BETULINIC ACID, A NATURAL BIO-ACTIVE COMPOUND: PROFICIENT TO INDUCE PROGRAMMED CELL DEATH IN HUMAN MYELOID LEUKEMIA

Sandeep Kumar Dash¹, Sourav Chattopadhyay¹, Satyajit Tripathy¹, Shib Shankar Dash², Balaram Das¹, Debasis Mandal¹, Braja Gopal Bag², Somenath Roy¹*

¹Immunology and Microbiology Laboratory, Department of Human Physiology with Community Health, Vidyasagar University, Midnapore-721 102, West Bengal, India.
²Department of Chemistry and Chemical Technology, Vidyasagar University, Midnapore, 721 102, West Bengal, India.

ABSTRACT
Betulinic acid (BA), plant derived pentacyclic Triterpenoid, exhibit selective anticancer functions in human cancer cells without showing harmful effects towards normal cells. The present study demonstrates that BA is highly effective against the human myeloid leukemia cell lines (KG-1A and K562). Selective anti-leukemic efficacy study of SA-BA was detected by time and dose dependent cell viability assay and based on this result 25µg/ml dose with 48 hours treatment period was selected for further experiments. It was observed that BA treatment significantly degrade the cellular redox state of KG-1A and K562 cells by elevating reactive oxygen species levels, which caused DNA damage and ultimately induced Apoptosis, confirmed by Annexin V-FITC + PI (Propidium iodide) dual staining using FACS. Involvement of caspase-3 was also noted for BA mediated apoptosis in KG-1A and K562 cells. Surprisingly, it was noted that BA treatment did not show harmful effects was noted for human blood lymphocytes.

KEY WORDS: Betulinic acid, leukemia, reactive oxygen species, apoptosis.
Abbreviations

BA: Betulinic acid
DMSO: Dimethyl sulfoxide
DTNB: 5, 5'-Dithiobis-(2-Nitrobenzoic Acid)
GSH: Reduced Glutathione
GSSG: Glutathione disulfide
H$_2$DCFDA: 2',7'-Dichlorodihydrofluorescein diacetate
HEPES: N-(2-hydroxyethyl)-piperazine-N-(2-ethanesulfonic acid)
MDA: Malondialdehyde
MMP: Mitochondrial membrane potential
NAC: N-acetyl-l-cysteine
PI: Propidium iodide
Rh-B: Rhodamine B
Rh-123: Rhodamine 123
ROS: Reactive oxygen species
TNF-α: Tumor necrosis factor alpha

INTRODUCTION

Secondary metabolites of various plants have been used to combat human diseases for several years, as they exhibit a wide range of biological properties that can be exploited for medical application.\(^1\) Triterpenes represent a varied class of natural products. Among them one of the most bioactive compound is betulinic acid (6-6-6-6-5 Pentacyclic Monohydroxy Triterpenic acid), showed diverse therapeutic potentials. Betulin, the reduced form of Betulinic acid (BA) was first isolated from plants in 1788 by Johann Tobias Lowitz and found to be a prominent constituent of the outer-bark of white-barked birch trees. Later on BA was extracted from heavy wood powder of *Ziziphus jujubae* tree and purified by column chromatography.\(^2\) A variety of biological activities have been described to BA including anti-inflammatory, anti-malarial and anti-HIV-1 effects. However, BA is most highly regarded for its activity and specific cytotoxicity against a variety of tumor cell lines, including melanomas, small and non-small cell lung carcinomas, ovarian, prostate and cervical carcinomas.\(^3\)

Leukemia is a type of cancer of the blood or bone marrow characterized by an abnormal increase of immature white blood cells called "blasts". Acute myelogenous leukemia (AML)
is a fast growing fatal form of leukemia produces immature white blood cells, initiates in bone marrow cells and quickly spreads into the blood system. Chronic myelogenous leukemia (CML) is an uncommon type of leukemia, making up about 15% of all cases of leukemia among adults, the consequences of a somatic mutation in the pluripotential lymphohematopoietic cell and thereby produces large no of white blood cells. Previous study showed that BA induce apoptosis upto 20µg/ml in human chronic myeloid leukemia cell line (K562) without altering the expression of Bcr-Abl gene and increasing DNA fragmentation followed by decreasing mitochondrial membrane potential (MMP).\(^4\) Another study at China showed that BA able to arrest the proliferation of K562 cells with an IC50 of 21.26 µg/ml at 24 h. BA treatment also increased the expression of the pro-apoptotic proteins Bax and caspase-3, was also found as a potent inhibitor of DNA Topo II enzyme on A549 cancer cell line with an ED50 value of 7.19 µM.\(^3\) Triterpenic acid is completely resistant to human peripheral blood mononuclear cells (PBMCs) with IC50 >30 µg/ml, but in case of HL-60 cell line the IC50 value was 20.70 ± 5.39 at 24 hr. This study also showed that BA induces time dependent increase in the sub G1 peak indicating the apoptotic phenomenon as obtained from the DNA content histogram analysis.\(^5\) Based on the above information, it can be clearly manifest that Severe side effects of various traditional and conventional chemotherapeutic is one of the major problem for cancer therapy. Thus, to overcome this important issue, phytochemical based anticancer drug development becomes the major thrust area of many researchers. BA, the naturally occurring bioactive agent, is an effective agent for cancer treatment. But till now evaluation of BA mediated anti-leukemic efficacy, especially towards human myeloid leukemia is not clearly studied. Thus, this study accentuates in depth evaluation of BA mediated selective cytotoxicity towards human acute myeloid leukemia cells (KG-1A) and chronic myeloid leukemia cells (K562) in in vitro experimental settings. Doxorubicin, the most potent chemotherapeutic agent, was taken as positive control for the study. Side-by-side, human peripheral blood lymphocyte (PBL) was taken as normal cell and any toxicity due to BA treatment was carefully investigated.

