Induction of apoptosis by a potent Betulinic acid derivative in Human colon carcinoma HT-29 cells

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Abstract- Traditional medicines or herbal formulations can serve as the source of potential new chemotherapeutic drugs. Nowadays novel plant derived natural products have been designed to determine biological structure-activity relationships. A new family of betulinic acid derivatives were synthesized and the structure-activity relationship of the compounds was initially evaluated by comparing their in vitro cytotoxicity against 4 human cancer cell lines U937, HepG2, HT29, MCF-7 and non cancerous human PBMC. Betulinic Acid derivative, BC-SM-05 was the most potent inducer of apoptosis and the most effective inhibitor of cell growth and proliferation with IC_{50} value 19.6 µM for colon carcinoma cell line HT-29. In addition, BC-SM-05 at IC_{50} concentration was also found to generate increased ROS production and caspases activation. Moreover, HT-29 cells treated with BC-SM-05 and stained with Hoechst 33258 showed formation of apoptotic bodies and DNA degradation whereas untreated cells had intact nuclei. All together our data indicates that BC-SM-05 induces apoptosis in HT-29 cells via mitochondrial dependent pathway may prove itself to be a potential therapeutic agent for colon cancer, providing a basis for the development of the compound as a novel anticancer agent.

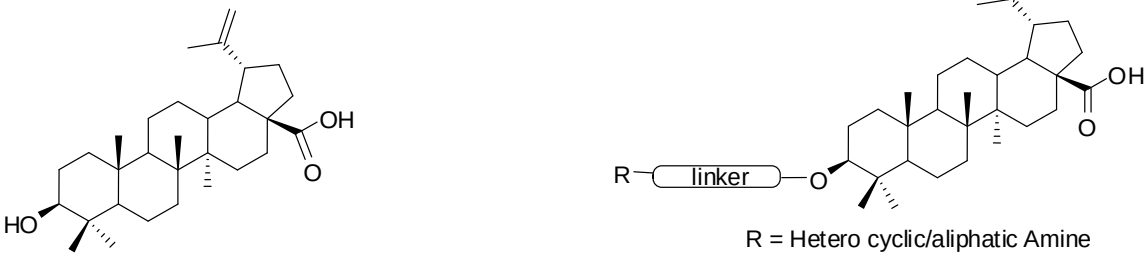
Index Terms- Triterpenoids, Betulinic acid, Cytotoxic activities, Apoptosis, ROS, Caspase activation, DNA fragmentation.

I. INTRODUCTION

Cancer, one of the threatened most disease is a leading cause of death in the developed world and the second leading cause of death in the developing world (1). According to a recent report by the World Health Organization, there are now more than 10 million cases of cancer per year worldwide. Chemotherapy is still far from producing a satisfactory result especially in underdeveloped countries due to its adverse side effects, high cost, prolonged treatment protocol and alarming increase in the incidence of drug resistance. Traditional medicines or herbal formulations can serve as the source of potential new chemotherapeutic drugs. So, development of novel plant derived natural products and their derivatives for anticancer activity are increasing day by day. The present study is designed to determine anticancer activity of some newly synthesized derivatives of natural triterpenoid compound (Betulinic Acid). Several triterpenoids, including ursolic and oleanolic acid, betulinic acid, celastrol, pristimerin, lupeol and avicins possess antitumor and anti-inflammatory properties. Some synthetic triterpenoid derivatives have been synthesized to improve antitumor activity such as cyano-3, 12-dioxooleana-1, 9 (11)-dien-28-oic (CDDO), its methyl ester (CDDO-Me), and imidazolide (CDDO-Im) derivatives. An increasing number of triterpenoids have been reported to exhibit cytotoxicity against a variety of cancer cells without manifesting any toxicity in normal cells. (2, 3, 4) Among them pentacyclic lupane type of triterpenoids are very important classes of natural products. The compounds of this class which includes betulin and betulinic acid were found to show significant antitumor activity on a broad panel of cancers (5, 6, 7). Betulinic acid was found to cause cancer cell death by induction of apoptosis involving caspases. Moreover it was demonstrated that betulinic acid was well tolerated in mice up to 500 mg/kg with no toxic effects (8). The induction of apoptosis is through the induction of changes in mitochondrial membrane potential, production of reactive oxygen species, and permeability of transition pore openings (9). These processes lead to the release of mitochondrial apoptogenic factors, activation of caspases, and DNA fragmentation (10, 11, 12). Betulinic acid, by an antioxidant mechanism tends to protect congenital melanocyte naevi cells from UV-C-induced DNA strand breakage independent of p53 and p21 (13). Betulinic acid induces weak inhibitory effects against topoisomerase I and IIa, but does not stabilize the topoisomerase IIa–DNA complex (14). Betulinic acid is active *in vivo* against TPA-induced tumors (15, 16).

