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Case Report

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BETULINIC ACID-MEDIATED CELL CYCLE ARREST, DECREASE IN MITOCHONDRIAL MEMBRANE POTENTIAL AND SUCCINATE DEHYDROGENASE ACTIVITY IN MURINE ASCITES DALTON'S LYMPHOMA

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ABSTRACT

Betulinic acid is a pentacyclic triterpenoid originally isolated from the bark of the white birch *Betula pubescens*, from which it got its name. Betulinic acid exhibits cytotoxicity in several cancer cell lines and the antitumor potential of betulinic acid against murine ascites Dalton's lymphoma has been earlier reported by us. Present studies were undertaken to further explore and evaluate the possible mechanism of antitumor activity of betulinic acid with reference to cell cycle analysis, changes in mitochondrial membrane potential and succinate dehydrogenase activity in Dalton's lymphoma cells. Cell cycle analysis using flow cytometry, changes in mitochondrial membrane potential and succinate dehydrogenase activity in Dalton's lymphoma cells were determined after betulinic aid treatment. *In silico* molecular docking study was done to scrutinize possible interaction of succinate dehydrogenase with betulinic acid. Betulinic acid treatment caused cell cycle arrest at S phase and also decreased mitochondrial membrane potential and succinate dehydrogenase activity in Dalton's lymphoma cells. Molecular docking study revealed the strong binding affinity of betulinic acid with succinate dehydrogenase. The decreased mitochondrial membrane potential and inhibition in succinate dehydrogenase activity in Dalton's lymphoma tumor cells after betulinic acid treatment may lead to the development of mitochondrial dysfunction and could be contributing to tumor cells death and should be imperative in its antitumor activity. It is suggested that mitochondria-targeted agents such as betulinic acid holds great promise as a novel anticancer drug.

KEYWORDS: Betulinic acid, Dalton's lymphoma. cell cycle, mitochondrial membrane potential, succinate dehydrogenase.

INTRODUCTION

Natural products, especially phytochemicals have been extensively studied in an attempt to explore anti-cancer agents.[1,2] Triterpenoids are ubiquitous in the plant kingdom and have been widely investigated for their anticancer activities due to their minimal/no toxicity and general availability.[3,4] Beneficial effects of triterpenoids against several types of cancers has been found.[5] Betulinic acid (BA) (Figure 1) is one such naturally occurring pentacyclic triterpene that has been isolated from the bark of white birch *Betula pubescens*, from which it derived its name. BA is also found in other plant sources such as *Tryphyllum peltaum*, *Ancistrocladus heyneaus*, *Zizyphus joazeiro*, *Diospyoros leucomelas*, *Tetracera boliviana* and *Syzygium formosanum.*[6] Initial reports suggested that BA exerts selective cytotoxicity against melanoma cell lines.[7] However, later studies revealed its anticancer activity against several types of cancers.[8,9] This anticancer activity has been attributed to its ability to trigger mitochondrial membrane permeabilization, a central event in the apoptotic process.[10] As betulinic acid has been shown to have selective cytotoxicity on tumor cells but not on normal cells, it has imperative therapeutic applicability.[11] Since, agents that exert a direct action on mitochondria may trigger cell death under circumstances in which standard chemotherapeutics fail, there is increasing interest to develop such compounds as cancer therapeutics.

Earlier, it has been reported by us that BA has potent antitumor activity against murine ascites Dalton's lymphoma (DL),[12] and induces apoptosis in Dalton's

lymphoma cells.[13] Based on this background of research findings, present studies were undertaken to further explore the mechanism of possible antitumor activity with reference to cell cycle analysis, changes in mitochondrial membrane potential, succinate dehydrogenase activity in DL cells. Cisplatin (*cis*–diamminedichloroplatinum (II) (CDDP), a well-known potent cancer chemotherapeutic agent, was used as a positive control.[14]

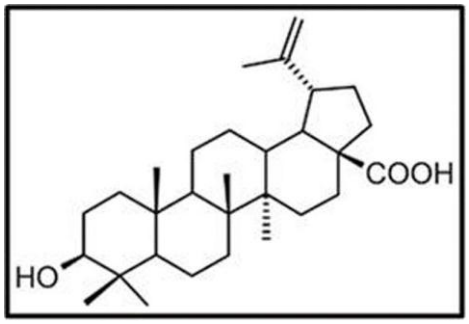

Figure 1: Chemical structure of betulinic acid.

MATERIALS AND METHODS

Chemicals

Betulinic acid (≥98% purity), [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium acid bromide] (MTT), rhodamine 123, propidium iodide were purchased from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals used in the experiments were of analytical grade and purchased within the country. Cisplatin solution (1 mg/ml of 0.9% NaCl) was obtained from Biochem Pharmaceutical Industries, Mumbai, India.