**MATERIALS AND METHODS**

*Culture Media and Chemicals*

N-acetyl-L-cysteine (NAC), Histopaque 1077, and Rhodamine B, RPMI 1640, penicillin, streptomycin, pentoxifylline (POF), doxorubicin were procured from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from GIBCO/Invitrogen. MTT was purchased from Himedia, India, Titron X-100, Tris–HCl, Tris buffer, Sodium dodecyl
sulphate (SDS), phenol, chloroform, iso-amyl alcohol, ethidium bromide (EtBr), 2-vinylpyridine were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. Commercially available dimethyl sulfoxide (DMSO) was procured from Hi-media, India, and was purified by vacuum distillation over KOH. All other chemicals were from Merck Ltd and SRL Pvt. Ltd. Mumbai, were of the highest purity grade available.

Synthesis and Purification of Betulinic Acid

Isolation, synthesis and purification of BA were already performed and reported previously. \[^2\] Briefly, the heavy wood bark powder of *Ziziphus jujuba* (1 Kg) was first extracted with petroleum ether (60°C-80°C) for 21 hours duration to remove the grease and non-polar materials. Then, the powdered material was extracted using diethyl ether (30 hrs) which produces a greenish white solid (18 g). After that, the crude product was dissolved in dehydrated methanol (1.8 liter) under hot conditions and subsequently decolorized with charcoal and filtered under hot condition. The volatile materials were removed under reduced pressure to afford again a greenish white solid (15.98 g). The purification process of the materials was done by column chromatography (silica gel, 100-200 mesh) using 5% EA/DCM as eluant afforded BA as a whitish solid (9.1 g, 0.91 % yield).

Physical Measurements

Purified BA was successfully characterized by \(^1\)H NMR, \(^{13}\)C NMR and Reversed-phase HPLC analysis. Self-assembled property of BA was examined by Optical polarized microscopic images (OPM), Scanning electron microscopy (SEM), Atomic force microscopy Images (AFM), X-Ray Diffraction (XRD) studies were previously reported. \[^2\]

In the present study, we have examined the anti-leukemic potential of the BA against two leukemic cell lines (KG-1A and K562).

Cell Lines Culture and Maintenance

The two leukemic cell lines, KG-1A (AML) and K562 (CML) cell lines were obtained from NCCS, Pune (India). These cell lines were cultivated and maintained in RPMI-1640 complete media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin under 5% CO\(_2\) and 95% humidified atmosphere at 37°C in CO\(_2\) incubator. Cells were cultured and maintained in exponential phase until number of cells reaches at 1.0 X 10\(^6\) cells/ml.
Selection of Human Subjects for Collection of Lymphocytes

For collection of human peripheral blood lymphocytes (PBL), six healthy human subjects were chosen to collect the blood sample for separation of lymphocytes. The selected subjects enrolled in this study were asymptomatic and none of them had abnormality on physical examinations and routine laboratory tests. The subjects were from same geographical area and same economic status, nonsmokers and non-alcoholic, and having same food habit. These subjects received no medication, including vitamin-E and vitamin C. All subjects gave informed consent. The selection excluded not only individuals with acute infections or chronic diseases, but also excluded healthy individuals undergoing supplementation with antioxidants. The study protocol was in accordance with the declaration of Helsinki, and was approved by the Institutional Ethical Committee (IEC) of Vidyasagar University. [6]

Isolation of Peripheral Blood Lymphocytes

Blood samples were collected from these six healthy human volunteers by vein-puncture in 5 ml heparin coated Vacutainers satisfying the method of Hudson and Hay [7]. Five milliliters of blood were diluted 1:1 with phosphate buffered saline (PBS) and layered onto Histopaque 1077 (Sigma) by using a Pasteur pipette and centrifuged at 400 x g (1500 rpm) for 40 min at room temperature. The upper monolayer of buffy coat i.e lymphocytes was transferred using a clean Pasteur pipette to a clean centrifuge tube and washed three times in balanced salt solution. The peripheral blood lymphocytes (PBL) were re-suspended in RPMI complete media supplemented with 10% FBS and incubated for a day at 37°C in a 95% air/5% CO₂ atmosphere in CO₂ incubator.

Drug Preparation

A 10 mg/ml stock of BA was prepared by dissolving 10 mg of BA in DMSO. Stock concentrations of BA were then serially diluted with RPMI media to prepare working concentrations. The amount of ethanol and DMSO for each concentration, was never exceeded >0.75%.

Experimental Design

Each type of cells was divided into 11 groups. Each group contained 6 petri dishes (2 X 10⁵ cells in each). The cells of each petri dish of control and experimental groups were maintained in RPMI 1640 media, supplemented with 10% FBS, 50 μg/ml gentamycin, 50 μg/ml penicillin and 50 μg/ml streptomycin at 37°C in a 95% air/5% CO₂ atmosphere in CO₂.
The following groups were considered for the experiment and cultured for 12, 24, 36, 48 and 72 hrs.

