APOTOTIC ACTIVITY OF ETHANOLIC EXTRACT OF
MORINGA OLEIFERA ROOT BARK ON HUMAN MYELOID
LEUKEMIA CELLS VIA ACTIVATION OF CASPASE CASCADE

Subhadeep Roy¹, Nilanjana Deb¹, Santanu Basu², Shila Elizabeth Besra¹*

¹Drug Development/Diagnostic & Biotechnology, CSIR, Indian Institute of Chemical
Biology, 4 Raja S.C. Mullick Road, Kolkata-700032, West Bengal, India.
²Employee State Insurance Hospital Sealdah, 301/3,A.P.C. Road, Kolkata –
700009, West Bengal, India.

ABSTRACT
Identification of cytotoxic compounds that induce apoptosis has been
the mainstay of anti-cancer therapeutics. The plant Moringa oleifera
Lam. (family: Moringaceae) is widely used in Indian traditional
medicine as a broad spectrum medicine. The ethanolic extract of
Moringa oleifera root bark (EMORB) and its fraction F1 & F2 showed
significant anti-leukemic activity in human leukemic cell lines with
EMORB- IC₅₀ of 30.22µg/ ml, and 28.29µg/ml, F1-9.73µg/ ml, and
10.23µg/ml and F2-8.56µg/ ml, and 9.12µg/ml in U937 & K562 cell
lines respectively. EMORB treated leukemic cells showed chromatin
condensation, apoptotic body formation and increased Caspase 9 and 3
production indicating apoptosis. Confocal microscopy images showed
externalization of phosphatidylserine from inner leaflet to outer leaflet
of the cell membrane in EMORB treated both the human myeloid
leukemic cell lines. Flow Cytometric analysis showed appreciable
number of cells in early apoptotic stage and the cells cycle study revealed that leukemic cells
accumulated in the G0/G₁ phase as well as in G2/M phase; the cell cycle was halted in this
phase and the DNA content decreased in other phases. The results obtained indicate that root
bark extract of Moringa oleifera plant and its fractions possess potent anti-leukemic activity
against U937 & K562 cell lines.

Key words: Moringa oleifera, Root Bark, Myeloid, Apoptosis, Caspase.
1. INTRODUCTION

Leukaemia refers to cancers that begin in the blood-forming cells of the body. These abnormal cells grow and multiply in an uncontrolled way. As the disease progresses, leukemic cells move through the bloodstream and invade other organs, such as the spleen, lymph nodes, liver, and central nervous system which are all known as haematological neoplasm. In the hematopoietic malignancies, chemotherapeutic approaches are widely applied in practice. Nowadays, several therapeutic approaches have been taken to overcome the complexities of different cancers. Drug discovery against cancer is ventured throughout the world especially from the natural products \[1\]. The greatest disadvantage in the presently available potent synthetic anti-cancer drugs lies in their toxicity, many side effects and reappearance of symptoms after discontinuation. To avoid the disease, it's essential to take advantage of this natural protection. Plants are therapeutic arsenals that have been playing significant roles in the healing processes, magic rituals, and religious practices of peoples from the five continents\[2,3\]. It would be extremely beneficial in the field of cancer chemotherapeutics, if a natural anti-cancer agent can be discovered. Our plant of interest, *Moringa oleifera* Lam. (family: Moringaceae) is a medicinal plant which has strong antimicrobial \[4\], anti-inflammatory, anti-spasmodic, diuretic \[5\], antibiotic \[6\], water purifying property \[7\], and antihypertensive \[8, 9\]. It also reduces cholesterol \[10\] & posses sympatholytic property \[11\]. The present communication is an approach to study the anti-leukemic property of ethanolic extract of root bark of *Moringa oleifera*, which has been investigated against acute and chronic leukemic cell lines: U937 (human leukemic monocytes lymphoma cell line) and K562 (human myelogenous leukaemia cell line).

2. MATERIALS AND METHODS

2.1. Chemicals

**Invitro evaluation**

This work was performed at the Drug Development/Diagnostic & Biotechnology, CSIR, Indian Institute of Chemical Biology. The design of this work mainly covers *in-vitro* study.