Ascites Dalton's lymphoma and its maintenance

Ascites Dalton's lymphoma (DL) has been commonly used murine malignant tumor in the screening of anticancer potentials of chemical agents as well as animals and plants extracts.[15]

Inbred Swiss albino mice colony was maintained under conventional laboratory conditions at room temperature (24±2ºC) with free access to food pellets (Amrut Laboratory, New Delhi) and water *ad libitum*, keeping 4-5 animals in a propylene cage. Ascites Dalton's lymphoma (DL) tumor was maintained *in vivo* in 10-12 weeks old mice of both sexes by serial intraperitoneal (i.p.) transplantation of viable tumor cells to the animals as per the established procedure.[16] Tumor-transplanted hosts usually survive for 19-21 days. Following tumor transplantation, an increase in abdomen size and body weight with sluggish movement of the animals was noted from 3rd-4th day onwards which was an early sign of tumor development. The maintenance, use of the animals and the experimental protocol of the present study was approved by the Institutional Animal Ethical Committee, North-Eastern Hill University, Shillong.

Drug treatment schedule

Betulinic acid was dissolved in dimethyl sulfoxide (DMSO) at a dose of 10 mg/ml before use. It was diluted in phosphate-buffered saline (PBS) to get the desired concentration and based on the earlier studies,[12,13,17] the therapeutic dose of betulinic acid was selected as 10 mg/kg body weight and 0.25 ml of the diluted drug was injected (i.p.) into mice. The day of tumor transplantation was taken as day '0'. Tumor-transplanted mice were randomly divided into three groups consisting of 10 mice in each group. Group-I mice served as control and received normal saline only. Group-II mice were injected with betulinic acid (i.p., 10 mg/kg body weight) on the 6th, 8th and 10th day post-tumor transplantation. Group-III mice were administered with cisplatin (i.p., 2 mg/kg body weight) on the 6th, 8th and 10th day post-tumor transplantation (Figure 2). Two animals were sacrificed by cervical dislocation after 24, 48, 72 and 96 h interval following last treatment with the drug (i.e. on the 11th, 12th, 13th and 14th day post tumor transplantation) and the tumor cells were collected for different studies. The experiments were repeated three times.

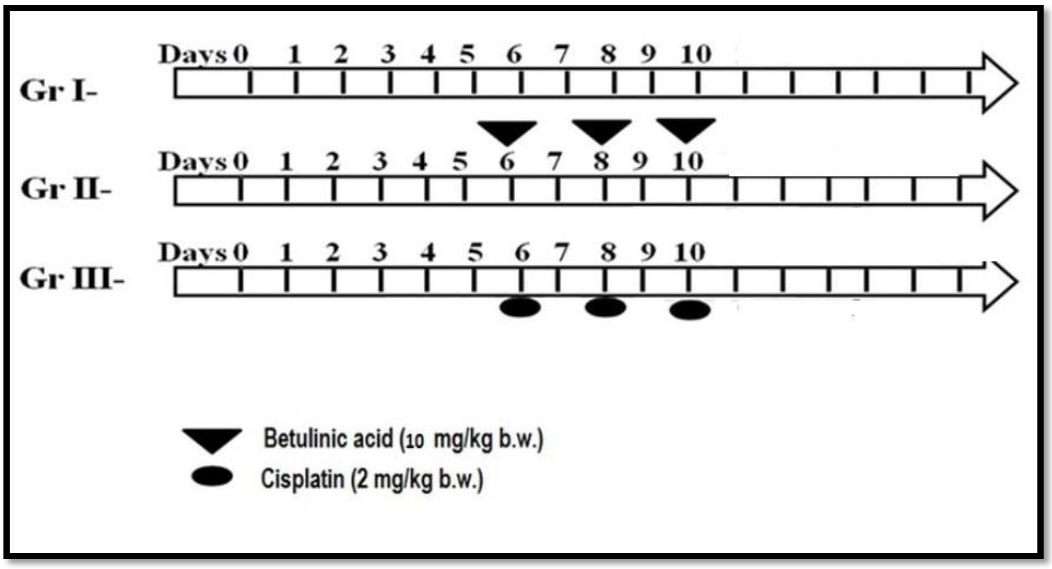

Figure 2: Schedule of drug treatment in tumor-bearing mice.

Tumor cell cytotoxicity

Cell multiplication inhibition was determined by MTT assay in *in vivo* and *in vitro* treatment conditions. MTT (3- (4, 5 dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide) assay is a non-radioactive colorimetric assay,[18] to measure cell cytotoxicity, proliferation or viability. The assay detects the reduction of MTT by mitochondrial dehydrogenase to purple formazan product, which reflects the normal functioning of mitochondria and hence cell viability. Briefly, 1x 10^6cells in 1 ml were seeded on 24 well plates and the cells were treated with different concentrations (20, 40, 60, 80 and 100 µg/ml) of betulinic acid or cisplatin for 12 h. The same doses were tested to determine IC50 value. Similarly, for *in vivo* cytotoxicity study tumor cells were collected from betulinic acid treated mice after 24-96 h of treatment. Depending upon the pilot study, cytotoxicity of betulinic acid was determined against normal splenocytes and DL cells. At the end of the incubation, medium was removed and MTT (5 mg/ml) was added and the cells were further incubated for 4 h after which the medium was removed. DMSO (100 µl) was added in each well to solubilise the formazan crystals. The absorbance was read at a wavelength of 595 nm. The percent cytotoxicity was calculated using the formula:

% Cytotoxicity = Absorbance of control- Absorbance of sample / Absorbance of control x 100