Group I: Control i.e., Cells + culture media; Group II: Cells + 1 μg/ml DOX in culture media; Group III: Cells + 1 μg/ml BA in culture media; Group IV: Cells + 5 μg/ml DOX in culture media; Group V: Cells + 5 μg/ml BA in culture media; Group VI: Cells + 10 μg/ml DOX in culture media; Group VII: Cells + 10 μg/ml BA in culture media; Group VIII: Cells + 25 μg/ml DOX in culture media; Group IX: Cells + 25 μg/ml BA in culture media; Group X: Cells + 50 μg/ml DOX in culture media; Group XI: Cells + 50 μg/ml BA in culture media.

After the treatment schedule the cells were collected from the petridishes separately and centrifuged at 2,200 rpm for 10 min at 4°C to separate cells and sups. The cells were washed twice with 50 mM PBS, pH 7.4. A required amount of cells were lysed using hypotonic lysis buffer (10 mM TRIS, 1 mM EDTA and Titron X-100, pH 8.0) for 45 min at 37°C and then processed for the biochemical estimation. Intact cells were used for mitochondrial membrane potential and different microscopic observations.

**Selection of Dose and Duration of BA by Cell Viability Assay**

The dose and duration dependent cytotoxicity of DOX and BA on PBL, KG-1A and K562 cell lines were quantitatively estimated by a non-radioactive, colorimetric assay system using tetrazolium salt, 3-[4,5-dimethylthiazol- 2-yl] -2,5-diphenil-tetrazolium bromide (MTT). The percentage of proliferation was calculated by using the following equation:

\[
\text{% Proliferation} = \frac{\text{OD sample} - \text{OD control}}{\text{OD control}} \times 100
\]

The potent, as well as a biocompatible dose of BA was selected based on the cell viability assay. All measurements were done in triplicate.

**In vitro Drug Uptake Assay**

The cellular internalization of BA in PBL, KG-1A and K562 were performed in vitro drug uptake assay using fluorescence microscopic imaging. Briefly, Rh-B labeled BA was prepared through the following process. Ten μg/ml BA dissolved in 10% DMSO was conjugated with 25μl (1 mg/ml) of Rh-B. This mixture was stirred for 24 hours at 37°C using magnetic starrier (REMI, India). Then, these fluoro-labeled BA was separated by centrifugation at 4°C. The obtained sediment was washed with de-ionized water and re-dispersed. This process was repeated three times to remove the un-reacted Rh-B. Finally, the
obtained Rh-B labeled BA (Rh-B-BA) was dispersed in culture medium for *in vitro* experiment.

PBL, KG-1A and K562 cells were plated at a density of 2× 10^4 cells/Petridis (35 mm) for 24 h. Rh-B tagged BA at 25 µg/ml dose were incubated for 6 hours at 37°C in a 95% air/5% CO₂ atmosphere in CO₂ incubator. After defined time, the cover slips were removed and the cells were washed 2 times with PBS (50 mM) and immediately observed in green light under the fluorescence microscope (NIKON ECLIPSE LV100POL) for uptake assessment. Images were acquired at 400X optical zoom and analysis was done using ImageJ software v.r. 1.43 (NIH).

**Determination of Reduced Glutathione (GSH) Level**

Reduced GSH estimation in the cell lysate was performed according to previously described method.\(^{[10]}\) The required amount (2X10^5) of all types of cell lysate was mixed with 25% of trichloroacetic acid and centrifuged at 2000 RPM for 15 min to settle the precipitated proteins. The supernatant was aspirated and diluted to 1 ml with 0.2 M sodium phosphate buffer (pH 8.0). Later, 2 ml of 0.6 mM DTNB was added. After 10 min the optical density of the yellow-colored complex formed by the reaction of GSH and DTNB (Ellman’s reagent) was measured at 405 nm. A standard curve was obtained with standard reduced glutathione. The levels of GSH were expressed as µg of GSH/mg protein. All measurements were done in triplicate.

**Determination of Oxidized Glutathione (GSSG) Level**

The oxidized glutathione level was measured after derivatization of GSH with 2-vinylpyidine according to the method of KarMahapatra et al.\(^{[10]}\) In brief, with 0.5ml sample, 2 µL of 2-vinylpyidine was added and incubated for 1 hour at 37°C. Then the mixture was deproteinized with 4% sulfosalicylic acid and centrifuged at 1,000×g for 10min to settle the precipitated proteins. The supernatant was aspirated, and GSSG level was estimated with the reaction of DTNB at 412 nm in spectrophotometer and calculated with standard GSSG curve. The levels of GSSG were expressed as µg of GSSG/mg protein. All measurements were done in triplicate.

**Intracellular Reactive Oxygen Species (ROS) Measurement**

Intracellular ROS measurement was performed using H₂DCFDA according to our previously reported method.\(^{[9]}\) In brief, normal PBL, KG-1A and K562 cell lines (2X 10^5 cells/ml) were
treated with BA at 25µg/ml for 48 hours. As a positive control, those cells were incubated with H₂O₂ (100 µM) for 30 min prior to the analysis. [11] After the specified treatment schedule cells were washed with culture media followed by incubation with 1 µg/ml H₂DCFDA for 30 min at 37°C. Then the cells were washed three times with fresh culture media. DCF fluorescence was determined at 485 nm excitation and 520 nm emission using a Hitachi F-7000 Fluorescence Spectrophotometer. All measurements were done in triplicate.

**Pre-Treatment with N-Acetyl- L-Cysteine**

To understand the involvement of ROS in BA induced leukemic cell death, PBL, KG-1A and K562 cells were seeded in a 96-well plate at 0.2 ml per well at a concentration of 2 X 10⁵ cells per milliliter. A stock solution of N-acetyl- L-cysteine (NAC; Sigma- Aldrich) was made with sterile water and added to cells at 5 and 10 mM for 1 h. After NAC pre-treatment, cells were treated with BA (25 µg/ml) for 48 h. Viability was determined by the MTT method. [9] All measurements were done in triplicate.

