Research Article

Cytotoxic Activity of Bark of *Oroxylon indicum* Benth against U937and K562 Human Leukemic Cell Lines

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ABSTRACT

Cancer, a leading cause of death, is a condition where cells have lost control over their mode of growth. We evaluated the anti-proliferative and cytotoxic and apoptogenic activity of methanolic extract of *Oroxylon indicum* Bark (MEOIB) on U937, K562 cells and normal lymphocytes. The cell viability was studied by MTT assay. Cell morphology was determined by Fluorescence, Confocal microscope and DNA fragmentation by agarose gel electrophoresis study. MEOIB inhibited the viability of cells in a time & concentration dependent manner. The Morphologically it was found that externalization of PS from inner leaflet to outer leaflet of the cell membrane and condensed chromatin, membrane blabbing, fragmentation of nuclei in all the treated cells than control and DNA bands appeared in the form of ladder after treatment with MEOIB. The present study reveals that the MEOIB possesses potent anti-leukemic activity against U937 and K562 cell line but there no toxicity in normal lymphocytes.

Keywords: Bark, *Oroxylon indicum*, Phosphatidylserine, Leukemia, Apoptosis.

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INTRODUCTION

Oroxylon indicum is a small or medium sized deciduous tree up to 12 m in height with soft light brown or grayish brown bark with corky lenticels.[1] *Oroxylon indicum* is a deciduous tree growing throughout India, South Asia, South East Asia, Sri Lanka, Philippines, Indonesia, China, Bhutan, Malaysia and Malacca. It is found up to an altitude of 1200 m mainly in ravines, in damp region and moist places in the forests.[2] In India, it is distributed in Himalayan foothills, Eastern and Western Ghats and North East India.[3] The fresh root bark is soft and juicy; it is sweet, becoming bitter later. On drying, the bark shrinks, adhere closely to the wood and becomes faintly fissured. Mother Nature stands as an inexhaustible source of novel chemo types and pharmacophores, and has been a source of medicinal agents for thousands of years, and an impressive number of modern drugs find their origin in natural products.[4] Cancer remains as one of the fatal disease throughout the world and there is renewed interest in the discovery of novel compounds that can be used to fight cancer. Many plant derived anti-cancer agents especially are widely used for treatment of cancer. We performed some in-vitro studies first in normal human cells and then in U937 (Human leukemic monocyte lymphoma cell line) and K562 (Human erythromyeloblastoid leukemia cell line) to investigate the anti- leukemic activity of MEOIB.

MATERIALS AND METHODS

Chemicals

RPMI 1640 was purchased from Gibco, USA., fetal bovine serum, streptomycin and penicillin, L-glutamine, HEPES, Trypan blue, MTT [3-(4,5-dimethylthiozol-2-il)-2,5-2,5-diphenyltetrazoliumbromide], cytosine-arabinoside (Ara-C), acridine orange, ethidium bromide, agarose, ethylene diamine tetra-acetic acid (EDTA), proteinase K, propidium iodide, Annexin-V FITC, RNase A were purchased from Sigma–Aldrich, USA. DMSO (Dimethyl Sulfoxide), Sodium bi-carbonate, Phenol, Chloroform, Isoamyl alcohol, methanol, Sodium Chloride, Potassium Chloride, Di-sodium hydrogen phosphate, Potassium di-hydrogen phosphate (Merck). All other chemicals and reagents were of analytical grade and supplied by CSIR, Indian Institute of Chemical Biology, Kolkata.

Cell lines

Human leukemic cell lines U937 and k562 were purchased from National Facility for animal tissue and cell culture, Pune, India and supplied from Indian Institute of Chemical Biology for *In-vitro* studies. Cells were sub cultured weekly at an initial concentration of $1x10^5$ cells/ml and maintained in sterile RPMI 1640 medium supplemented with 10% heat inactivated FBS. Cultures were maintained at 37ºC in a humidified atmosphere containing 5% CO_2 in air.

Normal lymphocytes were extracted from a freshly batch of normal blood provided by a willing volunteer for *In-vitro* studies. Cells were sub cultured weekly at an initial

concentration of $1x10^5$ cells/ml and maintained in sterile RPMI 1640 medium supplemented with 10% heat inactivated FBS. Cultures were maintained at 37ºC in a humidified atmosphere containing 5% CO_2 in air.