Cell cycle analysis using flow cytometry

After treatment with BA for 24, 48, 72 and 96 h, the DL cells were collected from mice and fixed with 70% ethanol. The cells were stained with propidium iodide (PI) buffer (4 µg/ml PI, 1% Triton X-100, 0.5 mg/ml RNaseA in PBS) for 30 min in the dark at room temperature and then filtered through a 40 µm nylon filter. The apoptosis and cell cycle distribution was analyzed for 50,000 cells by a Becton-Dickinson FACS Calibur, San Joes, CA, USA that uses the cell quest acquisition and analysis program. The apoptotic cells with hypodiploid DNA content were detected in the sub-G_1region or percentages of cells in G_1 or sub-G_1 were calculated. All the results were obtained from three independent experiments.

Mitochondrial membrane potential (ΔΨm) study

a. Laser-scanning confocal microscopy

DL cells from mice in different groups were collected, washed twice with PBS and 1x10^7 cells were incubated with cationic lipophilic rhodamine-123 (1µM, final concentration) to evaluate ΔΨm.[19] Green flouresent cells (reflecting high-polarized and low-polarized mitochondria) were quantified under the laser-scanning confocal microscope (Leica, TCS SP5, Germany). Flourescent images of the cells were thoroughly examined and photographed. Acquired signals were analyzed with ImageJ software and a minimum of ten fields were analyzed and average rhodamine intensity for each region was quantified.

b. Flow cytometry

The same cells were also analysed in a FACS Calibur, Becton, Dickinson flow cytometer with excitation at 488 nm and emission at 595 nm and 640 nm. At least 10,000 events were collected for each sample. Cells were maintained in dark and in aseptic condition throughout the whole experimental period.

Succinate dehydrogenase (SDH) (EC 1.3.5.1) assay

Ascites tumor was centrifuged at 1000xg for 10 min to collect DL cells pellet. DL cells pellet collected from the experimental animals under different treatment conditions were homogenized (10% homogenate) in ice-cold 0.25 M sucrose in a Teflon-pestle tissue homogenizer and centrifuged at 800xg at 4ºC for 10 min. The supernatant was collected and centrifuged at 14000xg at 4ºC for 20 min. The mitochondrial pellet was collected and washed in cold sucrose solution. The pellet was weighed, suspended again in sucrose solution, and sonicated (Soniprep-150) for determination of succinate dehydrogenase (SDH) activity. Succinate dehydrogenase activity was assayed in DL cells as per the procedure.[20] The unit of enzyme activity was expressed as nanomoles succinate oxidised per minute and the specific activity as units/mg protein.

***In Silico* molecular docking studies**

Molecular docking experiments were conducted to understand the predicted binding modes of betulinic acid at the SDH domain. The structures of betulinic acid (CID64971) and cisplatin (CID2767) were retrieved from PubChem Database (https://pubchem.ncbi.nlm.nih.gov/). The structures were then optimized using MMFF force field with optimization parameters such as 500 steps of steepest descent algorithm and convergence criterion of 10e-7.[21] The optimized structures were used for molecular docking studies.

The three dimensional structure of enzyme succinate dehydrogenase, SDH (PDB ID: 2W8Q) was obtained from Protein Data Bank (PDB) (www.rcsb.org).The steps for preparation of proteins included (a) removal of heteroatoms (water, ions), (b) addition of polar hydrogens, and (c) assignment of Kollman charges. The active sites were defined by considering grid boxes of appropriate sizes around the bound co-crystal ligands encompassing the active site residues (Table 1).

Table 1: Details of grid box dimensions considered for molecular docking.

Protein	Co-crystal ligand	Grid box dimensions		
		No. of grid points (npts)	Center (xyz coordinates)	Grid point Spacing (Å)
Succinate dehydrogenase	Succinic acid (SIN)	60×60×60	18.309, -45.622, -66.201	0.375

The compounds were docked against the above mentioned enzyme using AutoDock4.2.[22] The parameters for unknown atom type Pt (II) was included in the AutoDock parameter file.[23,24] The docking experiment was performed using Lamarckian Genetic Algorithm, with an initial population of 250 randomly placed individuals, a maximum number of 10^6 energy evaluations, a mutation rate of 0.02, and a crossover rate of 0.8. One hundred independent docking runs were performed for each compound. Conformation were clustered considering root mean square deviation (RMSD) cut-off of 2.0 Å and the lowest free energy of binding (ΔG) represent the most favourable conformation. The most favourable binding conformation was selected and evaluated for molecular interaction with their receptors using Molegro Molecular viewer vs 2.5.[25,26]

Statistical analysis

The data were expressed as mean ± SD and all determinations were repeated three times. Comparisons between the control and treated groups were made by one way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA 01060 USA) with Tukey multiple comparison tests, $p \leq 0.05$ was considered as statistically significant in all the cases.

RESULTS

Tumor cell cytotoxicity

Betulinic acid treatment caused cytotoxicity in tumor cells in time as well as dose dependent manner. Its cytotoxic effect was less on spleen cells as compared to DL cells. The *in vitro* IC_{50}value of betulinic acid in DL cells and spleen cells were found to be 72.26 µg/ml and 211.4 µg/ml respectively. This indicates that normal cells (spleen cells) were less sensitive to betulinic acid as compared to DL cells (Figure 3).

