treated with increasing dose of FERN was observed as compared to control. FERN exerted 50% growth inhibition (IC₅₀) of U937 at concentrations of 38.72 µg/ml and in K562 cells 60.11 µg/ml respectively.

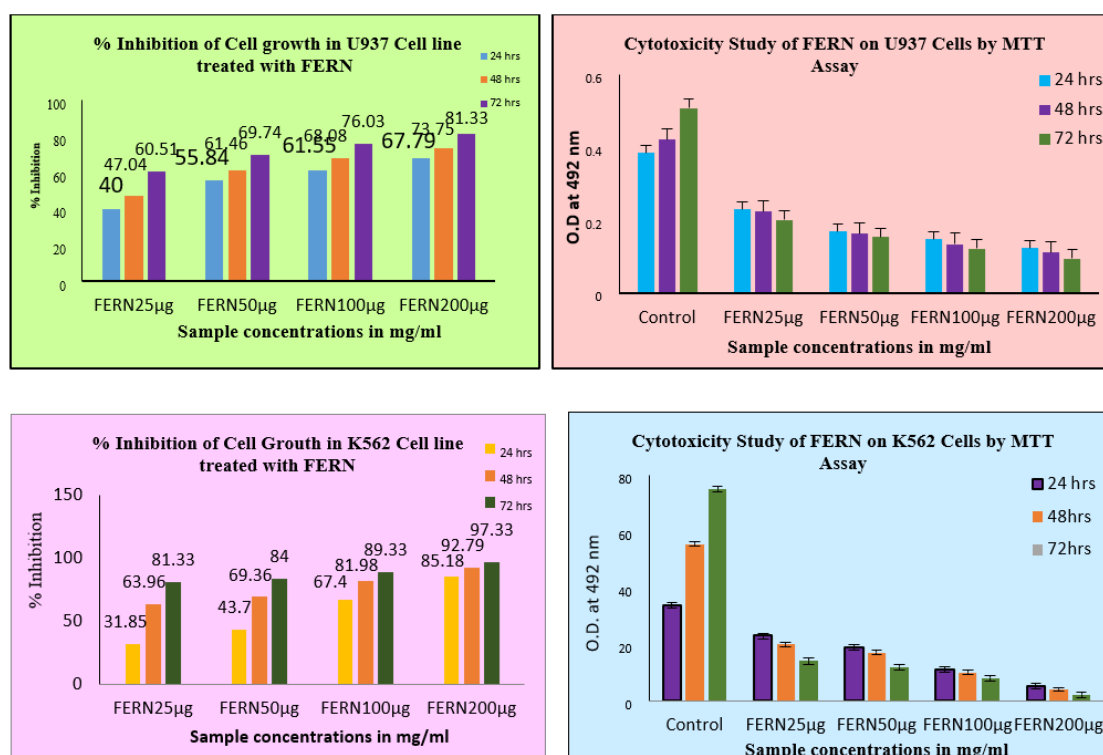


Figure 1: Histogram shows the effect of FERN on cell cytotoxicity by MTT Assay on U937 and K562 cell line after 24, 48 & 72 hours. The O.D at 492nm is compared to the untreated control cells. The change in percentage of inhibition is observed against time and concentration. The data shows significant rise in the % inhibition with respect to increase in time and concentration. Both the data showed decrease in O.D. from that of the control cells with respect to time and concentration dependent manner. Data are mean ±S.E.M.

Cell Morphology by Fluorescence Microscope

ME was found to produce cell death in the cell line by inducing apoptosis. Morphologically, apoptosis is characterized by cell shrinkage, plasma membrane blebbing, chromatin condensation and formation of apoptotic bodies. Figure 3 shows the morphology of U937

& K562 cell treated with FERN and stained with acridine orange and/or ethidium bromide. The fluorescent images showed that the untreated cells possessed intact nuclei and showed green fluorescence by taking the colour of acridine orange. Treatment with FERN caused chromatin condensation, membrane blebbing and formation of

apoptotic bodies and showed orange fluorescence also by taking the stain of ethidium bromide (DNA intercalates).