It is found in the bark of several species of plants, principally the white birch (*Betula pubescens* syn. *alba*) (17) from which it gets its name. In 1995, betulinic acid was reported as a selective inhibitor of human melanoma. (18) Then it was demonstrated to induce apoptosis in human neuroblastoma *in vitro* and *in vivo* in model systems.(19) Currently, it is undergoing development with assistance from the Rapid Access to Intervention Development program of the National Cancer Institute.(20)

In this study, Betulinic acid was extracted from *Dillenia indica* which is also called Elephant apple.



Betulinic acid (BA) **Derivative of betulinic acid**

Scheme 1: **Betulinic acid and its designed Derivative**

The diversity centres in betulinic acid are C-1, C-2, C-3, C-4, C-20 and C-28 positions and the derivatives resulted on various structural modifications at these positions screened for their anticancer activity.

In order to achieve the designed derivatives of betulinic acid, various nitrogenous heterocyclic (BC-SM-01 to BC-SM-04) or aliphatic amine (BC-SM-05) moieties were attached through linker with the hydroxyl group located at C3 position of betulinic acid and assessed the potency of these compounds to act as an inducer of cell death in different cancer cell lines and normal epithelial cell line.

II. Materials and Methods

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) except 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (USB Corporation, USA), HEPES (1X) liquid (Gibco® BRL, USA), Heat Inactivated Fetal bovine serum (Gibco® BRL, USA), DMSO (Merck, India), RPMI 1640 (Molecular Probes, Life Technologies Corp., USA), Hoechst 33258 (Calbiochem, USA) and CM-H2DCFDA 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (Invitrogen, USA). All the tissue culture ware was obtained from Tarsons.

Ethics Statement

Ethical approvals for the study and consent procedure were obtained from Internal Review Board (Ethical Committee on Human Subjects) of CSIR-Indian Institute of Chemical Biology. All clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki.

Cell Lines

Four human cell lines U937-leukemic monocytic lymphoma, HT-29-colon carcinoma, MCF-7-breast adenocarcinoma, HepG2-Hepato carcinoma were collecteded from National Centre for Cell Sciences, Pune, India. All of cells were maintained in RPMI-1640 medium except HepG2 which was maintained in high glucose DMEM. Both the media were supplemented with 10% FBS and antibiotics (50 IU/ml penicillin G and 50 µg/ml streptomycin). The cells were incubated at 37^0C in a humidified incubator containing 5% CO_2 and subcultured every 72 h using an inoculum of 5 X 10^5 cells /ml. Cell viability (>95%) was confirmed by trypan blue exclusion.

Isolation of Human Peripheral Blood Mononuclear Cells (PBMC)

PBMC were isolated from anticoagulated blood of six healthy donors by density gradient centrifugation using an equal volume of Ficoll-Hypaque (Histopaque-1077) at 400 x g for 30 mins. Then, PBMC were harvested from the interface and washed twice in phosphate buffered saline (PBS, 0.01 M, pH 7.4). After that resuspended in RPMI-1640 medium supplemented with penicillin (50 IU/ml), streptomycin (50 µg/ml) and 10% FBS. Cell viability was confirmed by trypan blue exclusion (>95%).

Preparation of Betulinic Acid Derivatives

Methanolic extracts of Betulinic acid (BA) was prepared from of the *Dillenia indica* fruits according to literature procedure. (21) An analogue library was prepared by our chemistry collaborators based on betulinic acid and their structure-activity relationship (SAR) studies in cancer cells. Towards this objective, functionalisation at C-3 hydroxy of betulinic acid was carried out followed by "click reaction" resulting in the formation of new derivatives. These compounds were purified (>98% purity) by HPLC separations and identified using spectral analysis.