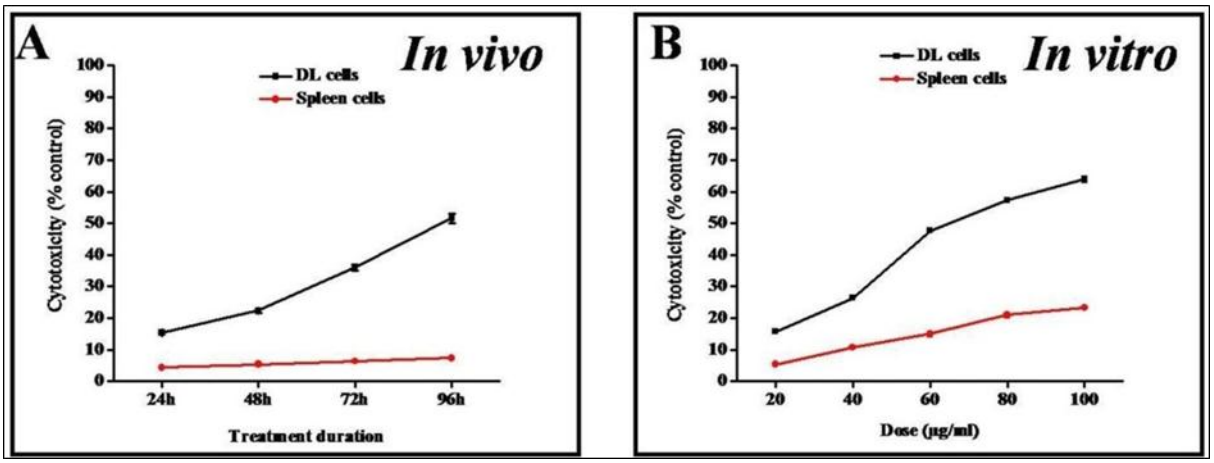

Figure 3: (A) *In vivo* and (B) *In vitro* cytotoxicity study in DL cells and spleen cells using MTT assay. DL cells showed a time and dose-dependent increase in cytotoxicity after BA treatment. However, there was less cytotoxic effect on spleen cells (normal cells) as compared to DL cells at the same time and concentration. The results were expressed as mean ± S.D., n=3.

Flow cytometric analysis of cell cycle

Flow cytometry analysis with propidium iodide (PI) staining revealed that as compared to control there was an increase in the S phase peak suggesting an increase in the percentage of cells in S phase after BA treatment. Cisplatin treatment for 96 h of also showed a significant increase in the percentage of cells in S phase as compared to control (Figure 4, 5).