Collection, extraction and preparation of the sample

The bark of *Oroxylon indicum* was collected from hills of Dumka, Jharkhand, India in the month of February, 2012. The bark of *Oroxylon indicum* powder (200gm) was soaked in about 1000ml methanol for three days at room temperature with occasional shaking. The mixture was then filtered by filter paper and the solvent was evaporated by rotary vacuum evaporator to produce the methanol free extract. Thus 2gm of methanol extract dark grey colour powder was obtained finally. This was stored at 4° C in airtight container and was designated as methanolic extract of *Oroxylon indicum* Benth bark(MEOIB) for the experiments. Stock solution was prepared as 1mg/ml from which desired doses were tested.

METHODS

Cell cytotoxicity study by MTT Assay

For cytotoxicity studies U937and K562 human leukemic cells $1x10^5$, 100µl cell suspension per well in a log phase were seeded in 96 well tissue culture plates. Cells are treated with freshly prepared 1mg/ml stock solution of MEOIB at different concentration and compared with standard anticancer drug Ara-c for a period 24,48 & 72hrs and kept at 37^0C in a humidified atmosphere containing 5% CO_2 in air untreated cells are served as control. At the end of treatment, 20µl of MTT [3-(4, 5-dimethylthiozol-2-il)-2,5-2,5- diphenyltetrazoliumbromide] 5mg/ml in PBS as stock solution was added to each well and incubated for another 4hrs. The MTT assay is colorimetric assays for measuring the activity of enzymes that reduce MTT to formazan dyes, giving a purple color. A solubilization solution DMSO (Dimethyl sulfoxide) 100µl is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance was taken at 492nm by micro plate manager (Reader type: Model 680 XR Bio-Rad laboratories lnc).[5]

Healthy human leukocytes culture and its viability study

After informed consent, 5 ml of blood was drawn (venipuncture) aseptically from healthy human volunteers (25-30 years of age) and transferred into sterile tubes containing 10U/ml heparin inside a laminar flow hood. Heparinized blood was diluted with equal volume of normal saline. 10 ml of diluted blood was carefully layered on 3 ml of Histopaque. It was then centrifuged at 1500 rpm for 20 min at room temperature. Leukocytes were collected from Histopaque-plasma interface and washed twice with normal saline. The cell pellet was resuspended in culture medium with density of $1x10^6$ cells/ml and cultured in a CO2 incubator with 95% O_2 and 5% CO_2 at 37°C for 24 h in presence and absence of MEOIB at the concentration of 10, 25 and 50µg/ml and standard anticancer drugs Ara-C (100µg/ml). Leukocytes were stimulated with 2.5 µg/ml of phytohemoagglutinin.[6] The extent of cytotoxicity was analyzed and expressed reduction of OD values in MTT assay.

Morphological studies for detection of apoptosis

Fluorescence microscopic studies

A fluorescence microscope was used to observe the changes in the cell membrane permeability and nuclear integrity of the leukemic cells after treatment with the MEOIB. U937 and K562 cells ($1x10^6$) were treated with 25 µg/ml of MEOIB for 24 h. The untreated control cells and the MEOIB treated cells were harvested separately, washed with PBS and then stained with acridine orange (100µg/ml) and ethidium bromide (100µg/ml) (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.

Confocal Microscopic studies

Leukemic cells ($1x10^6$) were treated with 25 µg/ml of MEOIB for 24 hrs. After 24 hrs the untreated control cells and MEOIB treated cells were harvested and washed with ice cold PBS. The cells were then stained with 10µg/ml of propidium iodide for 5 min. After mounting on slides the cells were observed to see the differences in nuclear morphology between the untreated and the BOIE treated leukemic cells under Confocal laser scanning microscope (Leica TCS-SP2 system, Leica Microsystems, Heidelberg, Germany) installed with an inverted microscope [Leica DM-7RB].[7] Images for propidium iodide were acquired from argon/krypton laser and UV laser line using 590 nm long pass filter for propidium iodide and 450 nm band pass filter for UV images.