**Cellular Morphology Analysis by Acridine Orange (AO)–Ethidium Bromide (ETBR) Double Staining**

To confirm the probable pathway of cell death, we analyzed both leukemic cells by EtBr-AO double staining method. A number of 2x10⁴ PBL, KG-1A and K562 cells were seeded into each well of a 6-well plate and incubated for 24 h at 37 °C in a humidified 5% CO₂ atmosphere. BA at 25 µg/ml dose, then added into the well for 48 hours. After incubation, cells were washed once with phosphate buffer saline (PBS). Ten microlitres of the cells were then put on a glass slide and mixed with 10 µl of acridine orange (50 µg/ml) and ethidium bromide (50 µg/ml). The cells were viewed under a fluorescence microscope (NIKON ECLIPSE LV100POL) with 400X magnification. [12]

**Assessment of Nuclear Morphological Changes by DAPI Staining**

For observation of nuclear morphological changes of PBL, KG-1A and K562 cells after BA treatment DAPI staining was used. In brief, all the test cells were seeded into six well plates and a number of 2X10⁵ cells/ml were treated with or without BA (0, and 25 µg/ml) for 48hr and were then isolated for DAPI staining according to the method of Lin et al [13] with some modification. After treatment, the cells were fixed with 2.5% glutaraldehyde for 15 min, permeabilized with 0.1% Triton X-100 and stained with 1 µg/ml DAPI for 5 min at 37°C. The cells were then washed with PBS and examined by fluorescence microscopy (NIKON ECLIPSE LV100POL).
DNA Damage Assay

After the treatment with BA, PBL, KG-1A and K562 cells were re-suspended in 270 µL pre-cooled lysis buffer (10mM Tris-HCl, 10mM NaCl, 10mM EDTA, pH 7.4) with 30µL SDS (10%). RNase A (final concentration 100 µg/ml) was then added, and incubation was continued at 45°C for 45 min. Subsequently, proteinaseK (final concentration 100 µg/ml) was added to the cellist, and incubation was continued at 50°C for overnight to complete digestion. DNA was isolated from the list using phenol/chloroform/ISO-amyl alcohol. Then, DNA was precipitated with one volume of 10M sterile ammonium acetate, and two volumes of absolute ethanol followed by centrifugation at 13000×g for 10min at 4°C. The extracted DNA samples were washed with 70% ethanol and dissolved in 50 µL TE buffer (10mM Tris-HCl, 1mM EDTA, pH-7.6). Gel loading buffer (10mM EDTA, 0.25% bromophenol blue, 30% glycerol) was then added to the DNA sample at 1:5 ratio and loaded onto a 1.2% agarose gel. The electrophoresis was carried out at 50V for 90min in TBE buffer (90mMTris-HCl, 2mMEDTA, 90mMboric acid, pH-8.0). After electrophoresis, DNA was visualized by soaking the gel in TBE buffer containing 1.5 µg/ml ethidium bromide in UV light, and the picture was captured in Bio-Rad gel documentation system. [10]

Assessment of Apoptotic Cell Population by Annexin V/PI Double-Staining Assay

PBL, KG-1A and K562 cells (1x10^6) were seeded in Petri discs and incubated for 24 hours at 37°C. Then, BA (0, and 25) was directly added to the dishes and incubated for an additional 48 hour, respectively. After the treatment with BA, PBL, KG-1A and K562 cells were collected, washed with PBS and re-suspended in PBS. Apoptotic cell death was confirmed by double staining with recombinant FITC-conjugated Annexin V and PI, using the Annexin V-FITC Apoptosis Detection kit (E-bioscience, India) according to the manufacturer's instructions. Flow cytometric analysis was performed immediately after dual staining. Data acquisition and analysis were performed in a Becton-Dickinson FACS verse flow cytometer using CellQuest software.

Caspase-3 Activity

Involvement of apoptosis was measured by measuring Caspase 3 level using ELISA according to manufacturer’s instructions (eBioscience, India). After the treatment with BA (25µg/ml for 48hr), PBL, KG-1A and K562 cells were lysed using lysis buffer and 10 µl of each treated and untreated cell extracts were taken and used as samples for Caspase 3 activity at 450 nm wave length [14]. All measurements were done in triplicate.
Protein Estimation

Protein was determined according to Lowry et al. \[15\] using bovine serum albumin as Standard.

Statistical Analysis

The data were expressed as mean ± SEM, n=6. Comparisons between the means of control and treated group were made by one-way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA 01060 USA) with multiple comparison t-tests, p < 0.05 as a limit of significance.