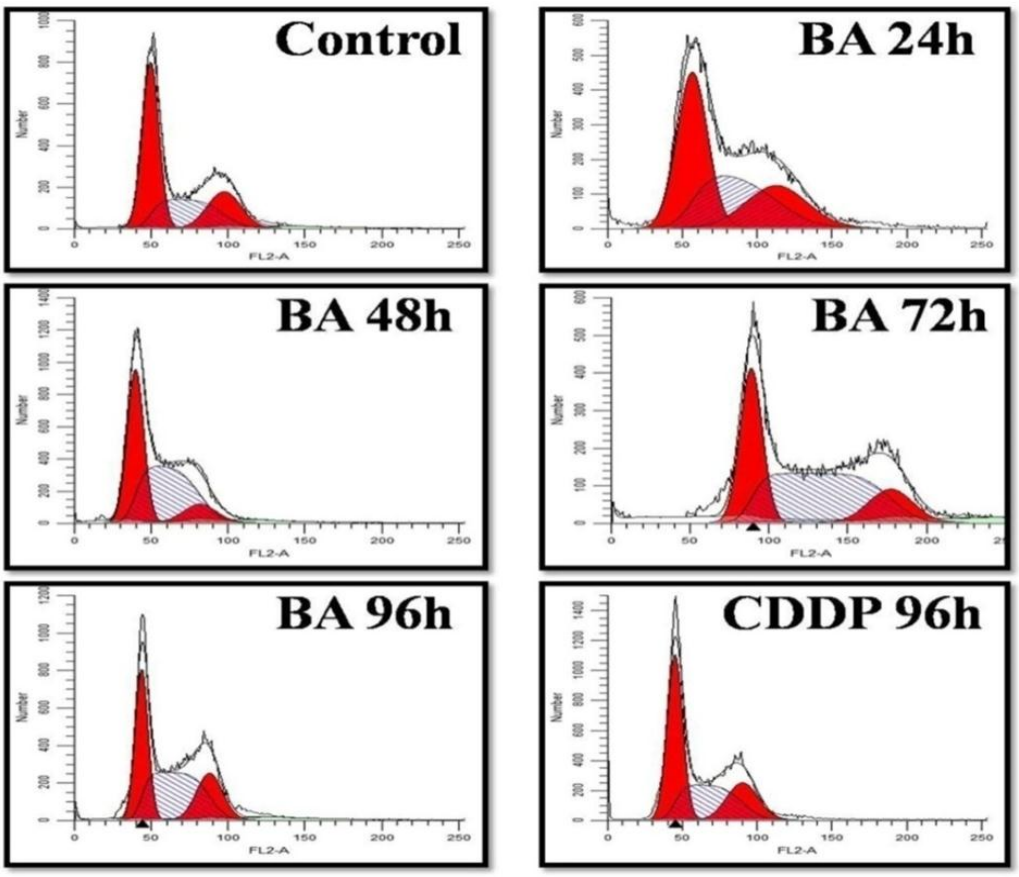

Figure 4: Flow cytometric analysis of betulinic acid-mediated apoptosis in DL cells. The cell cycle distributions are presented as cumulative proportions of cells within each of different cell cycle compartments (G0/G1, S and G2/M). BA treatment led to accumulation of fragmented DNA at S phase, which is an indication of apoptotic cell death. CDDP treatment also showed a significant increase in S phase cell population. Each experiment was repeated thrice. X axis: PI and Y axis: Cell count.

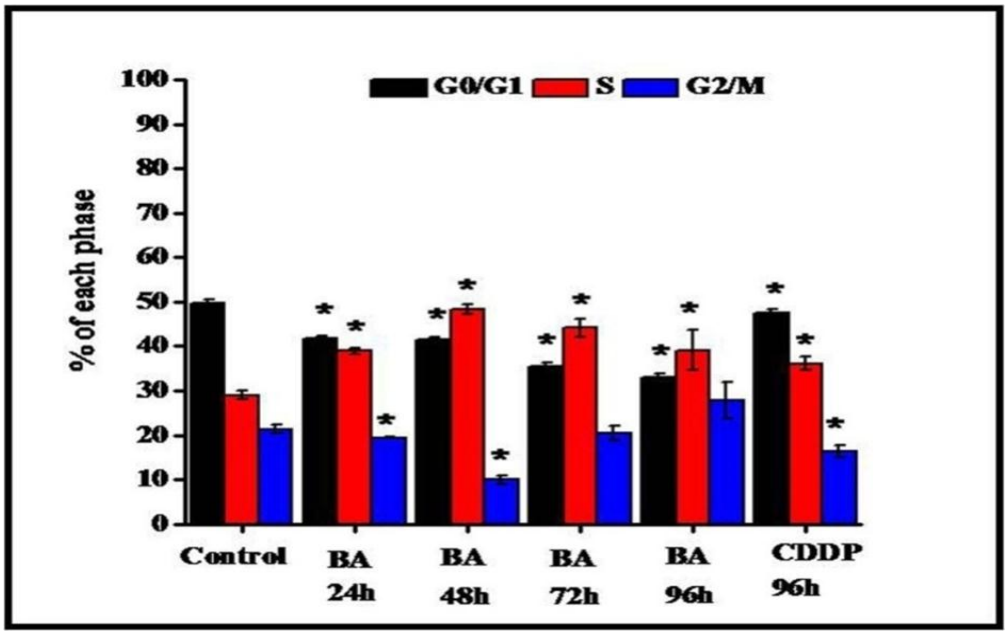

Figure 5: Quantitative analysis of different cell cycle compartments by flow cytometry. The results showed that the cells in S phase was increased significantly (*p≤0.05) from 24-96 h of BA treatment and subsequent decrease in G2/M phase occurred as compared to the control. Data were presented as mean ± S.D., ANOVA, n=3, *p≤0.05 as compared to control. CDDP, used for 96 h of treatment also showed significant (*p≤0.05) increase in the percentage (%) of cells in S phase.

Mitochondrial membrane potential

The ampholytic cationic fluorescence probe rhodamine-123 was used to monitor the betulinic acid-mediated changes in the mitochondrial transmembrane potential using confocal laser scanning microscope. Rhodamine-123 binding shows green fluorescence and propidium iodide used for nucleus staining displays red fluorescence under confocal microscope (Figure 6). There was a significant reduction in rhodamine florescence intensity and an increase in propidium iodide fluorescence intensity after BA treatment as compared to that of control (Figure 7). Quantification of the intensity profile of change in ΔΨm was determined from ten different randomly selected view fields under microscope in each group using ImageJ software. Flow cytometry results also showed a BA-mediated time dependent (24-96 h) decrease in mitochondrial transmembrane potential as shown by shifting of the fluorescence peaks towards left from the mean value of control (Figure 8).