Study of phosphatidylserine (PS) externalization

PS externalization was examined after treating the U937 and K562 human leukemic cells ($1x10^6$) with 25 µg/ml of for 2 MEOIB 4 hrs under Confocal laser scanning microscope (Leica TCS-SP2 system, Leica Microsystems, Heidelberg, Germany).The untreated and the BOIE treated cells were harvested separately, washed with ice cold PBS and Annexin V FITC binding buffer (10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$ $2H_2O$; 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.[8]

Detection of apoptosis by DNA fragmentation and agarose gel electrophoresis

U937 and K562 cells were treated with 25 µg/ml of MEOIB. The cells were harvested and washed twice with PBS. The cells were resuspended in 500µl of lysis buffer (50Mm Tris-HCl, Ph- 8.0, 10Mm EDTA, 0.5%SDS)100µg/ml of

Proteinase K was added incubation 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 4ºC overnight. To detect the DNA fragments the isolated DNA samples were electrophoresed overnight at 20V in1% agarose gel and stained with ethidium bromide. DNA fragmentation was observed in UV transilluminator.[9]

Statistical Analysis

All data are represented as arithmetic mean ± S.E.M. Statistical analysis was done by Student's t-test. A probability value of $p<0.05$ and $p<0.01$ were chosen as the criteria on of statistical significance.

Percentage of cell growth inhibition was calculated by the following formula: % Cell Inhibition = 100 x (OD of Control - OD of Treated / OD of Control), OD = Optical Density.

The percentage of cell viability was calculated as follows: Viable Cell (%) = (Total number of viable cells per ml / Total number of cells per ml) x 100

RESULTS

Cell Cytotoxicity study

In the cytotoxicity study or MTT assay of U937 and K562 cell lines, there was significant concentration and time dependent reduction in the O.D values compared to the untreated control cells after treating the cells with MEOIB. However, with same concentration of MEOIB on normal leukocytes insignificant reduction in the value was observed. With the same concentration, in normal human WBC cells, extract proved to be non-toxic to the cells, as confirmed by the O.D values. Thus, the studies showed an inhibition in cell viability in a concentration and time dependent manner.

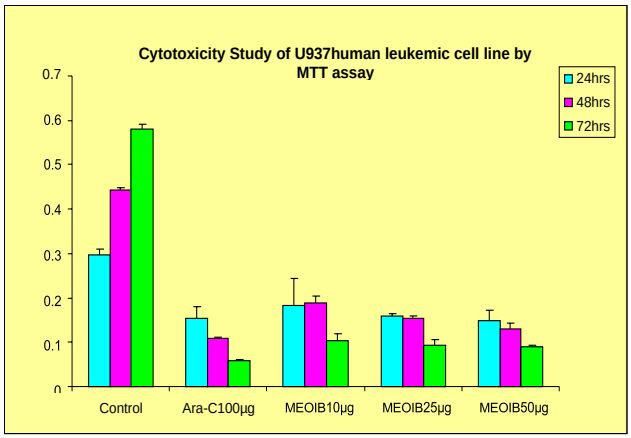

Figure 1: Histogram shows effect of MEOIB on U937 human leukemic cells after 24, 48 &72 hrs of treatment. The O.D Value is compared to the untreated control cells and standard drug (Ara-C) treated cells. Reduction in no. of cells is observed in a time and concentration dependent manner. Data are mean ± S.E.M.

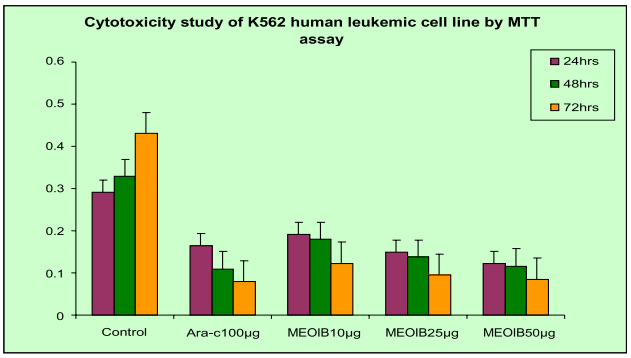

Figure 2: Histogram shows effect of MEOIB on K562human leukemic cells after 24,48&72 hrs of treatment. The O.D Value is compared to the untreated control cells and standard drug (Ara-C) treated cells. Reduction in no. of cells is observed in a time and concentration dependent manner. Data are mean ± S.E.M.