2.2. Cell Culture

Two human leukemic cell lines U937 and K562 were purchased from the National Facility for Animal Tissue and Cell Culture, Pune, India. The cells were maintained in RPMI 1640 medium (Gibco, USA), supplemented with 10% heat inactivated FCS, 100U/ml Penicillin (Biowest, Germany), and 100mg/ml Streptomycin (Biowest, Germany). 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. The peripheral blood of four normal individuals with prior ethical permission was also collected. From peripheral blood, only mononuclear cells were separated by Ficoll- Histopaque technique. The cells were then taken in RPMI 1640 medium with 5% FCS in aseptic condition. In all the experiments untreated leukemic cells as well as untreated cells were termed as control group.

### 2.3. Plant Material

The root bark of *Moringa oleifera* Lam. (family: Moringaceae) was collected from village of the Rajbandh, Dumka and Jharkhand, India in the month of April, 2011. The root bark of this plant was authenticated by Dr. V. P. Prasad, Scientist - C, Central National Herbarium, Botanical Survey of India, Ministry of Environment & Forests, and Government of India at Howrah, West Bengal. A voucher specimen No is **BSI/CNH/SF/Tech./2011** was deposited in the Drug Development/Diagnostic & Biotechnology Division, CSIR, Indian Institute of Chemical Biology, Kolkata.

### 2.4. Preparation of *Moringa oleifera* bark extracts

The barks of *Moringa oleifera* Lam. were harvested during dry season and air dried, then grinded into powder (200 gm) and soaked in about 350 ml petroleum ether (Merck) in room temperature and was repeated 2 times. Petroleum ether extract was obtained. Then, it was dissolved in 500 ml of ethanol (Merck) in room temperature for 7 days with occasional shaking. The mixture was then filtered by filter paper and the filtrate was evaporated by Rotary Vacuum Evaporator and lyophilized to produce ethanol free extract. 2.7gm of ethanolic extract of *Moringa oleifera* root bark (EMORB) was obtained finally. The extract was further dissolved in 100 ml water and then partitioned into two parts. One part was dissolved in 200 ml ethyl acetate to obtain ethyl acetate fraction and rest was dissolved in n-butanol to obtain the n-butanol fraction. The Ethyl acetate fraction of *Moringa oleifera* of root bark was termed as (F1), n-butanol fraction of *Moringa oleifera* of root bark (F2) Stock solution was prepared as (1mg/ml concentration) in phosphate buffer saline (PBS) from which desired doses were tested for different experimentation.

### 2.5. Phytochemical screening of *Moringa oleifera* bark extract

The phytochemicals present in *Moringa oleifera* barks were qualitatively identified in the ethanol extracts and have been reported earlier. Different types of test have been performed to establish the presence of different chemicals. Shinoda and Zinc chloride test for
flavonoid, Liebermann Burchard, Salkowski and Liebermann test for Steroid, Dragendorff and Hagger test for alkaloid have been performed and the chemical test confirmed the presence of Flavonoid, Steroid and Alkaloid. The ethanol extract of *M. oleifera* barks; contain steroids and triterpenoids, amino acids, saponins, alkaloids and carbohydrates. The administration of *M. oleifera* ethanol extract is safe & has no adverse effect on growth related and biochemical parameters indicating its safety.

### 2.6. Cytotoxicity Study

Log phase cells (U937 & K562) at a concentration of 1x10⁵ cells (100µl cell suspension) were seeded in 96 well tissue culture plates. They were treated with freshly prepared 1mg/ml stock solution of EMORB t in various concentrations between 5µg - 200µg for 24, 48, 72 hrs at 37°C in a humidified atmosphere containing 5% CO₂ in air. Untreated cells served as control. The cytotoxicity studies were performed by MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-2,5-diphenyltetrazoliumbromide] assay & the absorbance of the colour solution was measured at a wavelength of 492 nm by microplate manager (Reader type: Model 680 XR Bio-Rad laboratories Inc) [13] IC₅₀ values were obtained for 24 hrs. A comparative cytotoxicity study was performed between n-butanol fraction (F1) and ethyl acetate fraction of (F2) of EMORB, at doses between 5µg -100µg in both the cell lines for 24 hrs & corresponding IC₅₀ was calculated. Standard used: 100µg Ara-C (Cytosine arabinoside) was used for U937 and 0.372µg of Imatinib Mesylate (Brand name: Gleevec 100mg, Novartis) for K562 cell line.