Cell Viability Assay

The half maximal inhibitory concentration (IC_{50}) is a measure of a compound effectiveness to inhibit biological or biochemical function. The MTT assay is based on the bio-reduction of the tetrazolium salt MTT into soluble purple formazan by mitochondrial dehydrogenase active enzymes, expressed only in metabolically active cells and therefore viable cells. This product can be assayed calorimetrically. The amount of formazan produced is therefore directly proportional to the number of viable cells present.

Mitochondrial dehydrogenase

MTT (Yellow) ⟶ **Formazan (Purple)**

The cytotoxic activity of all betulinic acid derivatives dissolved in DMSO (final DMSO concentration <0.1%) were evaluated in U937, HT 29, HepG2, MCF-7 and PBMC using

MTT assay. Briefly, cells (1.25–2.5 X 10^4 cells/100 µl of RPMI 1640 or high glucose DMEM medium/well) were seeded in 96-well tissue culture plates and incubated with DMSO dissolved betulinic acid derivatives (using 0-50 µM concentration) for 48 h at 37^0C, 5% CO_2. A solution of MTT (5 mg/ml) was prepared in 1X PBS (0.02 M, pH 7.2) and stored at -20 ºC until used. It was stored in the dark. After treatment, cell viability was measured by adding 20 µl MTT (5 mg/ml in PBS) and incubated for 4 h at 37^0C. Then 100 µl DMSO was added to each well, resultant optical densities were measured at 540 nm in an ELISA Reader (BIO RAD, CA, USA). According to the specific absorbance that represented formazan production was calculated by subtraction of background absorbance from total absorbance. The mean percentage viability was calculated as follows:

$$\frac{\text{Mean specific absorbance of treated cells}}{\text{Mean specific absorbance of untreated cells}} \times 100$$

The results were expected as IC_{50} values which was enumerated by graphical extrapolation using Graph Pad Prism software (version 5, Graph Pad Prism software Inc, San Diego, CA, USA). Each experiment was performed at least three times and in duplicate.

Measurement of Intracellular Reactive Oxygen Species (ROS)

Elevated level of ROS generation can damage DNA, protein and lipids and ultimately causes apoptosis of cancer cells. To investigate the effect of BC-SM-05 i.e. the lead molecule on generation of ROS, CM- H2DCFDA–lipid soluble, membrane permeable non-fluorescent reduced derivative of 2, 7-dichlorofluorescein was used. This When chemically reduced, the dye remains non fluorescent but after cellular oxidation and removal of acetate groups by cellular esterase it becomes fluorescent and is measured by flow cytometry at an excitation wavelength of 485 nm and emits at 530 nm.

After treatment with IC_{50} concentration of BC-SM-05 for 0-2 h, cells were washed with PBS (2000 x g, 5 min), resuspended in PBS and then incubated with CM-H2DCFDA (5 µM in1X PBS) for 30 min at 37^0C in dark. Then, cells were again washed and resuspended in 500 µl of PBS. DCF fluorescence was determined by flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. (FACS Calibur, Becton Dickinson, USA) using forward vs. side scatter to gate the cell population and a FL1 histogram to quantify fluorescence of viable cells. The subsequent analyses were done using BD CellQuest Pro software. To confirm the elevated levels of ROS induced by BC-SM-05 for the inhibition of ROS generation, cells were pre-incubated with N-Acetyl cysteine (NAC), an established anti-oxidant (2.5 mM) for 3 h before treatment with BC-SM-05 and analyzed. Mean fluorescence intensities (MFI) were obtained using the FACS Diva software.

Caspase activity analysis

A family of cysteine proteases, caspases play the key role for activation of apoptotic pathway. Active caspases cleave the chromophore from the enzyme substrate which is measured as a proof of caspase activation. The enzymatic activity of caspase 8, 9, 3 was assayed in cell lysates (100 µg protein in 50 µl lysis buffer) using colorimetric assay kits as per the manufacturer's instructions. Briefly, control and BC-SM-05 treated (19.6 µM; 0–48 h) HT29 cell ($2.5x10^5$/ml) were washed with ice cold PBS, cell lysates were prepared and subsequently protein concentration was estimated. Lysates were combined with reaction buffer and incubated with specific colorimetric peptide substrates (Ac-IETD-pNA for Caspase-8, Ac-LEHD-pNA for Caspase-9, Ac-DEVD-pNA for Caspase-3; 4 mM, 5 µl) at 37°C for 6 h. The emission of paranitroanilide (pNA) was measured at 405 nm in an ELISA reader every 30 minutes for 6 h.