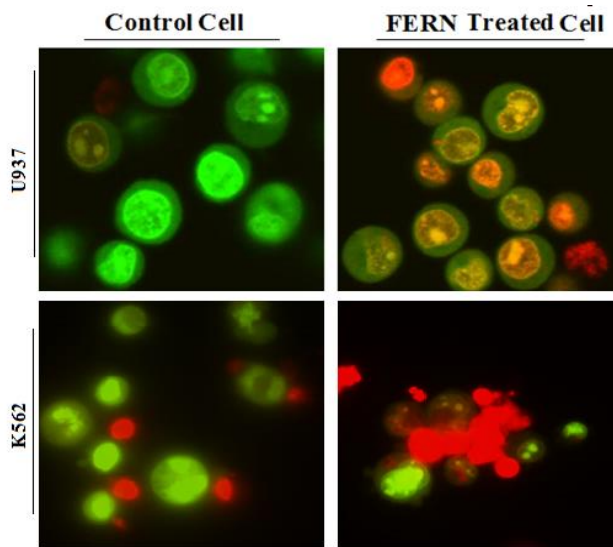


Figure 2: Fluorescence Microscopic images of U937 & K562 cell lines showing the apoptosis inducing activity of FERN. First image is showing control cells containing intact nuclei have taken the stain of acridine orange, second image contains the cells treated with FERN having stain of both acridine orange and ethidium bromide.

Agarose Gel Electrophoresis Study

After Agarose Gel Electrophoresis, the DNA samples isolated from untreated control U937 cells showed intact DNA bands whereas the DNA samples isolated from FERN treated cells showed fragmented DNA bands in the form of ladders. So the observation confirmed that the treatment with FERN caused apoptosis in all the leukemia cells.

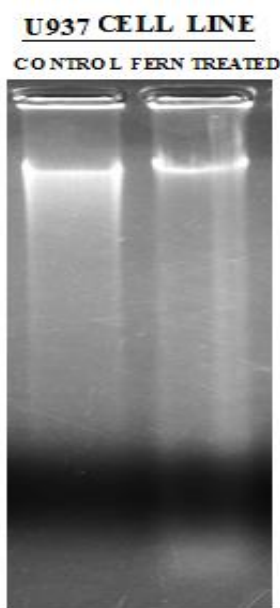


Figure 3: Showing agarose gel images of control vs treated cell line of U937 with IC₅₀ dosage. Lane 1 represents control of U937 respectively. Lane 2 represents FERN with IC₅₀ dosage on U937 cell line respectively.

Cell Cycle Arrest Study

Flow cytometric evaluation showed that after 24hrs treatment of U937 cell line DNA content increased in G1 phase (56.1% against 31.5%). In case of K562 cell line DNA content increased in G1 phase (59.7% against 29.3%). This observation demonstrate that FERN significantly inhibited the cell growth of U937and K562 cell line by arresting the cell populations in the sub-G0/G1 phase of the cell cycle.

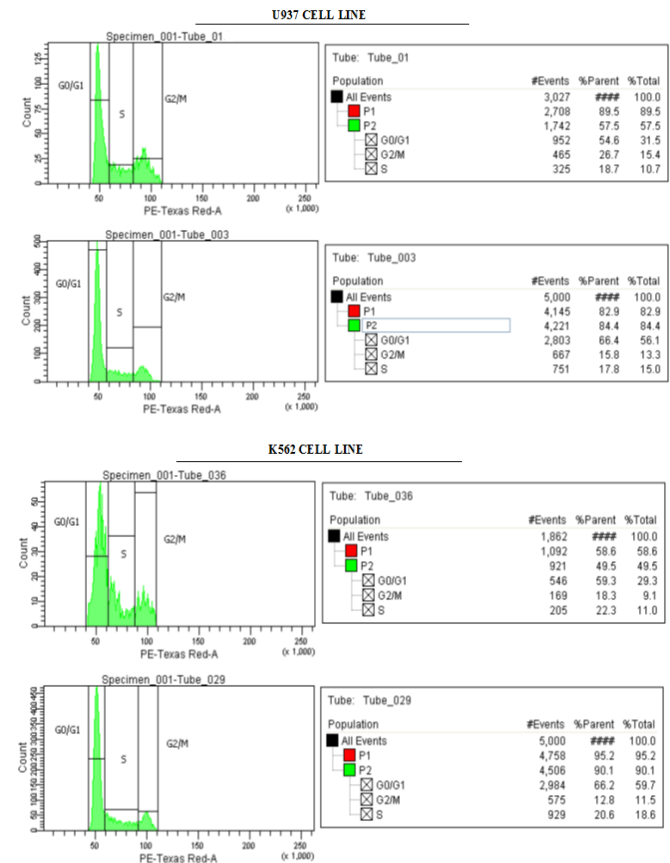


Figure 4: Flow cytometric evaluation of cell cycle phase distribution in controls and treated FERN at IC₅₀ dosage on U937& K562 cell after 24 hrs of treatment. Histograms represent various contents of DNA with actual number of cells (x-axis denotes fluorescence intensity of PE- Texas red and y-axis denotes count).