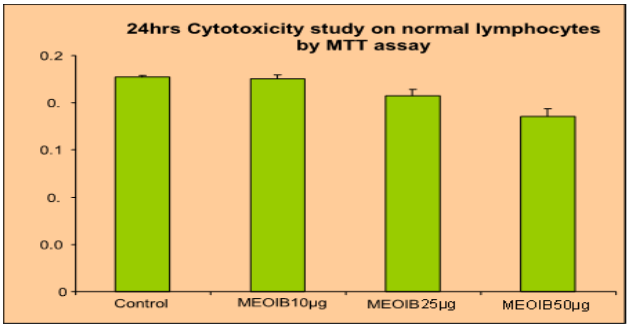

Figure 3: Histogram shows effect of MEOIB on normal human WBC after 24 hrs of treatment. Data are mean ± S.E.M.

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 MEOIB caused externalization of phosphatidylserine which after binding with Annexin V FITC gave green fluorescence.

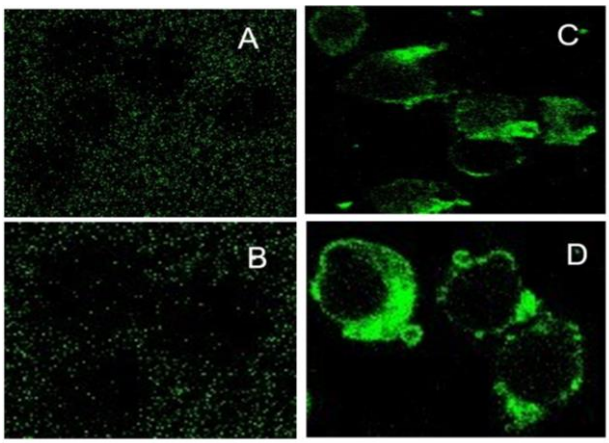

Figure 4: A & B control cells stained with Annexin –V-FITC and C & D represents methanolic extract of *Oroxylon indicum* Bark (MEOIB**)** treated U937 and K562 human leukemic cell lines. The MEBOI treated cells showed

green fluorescent rings of externalized phosphatidylserine indicating sign of apoptosis, after 24 hrs of MEBOI treatment as indicated by arrowheads.

Fluorescence microscopic studies

MEOIB treated U937& K562 cells stained with ethidium bromide and acridine orange, revealed the presence of apoptotic cell (early and late) as compared to the control cells. The fragmented nuclei and blabbed membrane were clearly observed in both the cell lines. Heterogeneous green fluorescence at greenish background and orange fluorescence at greenish background in BOIE treated cells were considered as early and late apoptotic cells.

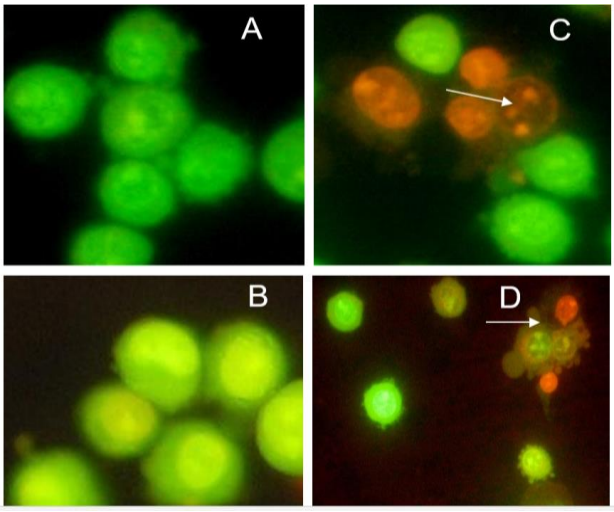

Figure 5: A and B represents control cells and C and D represents treated cells stained with DNA binding dye Acridine orange and Ethidium bromide.

Confocal Microscopic studies

MEOIB induced apoptotic changes in both the three leukemic cells after 24 hrs of treatment showing chromatin disintegration and formation of apoptotic bodies whereas the untreated control cells were with intact nuclei.