Cellular and Nuclear Morphology Analysis

The Hoechst, a fluorescent stains are also commonly used to visualize nuclear damage. Hoechst 33258 is excited at around 350 nm and emits blue/cyan fluorescence light around an emission maximum at 461 nm. HT 29 cells were seeded (2.5 x 10^5 cells/ ml) in a 6 well tissue culture plate. Drugs (BC-SM-05, IC_{50} = 19.6 µM) were added. Plates were incubated for 48 hours at 37^0C in a humidified 5% CO2 incubator. Hoechst 33258 was prepared in MilliQ water at a concentration of 5µg/ml. Cells were washed twice in PBS. Cell were fixed in 4% paraformaldehyde and incubated at 4^0C for 30 minutes. Cells were again washed twice in PBS. Hoechst 33258 was added at a final concentration of 5µg/ml and incubated at 37^0C for 30 minutes under dark conditions. Cells were again washed twice in PBS. (22) This was spotted on to poly-L-lysine coated slides and mounted using 50% glycerol. Slides were then analyzed in a laser scanning confocal microscope (Leica TCS SP2 System Leica Microsystem, Heidelberg, Germany, 100X). At least 20 randomly selected microscopic fields were observed per sample.

III. RESULTS

Cytotoxic activity of Betulinic Acid derivatives:

The cytotoxic activity of betulinic acid derivatives (BC-SM-01 to BC-SM-05) were evaluated by MTT assay on various cancer cells such as U937, HT-29, HepG2, MCF-7 as well as normal human PBMC (Table 1) using different concentrations (0-50 µM) of betulinic acid derivatives for 48 h.

As shown in Table 1, derivative BC-SM-05 exhibited minimum IC50 value (19.6 µM) among all the betulinic acid derivatives. So, we may conclude that derivative BC-SM-05 was the most potent inhibitor of HT-29 cells with IC50 value 19.6 µM. DMSO concentration used to solubilize these compounds was 0.1% which had no effect on cell viability.

For our study, we synthesized a number of betulinic acid derivatives (BC-SM-01 to BC-SM-05) based on modifications (heterocyclic or aliphatic amine) at C3 position of betulinic acid . Interestingly, when methyl amine (-CH_2NH_2) group was used for this derivative preparation (BC-SM-05), the highest cytotoxicity was observed against Human colorectal adenocarcinoma, HT-29 cancer cell line whereas negligible cytotoxic to normal human PBMC. Hereafter, we studied apoptotic pathway on HT-29 cell line only with BC-SM-05 as we found it as lead molecule.

Compound BC-SM-05 causes excessive ROS generation:

Enhanced ROS generation is an established hallmark of apoptotic cells death that results in oxidative stress and cellular

damage. They are a by-product of a number of natural cellular processes such as oxygen metabolism and inflammation. Excessive ROS generation beyond a certain threshold level render cancer cells towards apoptosis. To determine whether BC-SM-05 induced apoptosis is mediated by oxidative stress; the intracellular level of ROS was measured in HT-29 cells. We found that BC-SM-05 (19.6 µM for 0–2 h) was capable of producing ROS within 30 min in HT-29 cells and highest ROS generation found at 240 min (Fig. 1). To determine whether BC-SM-05 was the only factor responsible for excessive ROS generation, cells were pre-incubated with NAC (2.5 mM) for 3 h before treatment with BC-SM-05 (19.6 µM; 30 min) and ROS was similarly quantified and then ROS level was found to near about control cells (Fig. 1). Values are expressed as Mean Fluorescence Intensity±SEM of three independent experiments (**p,0.01, ***p,0.001).