RESULTS

Cell Viability Assay

The toxicity of the BA and DOX was checked towards normal lymphocytes KG-1A and K562 cell lines in vitro. BA mediated cytotoxicity to these normal cells and cell lines were measured by MTT assay. Fig. 1A showed that BA did not show any significant toxic effect on normal human lymphocytes upto 25µg/ml dose. Only very low amount of dead PBL cells was observed at 50 µg/ml dose but it is not significant. Whereas DOX killed the lymphocytes significantly (p<0.05) by 62.87% at 5 µg/ml dose and reached 81.86 % at the last dose used. BA killed the KG-1A and K562 cell lines in a dose dependent fashion. It was observed that BA significantly (p<0.05) decreased the viability of KG-1A cells from 5µg/ml dose and reached at 67.58% when 50 µg/ml as compared with untreated cells. In K562 cells, BA also showed significant cytotoxic effects. The viability of K562 cells were significantly (p<0.05) decreased by 40.84% at a 5µg / ml dose and reached at 61.15% when 50 µg/ml dose was used as compared with untreated cells. In the same line of treatment, DOX killed the KG-1A cell and K562 cell by 38.15%, 52.36%, 67.98%, 75.82%, 82.07% and 32.43%, 51.75%, 70.02%, 79.89%, 87.54% respectively at a dose of 1, 5, 10, 25 and 50µg/ml; whereas all these changes were significant at the level of p<0.05as compared with untreated cells (Fig. 1A).

As it was found that up to 25µg/ml dose of BA is completely biocompatible dose, so this dose was selected for the rest of the experiments. In, selection of the proper duration of treatment, this dose was selected and incubation time varied incubation period were tasted. Time dependent killing kinetic assay showed that 25 µg/ml dose of BA for 48 hour incubation played maximal anti-leukemic effects on KG-1A and K562 cells. Beyond this time interval no significant increase of leukemic cell death was observed (Fig. 1B). So, from
the time dependent killing kinetic assay 48 hour duration was selected for the rest of the experiments.

**Drug Uptake Assay**

Cellular internalization of BA was performed by fluorescence microscopy. Successful tagging of BA with Rh-B was applied for this purpose. The fluorescence imaging revealed that Rh-B labeled BA was successfully taken up by KG-1A (Fig 2C-D) and K562 cells (Fig 2E-F). A very little amount of fluoro-labeled BA was internalized in PBL (Fig 2A-B). It was observed that BA was distributed throughout the cells, indicating cellular uptake instead of adhering to the cell surfaces. This selective internalization may be due to receptor mediated endocytosis.

**Examination of Cellular Redox Status (GSH and GSSG level)**

From Fig 3 and Fig 4 it was found that in PBL, DOX (25 µg/ml) treatment significantly (p<0.05) decreased GSH level by 55.94% and significantly (p<0.05) elevated GSSG level by 148.306% as compared to control. But treatment with BA (25 µg/ml) in PBL showed completely reverse action. Here, BA treatment increased GSH level by 28.87% and decreased GSSG level by 28.99% as compared with control. Alteration of GSH and GSSG level in leukemic cells was observed to be same manner. DOX treatment significantly (p<0.05) diminished the level of GSH by 74.25% and increased GSSG level by 183.57% in KG-1A cells; in case of K562 cells DOX treatment significantly (p<0.05) decreased GSH level by 60.61% and significantly (p<0.05) increased GSSG level by 107.20% compared with the control group. BA exposure significantly (p<0.05) diminished GSH level by 67.92% in KG-1A cells and by 67.73% in K562 cells. GSSG levels of BA treated KG-1A cells was increased by 128.50% and also increased by 62.71% in K562 cells at the effective dose compared with the control group.

**Estimation of Cellular ROS Level**

Intracellular reactive oxygen species level was shown in Fig. 5. From Fig 5A, it was found that treatment with H$_2$O$_2$ (100 µM) elevated cellular ROS level by 3.81, 5.19 and 4.71 fold in PBL, KG-1A and K562 cells respectively. BA treatment has not significantly altered ROS level in PBL, but in both leukemic cells the intracellular ROS level was highly significant (P<0.05). BA treatment was able to significantly (p<0.05) increased ROS level by 3.28 fold in KG-1A cells and by 2.87 fold in K562 cells as compared with the control group.
To uncover whether ROS played crucial role in BA persuaded leukemic cell death, we pre-treated KG-1A and K562 cells by 2 and 5mM of NAC, a potent ROS inhibitor, for 4 hours before BA exposure and cell viability was estimated by MTT method. It was observed that pre-treatment with 5mM NAC effectively protected the cells from BA induced cytotoxicity. Cell viability of KG-1A cells was reached to 91.94% and to 93.82% in K562 cells compared with control group (Fig. 5B).

**Et Br-AO Double Staining for Cell Morphology Study**

Cell morphology study after BA treatment using Et Br-AO double staining is shown in Fig.6. From the data it was clear that BA treatment was able to decrease the number of viable KG-1A and K562 cells tremendously. Most of the cells exhibited typical characteristics of apoptotic cells like plasma membrane blebbing and formation of apoptotic bodies. The significant number of cells stained with orange colour was increased. A very small amount of cells were stained as red colour. These indicate that most of the cells were not undergoing necrosis and cell death occurred primarily through apoptosis. In case of PBL, no significant apoptotic changes were observed upon treatment with BA (Fig 6A-6I).

**Nuclear Morphological Changes Using DAPI Staining**

The nuclear specific stain, DAPI preferentially stains the nucleus by binding strongly to A-T rich regions in DNA, which is observed as blue fluorescence when excited under fluorescence microscope (excitation 330–380 nm, emission 430–460 nm). In our present study, DAPI staining exposed the changes associated with apoptosis in KG-1A and K562 cells treated with the BA (Fig. 7). The morphological changes were occurring with characteristics of apoptosis such as chromatin condensation, nuclear fragmentation, and imagination of nuclei were manifest in KG-1A and K562 cells upon treatment with BA. In case of PBL, no significant apoptotic changes were observed upon treatment with BA.