### 2.6.1 Toxicity study on normal lymphocyte by MTT assay

Toxicity studies of EMORB were studied on normal human WBC by MTT assay. 100µl cell (1x10⁵) suspension per well in a log phase were seeded in 96 well tissue culture plates. Treatment regime of 10µg, 50µg, 100µg, 200µg doses of EMORB were used for a period of 24 hrs & incubated at 37°C in a humidified atmosphere containing 5% CO₂ in air, untreated cells served as control. The absorbance was measured at 492nm by micro-plate manager (Reader type: Model 680 XR Bio-Rad laboratories Inc).

### 2.7. Morphological Studies for Detection of Apoptosis

#### 2.7.1 Fluorescence Microscopic studies

U937 and K562 cells (1x10⁶) were treated with different IC₅₀ doses of EMORB (corresponding to each cell line) for 24 hrs and observed using a fluorescence microscope for determining morphological changes. The untreated control cells and EMORB treated cells
were harvested separately (centrifuged at 1000 rpm for 5 min), the pellets were washed twice with PBS and then stained with 100µg/ml of acridine orange (Sigma, USA) and 100µg/ml of ethidium bromide (Sigma, USA) in a ratio of 1:1. The cells were then immediately mounted on slides and observed under a fluorescence microscope for the morphological determination of the cells undergoing apoptosis.

2.7.2. Confocal Microscopic studies
U937 and K562 cells (1x10^6) were treated with different IC₅₀ doses of EMORB for 24 hrs. After 24 hrs the untreated control cells and EMORB treated cells were harvested and washed with ice cold PBS. The cells were then stained with 10µg/ml of Propidium iodide (Sigma) for 5 min. After mounting on slides the cells were observed to determine the differences in nuclear morphology between the untreated and EMORB treated leukemic cells under confocal laser scanning microscope (Leica TCS-SP2 system, Leica Microsystems, Heidelberg, Germany) installed with an inverted microscope (LeicaDM-7RB) [14]. Images for Hoescht 33342 was acquired from UV laser line using 450 nm band pass filter for UV for images.

2.7.3 Study of phosphatidylserine (PS) externalization
PS externalization was examined after treating the cells (1x10^6) with different IC₅₀ doses of EMORB for 24 hrs under confocal laser scanning microscope (Leica TCS-SP2 system, Leica Microsystems, Heidelberg, Germany). The untreated and the EMORB treated cells were harvested separately, washed with ice cold PBS and with Annexin- V FITC binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM CaCl₂ 2H₂O; pH 7.4) respectively and they were then stained with 5 µl of Annexin-V- FITC for 10 minutes at room temperature. The cells were mounted on slides and the images were captured to observe the cells undergoing early apoptosis.

2.8. Agarose gel electrophoresis study
U937 & K562 cells were treated with 20µg/ml of EMORB and Ara-C (Std drug for U937) and Imatinib Mesylate (Std drug for K562). The cells were resuspended in 500µl of lysis buffer (50 mM Tris- HCl, pH -8.0, 10 mM EDTA, 0.5% SDS), 100µg/ml of proteinase K was added and incubation was done at 50°C for 1 h 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 20 V in 1% Agarose gel and stained with ethidium bromide. DNA fragmentation was observed in UV transilluminator.\textsuperscript{[15]}

2.9. Detection of Apoptosis by Flow Cytometry

In order to investigate the type of cell death induced by EMORB, flow Cytometric analysis was done by performing dot plot assay. The U937 and K562 cells (1x 10\textsuperscript{6}) were treated with individual IC\textsubscript{50} dose (18 hrs) of EMORB for 18 hrs. The cells were centrifuged at 2000 rpm for 8 min at 4 °C and pelleted down. Then washed with AnnexinV- FITC binding buffer provided in apoptosis kit (Sigma). Again after centrifuging at 2000 rpm at 4 °C, the cell pellets were dissolved in AnnexinV- FITC binding buffer containing AnnexinV- FITC and Propidium iodide. After 15 min incubation in dark at room temperature flow Cytometric analysis was done. All data were acquired with a Becton-Dickinson FACS LSR Fortessa 4 laser Cytometry. Flow-Cytometry reading was taken using 488 nm excitation and band pass filters of 530/30 nm (for FITC detection) and 585/42 nm (for PI detection). Live statistics were used to align the X and Y mean values of the Annexin-V FITC or PI stained quadrant populations by compensation. Data analysis was performed with BD FACS Diva software program\textsuperscript{[16]}.  