Caspases activation by BC-SM-05

During apoptosis cytochrome c is released from depolarized mitochondria. When cytochrome c is reached at cytosol, it causes activation of caspases, the main mediator of apoptosis. Caspase activation is a confirmatory evidence towards intrinsic pathway of apoptosis. Activation of caspases-8, -9 and -3 were measured using quantitative detection of colorimetric tetrapeptide substrates. As shown in Fig. 2A and 2B, activities of caspases -3 and -9 respectively increased significantly after 48 h treatment with BC-SM-05. In contrast, caspase-8 (Fig. 2C) did not increase significantly even after 48 h treatment of derivative BC-SM-05 as compared to control.

Compound BC-SM-05 causes DNA fragmentations

DNA degradation is the main characteristics of apoptosis. So, we have checked the nuclear fragmentation level of BC-SM-05 treated HT-29 cells using Hoechst 33258 dye. As shown in Fig:3, HT-29 cells treated with IC_{50} (19.6 µM) concentration of derivative BC-SM-05 resulted in gradual degradation of DNA and formation of apoptotic bodies in a time dependent fashion.

IV. Discussion

Cancer cells that avoid apoptosis continue to proliferate uncontrollably, which results in an increased tumor mass. So, induing apoptosis in cancer cells without affecting the normal cells is a key process to control cancer development and progression. As natural products are potential alternative source of safer chemicals to fight against cancer. We designed five derivatives of betulinic acid (BC-SM-01 to BC-SM-05) modified at its C3 position and found when methyl amine ($-CH_2NH_2$) group was attached to C3 position of betulinic acid (BC-SM-05), the highest cytotoxicity was shown against HT-29 cancer cells whereas negligible cytotoxic to normal human PBMC. Then we investigated that whether BC-SM-05 induce apoptosis in HT-29 cells by set up different hallmarked experiments on apoptosis like ROS measurement, Caspase Activity Assay and Nuclear morphology study.

V. Conclusion

We found BC-SM-05, modified with methyl amine ($-CH_2NH_2$) group attached at C-3 position of betulinic acid as the most potent inducer of apoptosis in HT-29 cells. We showed that BC-SM-05 was key factor responsible for elevated level of ROS generation in HT-29 cells, also causes caspases activation. Furthermore, it triggered DNA degradation. All together our findings suggest that BC-SM-05 induces mitochondrial pathway dependent apoptosis in HT-29 cells.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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Table 1: Cytotoxic activity of betulinic acid derivatives on different cancer cell lines

Compounds	Cell growth inhibition in terms of IC_{50} (μM)				
	U937	HT29	HepG2	MCF-7	PBMC
BC-SM-01	>50	38.2±0.9	49.8±0.5	>50	>50
BC-SM-02	39.98±0.7	>50	>50	>50	>50
BC-SM-03	45.8±0.4	36.9±1.3	>50	>50	>50
BC-SM-04	>50	39.7±2.2	>50	>50	>50
BC-SM-05	29.65±1.1	19.6±0.2	39.8±0.6	45.76±1.2	>50