**DNA Damage Study**

In order to delineate the mechanism of leukemic cell death mediated by BA, we performed DNA damage assay, which is one of the strong characteristic of apoptosis. From DNA damage study by agarose gel electrophoresis a typical ladder pattern of internucleosomal damage was observed in both cancer cells. Smear like DNA damage were found at 25μg/ml of BA treatment. In case of PBL, BA treatment did not induce any significant DNA damage at the selected dose. These data suggest that BA is a potent inducer of apoptosis in both KG-1A and K562 cells (Fig 8).
Assessment of Apoptotic Cell Population by Flowcytometry

Double staining examination of apoptosis and necrosis using Annexin V+FITC fluorescence (FL1) and PI fluorescence (FL3) gave different cell populations wherein FITC negative and PI negative cells were selected as viable cells, FITC positive and PI negative were apoptotic and FITC positive and PI positive were defined as late apoptotic. Only PI positivity showed necrotic or completely dead cell populations. As it was manifest from Fig. 9, KG-1A and K562 cells treated with 25µg/ml BA, showed increased Annexin-V positivity. Maximum numbers of the cells (44.14% in KG-1A and 42.49% in K562 cells) gave both FITC and PI signals which suggested the cells undergoing late stage apoptosis. Lower number of the PI positive population was observed in both leukemic cells (1.42% in KG-1A and 2.82 % of K562 cells). In case of PBL, BA treatment did not show any significant apoptotic or necrotic cell population.

Induction of Caspase-3 Activity

Caspase-3 plays a regulatory role in the apoptotic pathway mediated cell death, which was induced following exposure to BA treatment in KG-1A and K562 cells (Fig. 10). When KG-1A and K562 cells were treated with BA (25µg/ml) for 48 hrs, caspase-3 activity increased significantly with compared to untreated cells. In case of PBL, Caspase-3 activation was not observed.

Figures
Figure 1

Figure 2

A

Gray Scale

B

Fluorescence

C

Overlay

D


E


F


G


H


I
Figure 3

Figure 4
Figure 5

Figure 6
Figure 7

Figure 8
Figure 9

Figure 10
Figure Legends

Figure 1: A. *In vitro* cell viability assay of DOX and BA treated PBL, KG-1A and K562 cell lines. Cells were treated with DOX and BA for 48 hours at 37 °C. Cell viability was measured by the MTT method as described in materials and methods. B: *In vitro* killing kinetic assay of BA on PBL, KG-1A and K562 cells. Values are expressed as mean ± SEM of three experiments; superscripts indicate significant differences (p < 0.05) compared with the control group.

Figure 2: A-I: Intracellular uptake of BA on PBL, KG-1A and K562 cells by fluorescence imaging. A required amount of cells was treated with Rhodamin-B labeled BA (25 µg ml⁻¹) for 6 h. Intracellular uptake was examined using fluorescence microscope. Here, A-C: BA treated PBL, D-F: BA treated KG-1A cells, G-I: BA treated K562.

Figure 3: Intracellular reduced glutathione (GSH) levels of DOX treated and BA treated PBL, KG-1A and K562 cell lines. The levels of GSH were expressed as µg of GSH/mg protein. Values are expressed as mean ± SEM of three experiments; superscripts indicate significant differences (p < 0.05) compared with the control group.

Figure 4: Intracellular oxidized glutathione (GSSG) levels of DOX treated and BA treated PBL, KG-1A and K562 cell lines. The levels of GSSG were expressed in term of µg of GSSG/mg protein. Values are expressed as mean ± SEM of three experiments; superscripts indicate significant differences (p < 0.05) compared with the control group.

Figure 5: A. Effects of DOX and BA on ROS induction in PBL, KG-1A and K562 cell lines. DCF fluorescence intensity was expressed in term of ROS production. Values are expressed as mean ± SEM of three experiments; superscripts indicate significant differences (p < 0.05) compared with the control group. Intensity of control cells was set to 1.00. Data is represented as fold change of the ROS level in the control group. B. Quenching of ROS rescues KG-1A and K562 cells from BA induced cytotoxicity. KG-1A and K562 cells were pre-treated with 2 and 5mM NAC for 4-6 hrs and then subsequently exposed to BA 25 µg/ml dose. Cell viability was estimated by MTT assay.

Figure 6: Qualitative characterization nuclear morphology by EtBr/AO dual staining using fluorescence microscopy. After the treatment schedule PBL and leukemic cells were incubated with EtBr/AO. At the end of EtBr/AO, cells were washed with PBS and they were
visualized by fluorescence microscopy at excitation/emission wave length 490/620 nm. Here, (A): PBL control, (B): PBL treated with BA; (C) KG-1A control, (D): BA treated KG-1A cells, (E): K562 control and (F): BA treated K562 cells.

**Figure 7:** Qualitative characterization nuclear morphology by DAPI staining using fluorescence microscopy. After the treatment schedule PBL and leukemic cells were incubated with DAPI. At the end of DAPI exposure, cells were washed with PBS and they were visualized by fluorescence microscopy at excitation 330–380 nm and emission 430–460 nm. Here, A: PBL control, B: BA treated PBL, C: KG-1A control, D: BA treated KG-1A cells, E: K562 control and F: BA treated K562 cells.