2.10. Study of Cell Cycle Arrest by Flow Cytometry

To assay the stage of cell cycle arrest in a flow cytometry\textsuperscript{[16]}, U937 and K562 (1x10\textsuperscript{6}) cells were treated with EMORB (IC\textsubscript{50} 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).

2.11. Caspase-9 assay

The assay was performed using a Caspase-9, Apoptosis Detection, Colorimetric BioAssay Kit (US Biological) according to the manufacturer’s protocol. U937 and K562 cells (1x10\textsuperscript{7}) were treated with IC\textsubscript{50} dose of EMORB for 24 h. The cells were pelleted down and resuspended in 50 μl of cell lysis buffer (supplied with the kit) and incubated on ice for 10 min. After centrifuging at 10,000 x g for one min, the supernatants (cytosolic extract) were
transferred to fresh tubes and kept on ice and the caspase-9 assay was performed according to the supplied kit protocol. 50 µl of 2X reaction buffer (containing 10 mM DTT) was added to each sample. 5 µl of LEHD-pNA substrate (4 mM) (200 µM final concentration) was added and incubation was done at 37 °C for 1-2 h. Absorbance was read at 405 nm and calculations were thereby done.

2.12. Caspase -3 assay
The assay was performed using a Caspase-3 Assay kit, Colorimetric (Sigma) according to the manufacturer’s protocol. U937 and K562 cells (1x10⁷) were treated with IC₅₀ dose of EMORB for 24 h. The untreated and the treated cells were pelleted down by centrifugation at 600 x g for 5 min at 4 °C. Supernatants were removed and the cell pellets were washed with 1 ml of PBS. The cells were again centrifuged and the supernatants were removed completely. The cell pellets were suspended in 100 µl of 1X lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT) and incubated on ice for 20 min. The lysed cells were centrifuged at 20,000 x g for 15 min at 4 °C and the supernatants (cell lysates) were analysed for the caspases-3 activity according to the manufacturer’s protocol. Cell lysates were incubated with 2 mM Caspase-3 substrate (Ac-DEVD-pNA) in 1X assay buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT) for 90 min at 37 °C. The absorbance was read at 405 nm and the results were calculated using a p-nitroaniline calibration curve.

2.13. Statistical Analysis
Statistical analysis was done by Student’s t-test. P < 0.05 was considered as significant.

The percentage cell inhibition was calculated by the following formula:- %Cell inhibition= 100 × (O.D of control - O.D of treated)/O.D of control 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) × 100.

3. RESULTS
3.1 Cytotoxicity study by MTT Assay
In the MTT assay EMORB at concentrations of 5µg to 200µg significantly inhibited the growth of U937 and K562 cells compared with that of the control cells after 24, 48 & 72 hrs of treatment in a concentration-dependent manner (Fig.1). These observations provided proof for cytotoxic nature of EMORB. The IC₅₀ calculated after MTT assay: EMORB in U937 is found to be 30.22µg/ ml, and in K562 it is 28.29µg/ml. The IC₅₀ value of F1 in U937 is found
to be 9.73µg/ml, and in K562 it is 10.23µg/ml. The IC$_{50}$ value of F2 in U937 is found to be 8.56µg/ml, and in K562 it is 9.12 µg/ml.

3.1.1 Toxicity study on normal lymphocyte by MTT assay

In the cytotoxicity study or MTT assay on normal lymphocytes, there was a no significant reduction in the O.D. values compared to that of the untreated control cells after treating the cells with EMORB at the doses of 10 µg, 50µg, 100µg, 200µg for 24 hrs. The O.D of the EMORB is almost similar to that of the control cells, thereby indicating that the EMORB has selectivity for leukemic cells and exerts no effect on the normal lymphocytes. (Fig.2)

![Fig. 1:](image)

Fig. 1: - The histogram shows the effect of EMORB on on cell cytotoxicity in U937 (A) & K562 (B) and its fractions F1 and F2 on U937 (C) and K562 (D) cell line. The cell growth is compared to the untreated control cells and standard Ara-C & Imatinib Mesylate treated cells for U937 & K562 cells respectively. Reduction in the number of cells is observed in a time and concentration dependent manner. Data are mean ± S.E.M. * denotes significant decrease in cell count from control values p<0.05.
3.2. Morphological Studies for Detection of Apoptosis