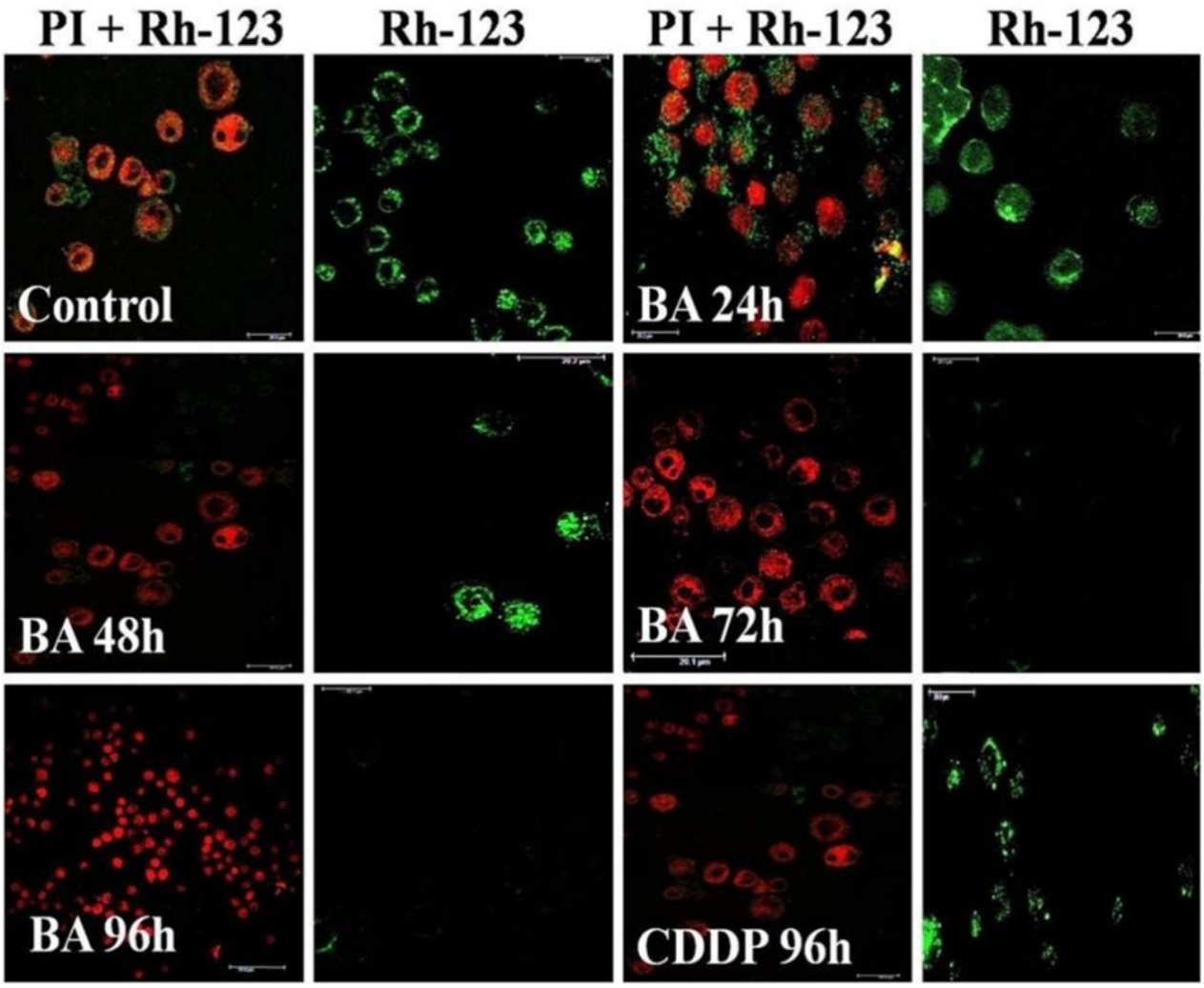

Figure 6: Determination of mitochondrial membrane potential (ΔΨm) in DL cells from mice in different treatment groups. Derease in mitochondrial membrane potential was noted after the BA as well as CDDP treatments. The scale bar indicated in the photographs is 20.1µm.