**Figure 8:** Determination of DNA fragmentation by agarose gel electrophoresis method. Here, C1: KG-1A control; T1: KG-1A (25 µg/ml BA treated); C2: K562 control; T2: K562 (25 µg/ml BA treated); C3: PBL control; T3: PBL (25 µg/ml BA treated);

**Figure 9:** Estimation of apoptotic cell population by FACS using Annexin-V/PI staining. The percentage of cell population of BA treated PBL, KG-1A and K562 cells at Lower Left (LL: viable cells), Lower Right (LR: Annexin V-FITC positive apoptotic cell), Upper Left (UL: Annexin V-FITC and PI dual positive), Upper Right (UR: Only PI positive necrotic cells) was estimated before and after treatment. Values are expressed as mean. Here, (A): PBL control, (B): BA treated PBL, (C): KG-1A control, (D): BA treated KG-1A cells, (E): K562 control and (F): BA treated K562.

**Figure 10:** Estimation of caspase-3 activity by ELISA technique. Values are expressed as mean ± SEM of three experiments; superscripts indicate significant differences (p < 0.05) compared with the control group.

**DISCUSSION**

Phytochemical and different microbial extracts from various biological origins are routinely screened for biological activities, and it is assessed that approximately 20% to 25% of new drugs are derived from natural products or their synthetic analogues [16] BA is a naturally occurring pentacyclic triterpenoid has been shown to possess a variety of biological activities, including the inhibitory effect of human immunodeficiency virus (HIV), anti-bacterial, anti-malarial, anti-inflammatory, anti-helmintic and anti-oxidant properties [17] Recent studies showed that BA had strong anti-proliferative actions on various kinds of tumor cells, [18] such
as prostate cancer, \cite{16} small cell lung cancer, non-small-cell lung cancer, oophoroma, and cancer of the cervix, but exerted no effect on normal cells. \cite{19}

This study showed that BA significantly killed the KG-1A and K562 cells selectively in a dose-dependent manner, with no toxic effects on PBLs (Fig. 1A). Cytotoxic have been defined as the cell killing property of a chemical compound independent from the mechanism of death. \cite{20} It was found that there was only a slight decrease of PBL viability treated with 50µg/ml BA was noted. Thus, before this dose, i.e. 25 µg/ml was selected for further experiments. The result obtained from killing kinetic assay (Fig 1B) clearly showed that BA produces potent anti-leukemic effects at 48 hours of exposure. Collectively, 25 µg/ml dose of BA with 48 hrs incubation period was selected. Comparative cytotoxicity study of BA with doxorubicin, the most potent anticancer drug showed that though BA killed lower amount of leukemic cells as compared to doxorubicin but BA showed good compatibility with PBL. The non-toxic effects of BA towards normal cell was previously observed by researchers \cite{21-22} and those finding supports the outcome of our present study.

Cellular uptake of bioactive drug is one of the important parameters for testing, bio efficacy of the compound. Adequate uptake of the bio-active compound is proportional to the relative bio-activity. In our study, Rh-B labeled BA was used \textit{in vitro} for examination of BA internalization in PBL, KG-1A and K562 cells. BA was found to be distributed in the cytoplasm of KG-1A and K562 cells. In PBL, a very little internalization of BA was observed. This indicated that cellular uptake was associated instead of adhering to the surface and the BA was preferentially targeted the cancer cells and were internalized. (Fig.2). Due to insignificant detectable internalization of BA in PBL, no toxic effects were found and on the other side, it maintained cellular redox balance which was confirmed by further experiments. Alteration of lipid metabolism is one of the hallmarks of cancer cells. Changes and dysregulated expression of lipid metabolizing enzymes are directed towards oncogenic signals and the proteins involved in this process are excellent chemotherapeutic targets for cancer treatment. \cite{23} In addition, tumor cell membranes contain a higher amount of phospholipids than normal cells. \cite{24-25} The presence of a high amount of phospholipids may influence the attachment of BA to the cancer cell membrane, entered the cell and allows apoptosis followed by necrosis by activation of different biochemical processes. This selective uptake of BA was responsible for selective cytotoxic effects which were found from cell viability study.
Glutathione, an important cellular reluctant, is involved in protection against different free radicals, cellular peroxides, and toxic compounds in cellular systems. [26-27] This study showed that GSH level in normal cells increased significantly (p<0.05) when treated with BA, but decreased significantly (p<0.05) when treated with DOX. It was clearly understood that BA maintains the normal cellular redox balance which is very much necessary for cellular metabolic process. From my result, it was found that GSH levels in cancer cell line were decreased significantly (p<0.05) when treated with BA and DOX. In case of cancer cell lines BA can alter the cellular redox balance and helps these cells towards the oxidative damage. From my experiment, it was observed that GSSG level in normal cells slightly decreased when treated with BA, but increased significantly (p<0.05) when treated with DOX. It was clearly understood that BA is less toxic to normal cells than DOX. From my experiment, it was observed that GSSG level in cancer cells increased significantly (p<0.05) when treated with BA and DOX.