3.2.1. Cell Morphology Studies by Fluorescence Microscopy

Fluorescence microscopic observations of the EMORB treated (individual IC50 dose/ h) U937, K562 cells stained with ethidium bromide and acridine orange (colour-red or orange), revealed the presence of apoptotic cells (early and late) as compared to the untreated control cells stained with only acridine orange (colour-green). Arrays of nuclear changes were observed including chromatin condensation and apoptotic body formation that are indicative of an apoptotic process comprising of both early and late apoptotic stages.(Fig.3)

![Fluorescence microscopic images](image)

**Fig: 3** Fluorescence microscopic images of untreated control U937 (A), & K562 (C) and EMORB treated U937 (B) and K562 (D) cells. The control cells showed with intact nuclei and gave bright green fluorescence whereas treated cells showed condensed chromatin, fragmented nuclei expressing the signs of apoptosis (indicated by the arrowheads).
3.2.2. Cellular and Nuclear Morphology Studies by Confocal Microscopy

EEMOB induced apoptotic changes in both the leukemic cells after 24 hrs. of treatment showing chromatin disintegration and formation of apoptotic bodies whereas the untreated control cells were with intact nuclei. (Fig.4)

Fig: 4. Confocal microscopic images of untreated control U937 & K562 (A & C) and EMOB treated U937 & K562 (B & D) cells. The control cells were with intact nuclei whereas EMOB treated cells indicated apoptotic changes like nuclear disintegration and formation of apoptotic bodies, shown by the arrowheads. Cells were stained with DNA binding dye Hoescht 33342.

Fig: 5 Confocal microscopic images of untreated control U937 (a) and K562 (c) EMOB treated U937 (b), K562 (d) cells. U937 & K562 cells showed green fluorescent rings of externalized phosphatidylserine indicating the sign of apoptosis after 24 hrs of treatment with EMOB.
3.2.3. Study of phosphatidylserine (PS) externalization: Phosphatidylserine is predominantly accumulated in the inner leaflet of plasma membrane of living cells but in the apoptotic cells, phosphatidylserine is translocated from inner to outer leaflet of plasma membrane. Treatment with EMORB caused externalization of phosphatidylserine which after binding with annexin V-FITC gave green fluorescence. (Fig.5)

3.3 Agarose gel electrophoresis study
After Agarose gel electrophoresis, the DNA samples isolated from the untreated control U937 and K562 showed intact DNA bands whereas the DNA samples from U937 and K562 cells treated with EMORB and standard showed fragmented DNA bands in the form of ladders. (Fig.6) So, the observations confirmed that the treatment with EMORB, caused apoptosis in both U937 and K562 human leukemic cell lines.

![Image of Agarose gel electrophoresis](image_url)

Fig 6:- Lane 1 represents U937 and K562 control cells, lane 2 Ara-C (100μg), Imatinib (.372 μg) Mesylate, lane 3 represents EMORB IC<sub>50</sub> dose for U937 & K562 cells respectively.

3.4. Detection of Apoptosis by Flow Cytometric Analysis
In the flow cytometric analysis, double labelling technique, using annexin V FITC and propidium iodide, was utilized. Lower left (LL) quadrant (annexin V-/PI-) is regarded as the population of live cells, lower right quadrant (LR) (annexin V+/PI-) is considered as the cell population at early apoptotic stage, upper right (UR) quadrant (annexin V+/PI+) represents the cell population at late apoptotic stage and extreme upper right (UR) & upper left (UL) quadrant (annexin V-/PI+) is considered as necrotic cell population. (Fig.7) The Flow
cytometric data analysis revealed that after 18 h of treatment with IC50 dose of EMORB showed the presence of 10.4% of U937, 17.1% of K562 cells in LR quadrant (early apoptotic stage).

Fig.7 – (A) & (C) are the U937 & K562 controls and (B) & (D) are the EMORB treated cells respectively. Flow cytometric data analysis after 18 h of treatment with IC50 dose of EMORB showed the presence of 10.4% of U937 cells and 17.1% of K562 cells in LR quadrant (early apoptotic stage).