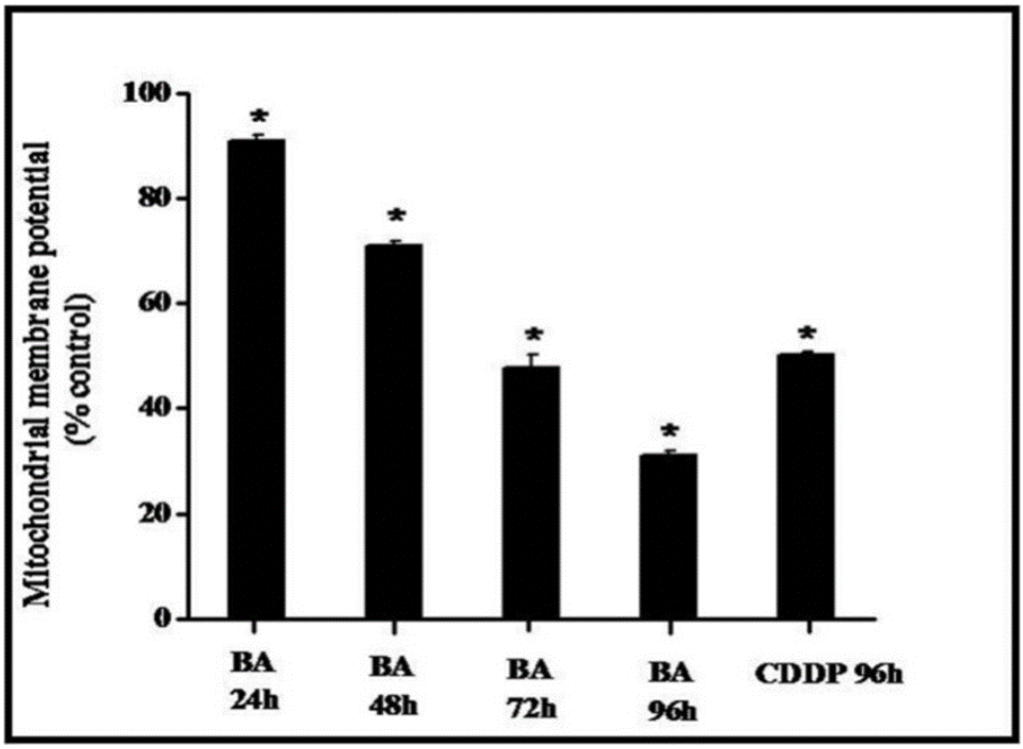

Figure 7: Percent changes in mitochondrial transmembane potential in DL cells. BA treatment caused a time-dependent decrease in high polarized mitochondria indicating decrease in mitochondrial membrane potential. Data were presented as mean ± S.D., ANOVA, n=3, *p≤0.05 as compared to control. BA- betulinic acid; CDDP- cisplatin.

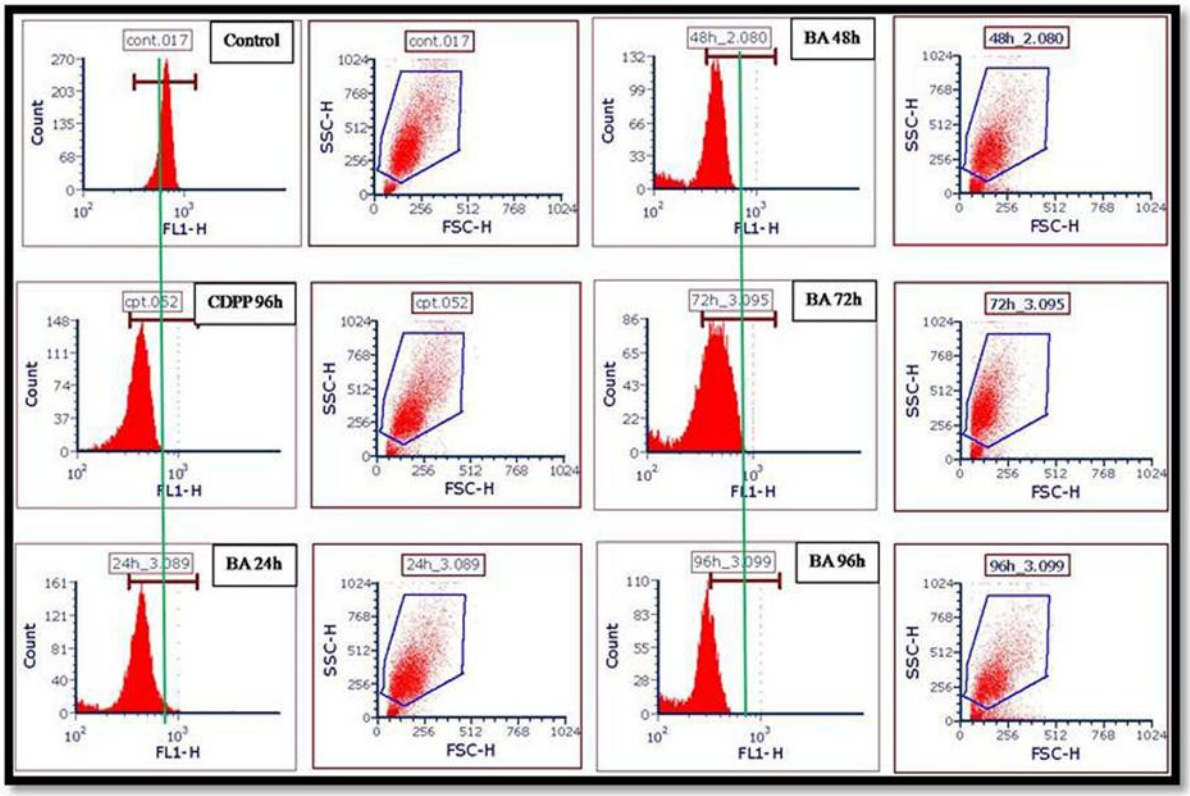

Figure 8: Flow cytometry analysis of mitochondrial membrane potential (ΔΨm) using rhodamine-123 dye. Mitochondrial staining profiles are shown as fluorescence pulse (FL1-H). Decrease in mitochondrial membrane potential was observed following BA treatment (24-96 h) as indicated by shifting of histogram peak towards left from the mean value (vertical line) of control. Mitochondria gates are shown in encircled scattered plot for each treatment groups. X-axis: green fluorescence, Y-axis: number of events. BA- betulinic acid; CDDP- cisplatin.

Succinate dehydrogenase (SDH; EC 1.3.5.1) activity
An overall increase in SDH activity was noted in DL cells during tumor growth.After BA and CDDP treatment there was a significant time-dependent decrease in SDH activity in DL cells (Figure 9).