ROS are the molecules and ions containing unpaired valence shell electrons and being a free radical it is highly active and shows an important role in cell signaling regulation, leading to oxidative cell damage and ultimately cell death. [28] The physiologically active cellular system normally develops lower amount of ROS during metabolism, which is effectively quenched by many antioxidant enzymes of glutathione system. In addition, intracellular ROS generation occurs by the mitochondrial respiratory chain reaction, membrane-bound superoxide-generating enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and arachidonic metabolic reaction. [29-30] In the present study disrupted cellular metabolic activity due to treatment of BA in KG-1A and K562 cells confirmed by MTT assay may deserve one of the suitable cause for ROS mediated leukemic cell death (Fig 5A). It was found that pretreatment with NAC significantly protected the KG-1A and K562 cells from BA induced toxicity (Fig. 5B). Restoration of leukemic cell viability >89% suggested that ROS is the main agent which played a crucial anti-leukemic potential caused by BA. Increased levels of ROS stimulate the release of different pro-inflammatory markers, including TNF-α. This TNF-α activates nuclear factor-κB (NF-κB) and c-Jun N-terminal kinase (c-Jun NH2-terminal kinase, JNK), as well as ultimately produces cell death by apoptotic and necrotic pathways. [31-32] To observe the probable pathway of cell death occurs due to apoptosis or necrosis, we analyzed the cells by EtBr-AO double staining method. The cellular morphology associates with BA treatment observed with Et Br-AO double staining are shown in Fig 6. This typical staining reveals that the viable cells with intact DNA and
nucleus give around and bright green nuclei, whereas early apoptotic cells will have fragmented DNA and gives several green colored nuclei. The late apoptotic and necrotic cell’s DNA would be fragmented and stained orange and red. In our study, it is evident that BA treatment drastically decreases the number of viable KG-1A and K562 cells, but did not show any apoptotic or necrotic effects on PBL. Most of the leukemic cells exhibit typical characteristics of apoptosis, including plasma membrane blabbing and formation of apoptotic bodies. The maximum population of KG-1A and K562 cells stained with an orange color and a very small amount of cells are stained as red color. Collectively, these results indicate that most of the leukemic cells of the two myeloid cell lines are not undergoing necrosis and cell death occurs primarily through apoptosis. To investigate changes of nuclear morphology of PBL, KG-1A and K562 cells due to treatment of BA, we stained the BA exposed cells by DAPI staining. It was found that KG-1A and K562 cells showed significant morphological changes in nuclear chromatin after BA treatment for 48 hrs (Fig 7). Chromatin condensation and fragmentation is one of the major characteristics of apoptosis. In case of PBL no such nuclear changes were observed and the PBLs conserved their normal intact nucleus which supports the compatibility of BA towards normal cells. DNA damage study on agarose gel electrophoresis revealed that BA shows potent DNA damaging activity on both leukemic cells supports the results obtained from previous experiments. The DNA damage is caused by the activation of an endogenous nuclear endonuclease that selectively and characteristically cleaves the double-stranded nuclear DNA at sites located between nucleosomal units (such as linker DNA), generating mono- and oligonucleosomal DNA fragments. The smear like DNA pattern was observed in the DNA of BA treated KG-1A and K562 cells in agarose gel electrophoresis. It has been suggested that DNA digestion in apoptotic cells is an ordered of multiple progression, which may involve the collaborative action of a number of endonucleases and proteases, and that the nature of the endonucleases activation may be cell type specific. On the other side, no-such damage of DNA was noted for BA treated PBL (Fig 8). The induction of apoptosis associates with BA treatment and estimation of exert population of viable cells, apoptotic cells and necrotic cells were confirmed by dual staining analysis of Annexin V+FITC. Annexin V+FITC fluorescence (FL1) and PI fluorescence (FL3) gave different cell populations wherein FITC negative and PI negative cells were selected as viable cells, FITC positive and PI negative were apoptotic and FITC positive and PI positive were defined as late apoptotic. In our study BA treatment significantly increased the number of late apoptotic cells in both leukemic cells. Induction of apoptosis was slightly higher for KG-1A cells than K562 cells (Fig 9). This may be due to the maturity
of the two leukemic cells. KG-1A cells are more immature than K562 cells.\textsuperscript{[36]} Side by side very small population (insignificant) of PBL was found for Annexin V+FITC positive, suggested that normal cells are resistant to BA. Involvements of caspases are key mediators of programmed cell death (apoptosis). Among the caspase proteins, caspase-3 is a commonly triggered death protease by catalyzing the specific cleavage of many key cellular protein associates with normal metabolism. In the present study, BA treatment was able to significant (P<0.05) elevation of caspase-3 activity in both leukemic cells as compared to untreated cells. Here also slightly higher caspase-3 activity was noted for KG-1A cell line. Caspase-3 is also essential for some typical hallmarks of apoptosis, and it is indispensable for apoptotic chromatin condensation and DNA fragmentation which was observed from Et Br/AO and DAPI staining. In case of PBL, caspase-3 elevation did not find effectively after BA treatment.\textsuperscript{[37]}

Moreover, in spite of the lower potency compared with DOX, BA seems to be selective inducer of myeloid leukemic cells, without showing any toxic impact on normal lymphocytes. Thus, this study supports the use of BA in further preclinical and clinical studies on a broad spectrum of malignant tumors.

**CONCLUSION**

In this study, we found that BA showed potent anti-leukemic activity towards KG-1A and K562 cell lines without showing toxic impact on human blood lymphocytes. Selectivity and biocompatibility are the two basic requirements of anti-cancer drug. Considering this fact, we observed that BA showed selective cytotoxicity on both leukemic cells occurred by the alteration of cellular redox balance, disruption of cellular oxidative stress and thereby induction of apoptosis phenomenon confirmed by FACS analysis. It was also revealed that generate ROS played a vital role behind the BA induced leukemic cell death. Excess production of ROS triggers the apoptosis process through activation of caspase 3 in both leukemic cell lines. Hence, this study elicited the probable mechanism of action of BA towards KG-1A and K562 cells, thus this molecule can be used as a lead molecule for generation of drugs in leukemia treatment and management.

**ACKNOWLEDGEMENT**

The authors express gratefulness to the USIC, Vidyasagar University, Midnapore, for providing the facilities to execute these studies. We are heartily thankful to all the healthy human volunteer’s for providing the blood samples.
Conflicts of Interests

The authors declare there are no conflicts of interest.

REFERENCES