3.5 Study of Cell Cycle Arrest by Flow Cytometric Analysis

Flow Cytometric analysis showed that after 24 h treatment of U937 with EMORB at IC50 dose, DNA content increased in G1 phase (67.5% against 53.2%). In case of K562 cell line, DNA content increased in G1 phase (66.6% against 55.0%) after EMORB treatment.( Fig.8) These results indicated that drug treatment arrested the cell cycle of both the cell lines at G0/G1 phase as well as in G2/M phase.
Fig. 8: Flow cytometric analysis of cell cycle phase distribution in controls and treated EMORB at IC₅₀ dose on U937 & K562 cells K562 cells after 24 hrs 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.

3.6 Caspase-9 assay
Caspase-9 is one of the main initiator caspases and has been linked to the mitochondrial death pathway. To investigate whether treatment with EMORB induced apoptosis via intrinsic pathway, caspase-9 assays were performed in U937 and K562 cells. The experiments revealed significant increase in the caspase-9 activity in the cytosolic extract of EMORB treated at concentration of 30.22 µg/ml for U937 cells and 28.29 µg/ml for K562 cells (IC₅₀ dose) compared with that of the untreated control U937 and K562 cells respectively (Fig.9), supporting the fact that apoptosis induced by EMORB treatment might be mediated through the intrinsic pathway.
Fig.9: Effect of EMORB treatment on Caspase-9 activity in U937 and K562 cells after 24hrs at IC\textsubscript{50} dose with respect to control.

3.7 Caspase-3 assay

Caspases have long been considered the pivotal executioners of programmed cell death. A change in the mitochondrial membrane permeability results in the release of apoptosis factors from mitochondria, which activate caspases\cite{17}. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. The primary target of the caspase-9 is procaspase-3, one of the most deleterious effector caspases. To observe whether caspase-9 activated caspase-3 after treatment of extract i.e., EMORB at concentration of 30.22 µg/ml for U937 and 28.29 µg/ml for K562 cells (IC\textsubscript{50} dose), caspase-3 assays were performed. Caspase-3 activation was clearly observed in EMORB treated U937 and K562 cells when compared with that of the untreated control cells (Fig.10).

Fig.10: Effect of EMORB treatment on Caspase-3 activity in U937 and K562 cells after 24hrs at IC\textsubscript{50} dose with respect to control.
4. DISCUSSION
A significant part of drug discovery in the last forty years has been focused on agents to prevent or treat cancer. This is not surprising because, in most developed countries and, to an increasing extent, in developing countries, cancer is amongst the three most common causes of death and morbidity. Treatment for cancer involves surgery, radiotherapy and chemotherapy and often a combination of two or all three is employed \(^{18}\). Natural products provide an appreciable percentage of new active lead molecules, clinical candidates and drugs despite competition from different methods of drug discovery. The number of natural product derived drugs present in the total drug launches from 1981 to 2002 was recently analyzed \(^{19}\) and it was concluded that natural products are still a significant source of new drugs, especially in the anti-cancer and anti-hypertensive therapeutic areas.

Those morphological changes are a consequence of characteristic molecular and biochemical events occurring in an apoptotic cell, most notably the activation of proteolytic enzymes which eventually mediate the cleavage of DNA into oligonucleosomal fragments as well as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles. Apoptosis is in contrast to the necrotic mode of cell-death in which case the cells suffer a major insult, resulting in a loss of membrane integrity, swelling and disrupter of the cells. During necrosis, the cellular contents are released uncontrolled into the cell's environment which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue \(^{20}\). Apoptosis or programmed cell death (PCD) is a genetically encoded cell elimination program which ensures equilibrium between cell proliferation and cell death and by which damaged or unwanted cells are eliminated \(^{20}\). Therefore it is important to stress that apoptosis is a normal physiological process \(^{21, 22}\).