Figure 1. Analogue BC-SM-05 triggers redox imbalance in HT 29 cells

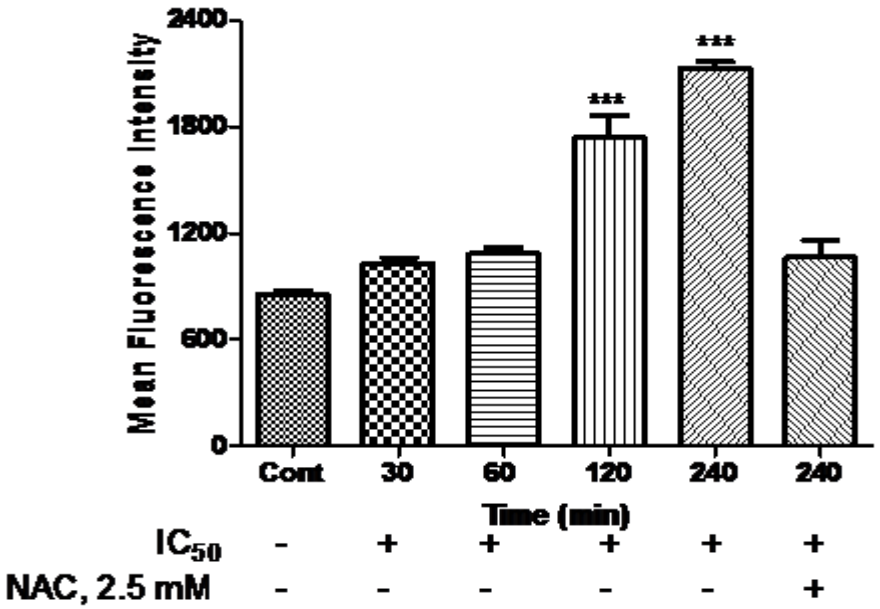
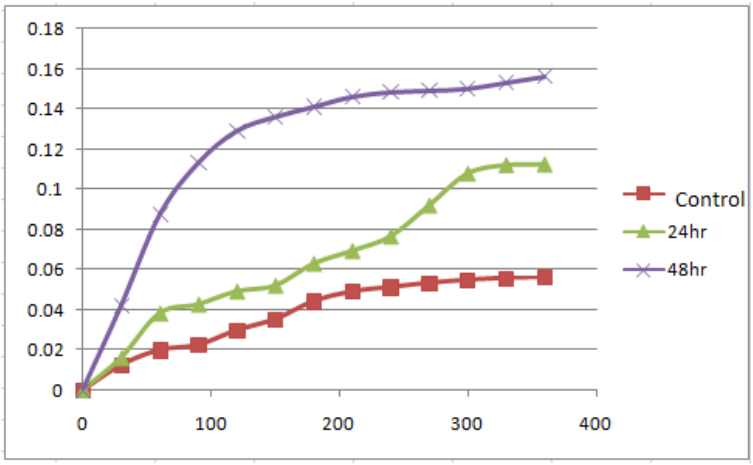
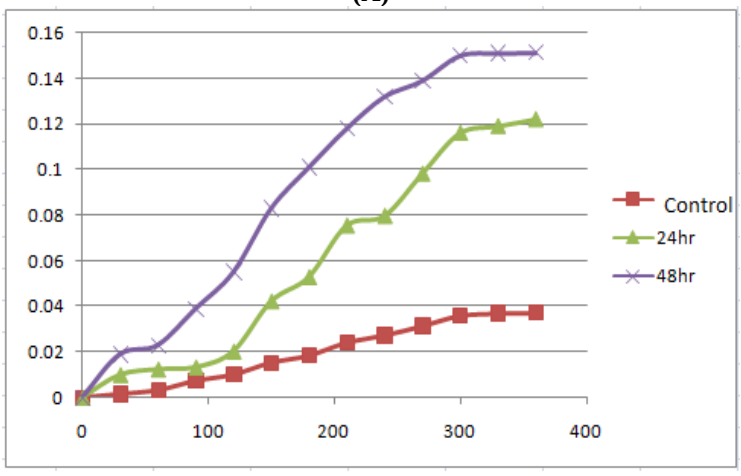


Figure 2. Activation of caspases by BC-SM-05



(A)



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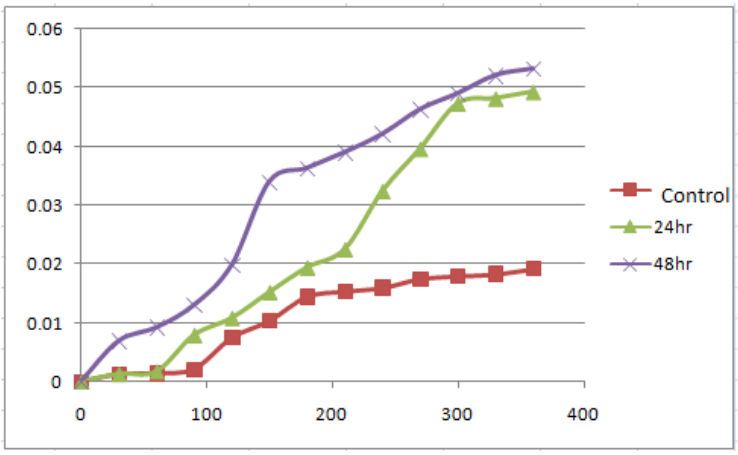

(C)

Figure 3: Nuclear fragmention induced by 2c BC-SM-05