DISCUSSION

Cancer is a group of diseases involving abnormal cell growth which is caused by mutation of genes involved in the cell division and its control. This mutation can either accelerate the rate of cell division or inhibit the normal control on the system like arrest of cell cycle or apoptosis. It has the potential to invade or spread to other parts of the body.¹⁴ In the most developed countries and the developing countries, cancer is among the three most common causes of death. Treatment of cancer involves chemotherapy, radiotherapy, surgery and often a combination of two or three.¹⁵ The lack of specificity and targeted efficacy of chemotherapy and synthetic drugs leads to drastic side effects. Humans have been using plants to treat various diseases for thousand years. The active anticancer agents

have been identified from many plants whose potency to inhibit the cancer metastasis is studied extensively.¹⁶ Medicinal plants maintain the health and vitality of individual and also cure various diseases including cancer without causing toxicity. Natural products discovered from medicinal plants have played an important role in treatment of cancer.¹⁷ Natural products are a potential group of anticancer agents in the current world. It has been effective in various types of cancer. Out of 136 drugs discovered in the last decade, 80% were that of natural origin. The fruits of *Rhamnus nepalensis*, Emodin (3-methyl-1,6,8-trihydroxyanthraquinone) present in it, provides excellent basis for development of novel chemopreventive agents against cancer. *Rhamnus* species is found to contain anthraquinones such as emodin or chrysophanol, their reduced forms such as emodin anthrone and chrysophanol anthrone, dimers such as chrysophanol dianthrones and emodin dianthrones and their glycosides such as prinoidin and some may contain flavonoids. Some of these anthraquinone have been found to possess anti-leukemic, cytotoxic, laxative, photosensitizing and vaso-relaxant properties. The Fruit extract had shown cytotoxicity to KB cells (HeLa cells).⁹ Therefore, the present study reveals that the fruit extract of *Rhamnus nepalensis* (FERN) possesses anti-leukemic activities. The cytotoxic activity was observed on human myeloid leukemia (U937 and K562) cell lines. The cytotoxic and apoptosis activities of FERN were supported by the observations in MTT assays and apoptotic studies respectively. The extract showed significant inhibition in cell growth and metabolic activities of both the cell line in a time and concentration dependant manner. IC₅₀ dose of FERN was 38.72 µg/ml on U937 and 60.11 µg/ml on K562 cells respectively. This reveals that FERN acts on human acute myeloid leukemic cell as well as on chronic myeloid leukemia cells. The process of apoptosis is characterized by several morphological changes such as cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies. The fluorescence microscopic images of both the cells clearly showed nuclear disintegration of FERN treated U937 and K562 cells compared with that of untreated control cells when stained with acridine orange and ethidium bromide. Further, the gel patterns of agarose gel electrophoresis of FERN treated leukemia cells support the anti-leukemia activity of FERN. The FERN treated leukemia cells showed degraded DNA bands in the form of ladders, evidence of apoptosis, whereas intact DNA bands was showed in untreated control cells when observed in UV transilluminator. It was also revealed in the Cell cycle analysis that the treatment with FERN arrested the cell populations in the sub G₀/G₁ phase of the cell cycle.

CONCLUSION

From the experimental studies carried out with the fruit extract of *Rhamnus nepalensis*, it can be concluded that the methanolic extract of FERN have anti-leukemic activity on acute myeloid leukemia cell line (U937) and multidrug resistant cell line (K562). Therefore, it can be stated that Fruit extract of *Rhamnus nepalensis* could be a probable

and potent sources of anti-cancer agent for the treatment of leukemia. Further study should be done to identify the active compound/compounds of *Rhamnus nepalensis* fruit which are responsible for anti-leukemic/anticancer activity on cancer cells.

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