The present investigation confirmed the cytotoxic activity and apoptosis inducing ability of Ethanolic extract of Moringa Oleifera root bark (EMORB) against two human leukemic cell lines- U937 and K562. The cytotoxic activities of EMORB and its fractions F1 & F2 were supported by the observations in MTT assays respectively. EMORB inhibited the growth of the metabolic activities of U937 & K562 cells in a concentration-dependent manner. The MTT assay performed on normal lymphocytes revealed that the EMORB shows insignificant cell toxicity thereby indicating specificity against only leukemic cell lines. Apoptogenic activity of EMORB was investigated by different morphological studies like fluorescence
microscopic, confocal microscopic and phosphatidylserine (PS) externalization studies. 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. Fluorescence microscopic images clearly showed nuclear disintegration of EMORB treated leukemic cells compared with that of the untreated control cells when stained with acridine orange and ethidium bromide. The untreated control cells showed bright green fluorescence as the live cells with intact membrane excluded ethidium bromide and only acridine orange could enter into them. On the contrary EMORB treated cells showed more intense orange-red fluorescence and reduced green fluorescence since apoptotic and necrotic cells could not exclude the dyes and gave a combination of orange-red and green fluorescence. So, the observations indicated that the treatment with EMORB was inducing apoptosis in the leukemic cells. Apoptogenic activity of EMORB was further evidenced from the confocal microscopic images of the treated leukemic cells when compared with that of the untreated control cells. After EMORB treatment, U937 and K562 cells showed several signs of apoptosis like chromatin condensation, nuclear fragmentation and formation of apoptotic bodies whereas the untreated control cells were with intact nuclei. Externalization of PS from inner leaflet to outer leaflet of the membrane is the hallmark of early phase of apoptosis. Externally translocated PS binds with annexin V in a calcium dependent manner. Fluorescence microscopic images of treated U937 and K562 cells showed bright green fluorescent rings of externalized PS, supporting the fact that treatment with EMORB induced apoptosis in the leukemic cells. Further evidence in support of the apoptogenic activity of EMORB was obtained from the gel patterns of agarose gel electrophoresis. EMORB treated cells showed degraded DNA bands in the form of ladders, a typical indication of apoptosis, whereas the untreated control cells showed intact DNA bands when observed in UV transilluminator. Dual staining with annexin V FITC and propidium iodide in dot plot assay made it possible to identify live, early apoptotic and late apoptotic cells. Experiments showed increased number of cells in the early and late apoptotic stage after treatment with EMORB implying the fact that apoptosis was triggered by the treatment with EMORB in U937 and K562 cells. Cell cycle analysis revealed that treatment with EMORB arrested the U937 and K562 cell populations in the G0/G1 phase as well as in G2/M phase of cell cycle. Caspases, a family of cysteine proteases have emerged as key enzymes in the regulation of apoptotic pathway. Activation of Caspase leads to the classical form of apoptotic pathway. There are two major apoptotic pathways known to date, initiated by either the mitochondria (the ‘intrinsic’ pathway) or the cell surface receptors (the
'extrinsic' pathway). Mitochondria-mediated apoptosis occurs in response to a wide range of death stimuli, including activation of tumor suppressor proteins (such as p38 and p53) and oncogenes (such as c-Myc), DNA damage, chemotherapeutic agents, serum starvation, and ultraviolet radiation[26]. In the intrinsic pathway, diverse proapoptotic signals converge at the mitochondrial level triggering, caspase-9 activation initiating a downstream caspase cascade through the complex formation with Apaf-1, dATP, and pro-caspase-9 in the cytosol, which ultimately lead to the activation of the executioner caspase-3 and finally cell death[27]. Caspase-9 and caspase-3 assays showed concentration-dependent increase in the activities of caspase-9 and caspase-3 respectively after EMORB treatment in U937, K562 cells.

CONCLUSION
In conclusion, our study provides evidence that ethanolic extract of root bark of Moringa oleifera (EMORB) preferentially kills leukemic cells (particularly those of histiocytic lymphoma and erythroblastic cells) by triggering programmed cell death. EMORB induced cytotoxicity involved phosphatidylserine exposure, leading to altered DNA fragmentation and enhanced caspase activation. Thus, these findings add a new avenue to root bark of Moringa oleifera induced cell death pathways and significant promise for future cancer therapeutics. Further studies are underway to isolate the active compounds from the fractions and to explore signal transduction pathways regulating apoptosis induced by the active compounds in quest of an interesting formulation which can be used in the treatment of cancer.

ACKNOWLEDGEMENT
The authors are very grateful to Indian Institute of Chemical Biology, Kolkata for providing facilities to perform the work. The authors are also thankful to CSIR for providing the necessary funding for the project.

REFERENCES