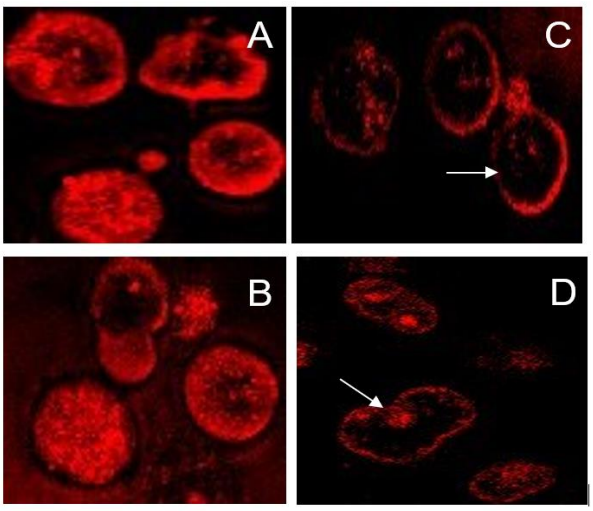

Figure 6: A and B represents the Confocal microscopic images of untreated control cells stained with Propidium iodide and C and D represents MEOIB treated U937 and K562 human leukemic cell lines.

DNA fragmentation by agarose gel electrophoresis

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 & K562 cells treated with MEOIB showed fragmentation. Therefore, the observations confirmed that the treatment with MEOIB, caused apoptosis in both the human leukemic cell lines.

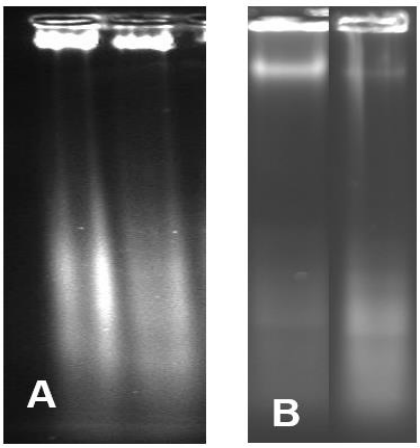

Figure 7: The gel pattern shows DNA ladder. DNA isolated from U937(A) and K562(B) human leukemic cell line. The lane-1 untreated control, lane 2-treated with MEOIB respectively.

DISCUSSION

A significant part of drug discovery in the last forty-five 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. Treatments for cancer may involve surgery, radiotherapy and chemotherapy and often a combination of two or all three is employed.[10] Death due to cancer is increasing worldwide though advancement in treatments is going on. There are some limitations in every mode of treatment available today for cancer. Pursuing drug clues against cancer from natural products constitutes one of the important areas in modern biology. The present study was conducted to explore the anticancer activity of the MEOIB on human leukemic cell lines U937 and K562. Cytotoxic effect of MEOIB was demonstrated by MTT assay. Fluorescence microscopic studies of the cell lines after drug treatment, using ethidium bromide and acridine orange, showed intense red and reduced green colour compared to untreated control which showed green fluorescence. Control cells have intact cell membrane and ethidium bromide could not penetrate the membrane. In MEOIB treated cells, cell membrane got ruptured to allow ethidium bromide to penetrate and give red fluorescence. Beside this, MEOIB treated leukemic cells showed chromatin condensation and

apoptotic body formation. So, the observations indicated that the treatment with MEOIB was inducing apoptosis in the leukemic cells. Apoptogenic activity of MEOIB was evidenced from the confocal microscopic images of the treated leukemic cells when compared with that of the untreated control cells. After MEOIB 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 calcium dependent manner. [11] Green, Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. During apoptosis or programmed cell death, a specific nuclease cleaved genomic DNA to generate DNA fragments and these fragmented DNA ladder is one of the hallmarks of apoptosis. [12] In the present study, MEOIB treated U937 and K562 cell lines showed DNA ladder indicating onset of apoptosis. We checked the effect of MEOIB on normal blood lymphocytes from healthy individuals. At same concentration uses on U937 and K562, MEOIB killed only about 3%, 5% and 10% of normal blood lymphocytes. So, it can be said that MEOIB at the same doses was not cytotoxic towards normal cells compared to leukemic cell lines.

CONCLUSION

From the above findings, it can be concluded that MEOIB exerts anti-leukemic activity in U937 and K562 cell lines through morphological changes and DNA fragmentation by apoptosis. As the bark of the plant, the source of the drug, are available in plenty, these may be exploited for getting the novel compound. Detailed studies to isolate, identify and establish the mechanism of action of MEOIB are however warranted.

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Authors Contributions

Moumita Ray, Subhodeep Roy and Dr. Chinmoy Choudhury contributed extraction of Test Sample and experiments. Dr. Shila Elizabeth Besra contributed as collection, identification of specimen, manuscript preparation, data curation and manuscript improvement.

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