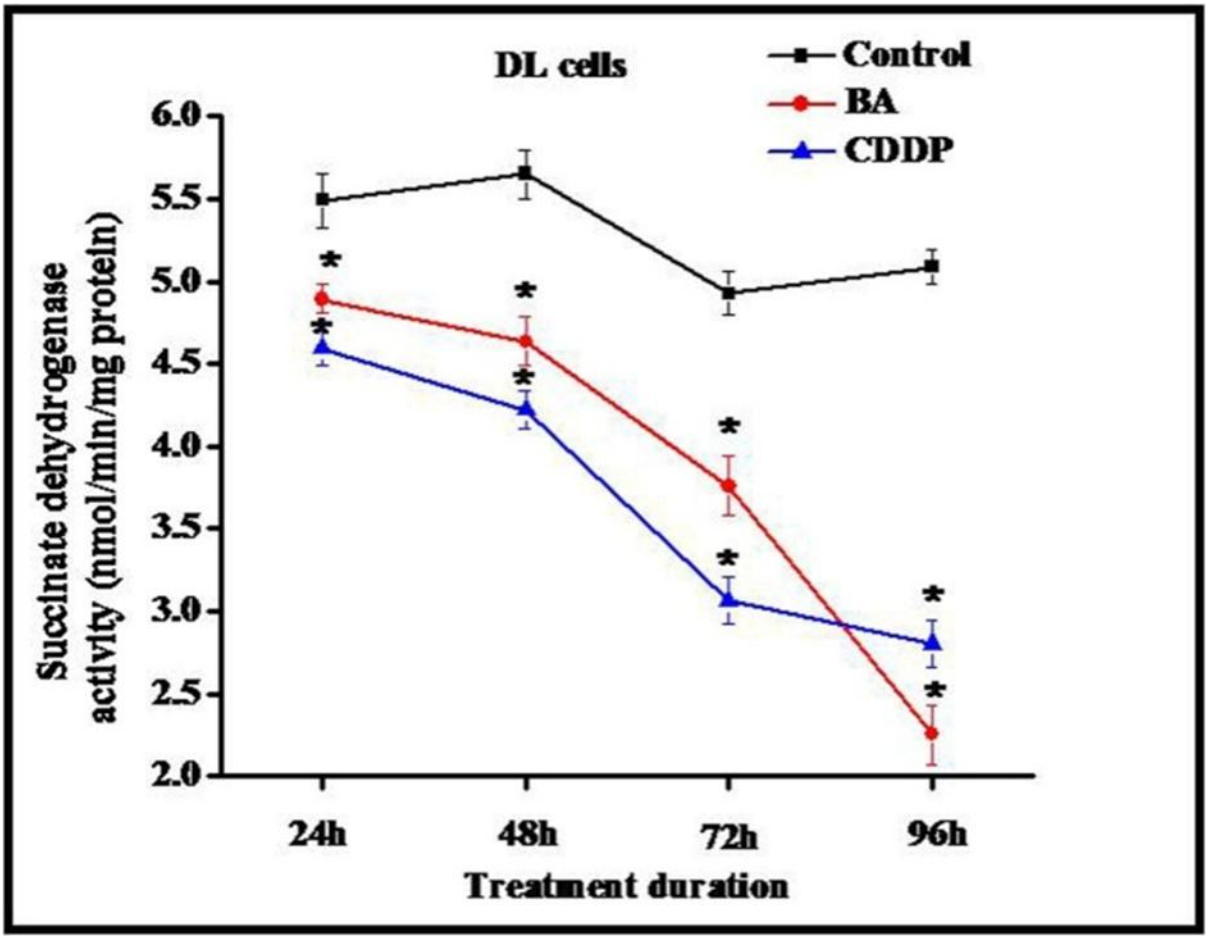

Figure 9: Changes in the succinate dehydrogenase (SDH) activity in DL cells of tumor-bearing mice under different treatment conditions. An overall decrease in SDH activity was observed in DL cells after BA treatment. Results were expressed as mean ± S.D., ANOVA, n=3, *p≤0.05 as compared to respective control at corresponding time point of treatment.

Molecular docking
Betulinic acid (BA) was capable of docking into the active site of SDH enzyme. This compound fitted well in the active site of SDH, similar to that of cisplatin as shown by binding affinity.The binding free energies (ΔG) of betulinic acid and cisplatin with SDH are presented in Table 2. Molecular docking experiment revealed that betulinic acid binds toSDHwith ΔG of -1.74 kcal/mol. Interestingly, no hydrogen bonds were established with the active site residues and the molecular interactions is only mediated through non-bonded contacts involve Lys148, Gly155, Glu156, Tyr159, Phe206, Glu494, Leu496 and Ser498 (Figure 10). Cisplatin displayed ΔG of -3.63 kcal/mol. It interacts with SDH through three hydrogen bonds of distances 2.65, 3.21 and 3.23 Å established with Tyr469, Gly495 and Ser498 respectively. The non-bonded contacts involve residues like Arg334, Val341, Leu496, Ile497 and Phe504 (Figure10).

Table 2: The binding free energy of the compounds from molecular docking.

Protein	Compounds	Binding Free Energy (ΔG)[kcal/mol]
Succinate dehydrogenase	Betulinic acid	-1.74
	Cisplatin	-3.63

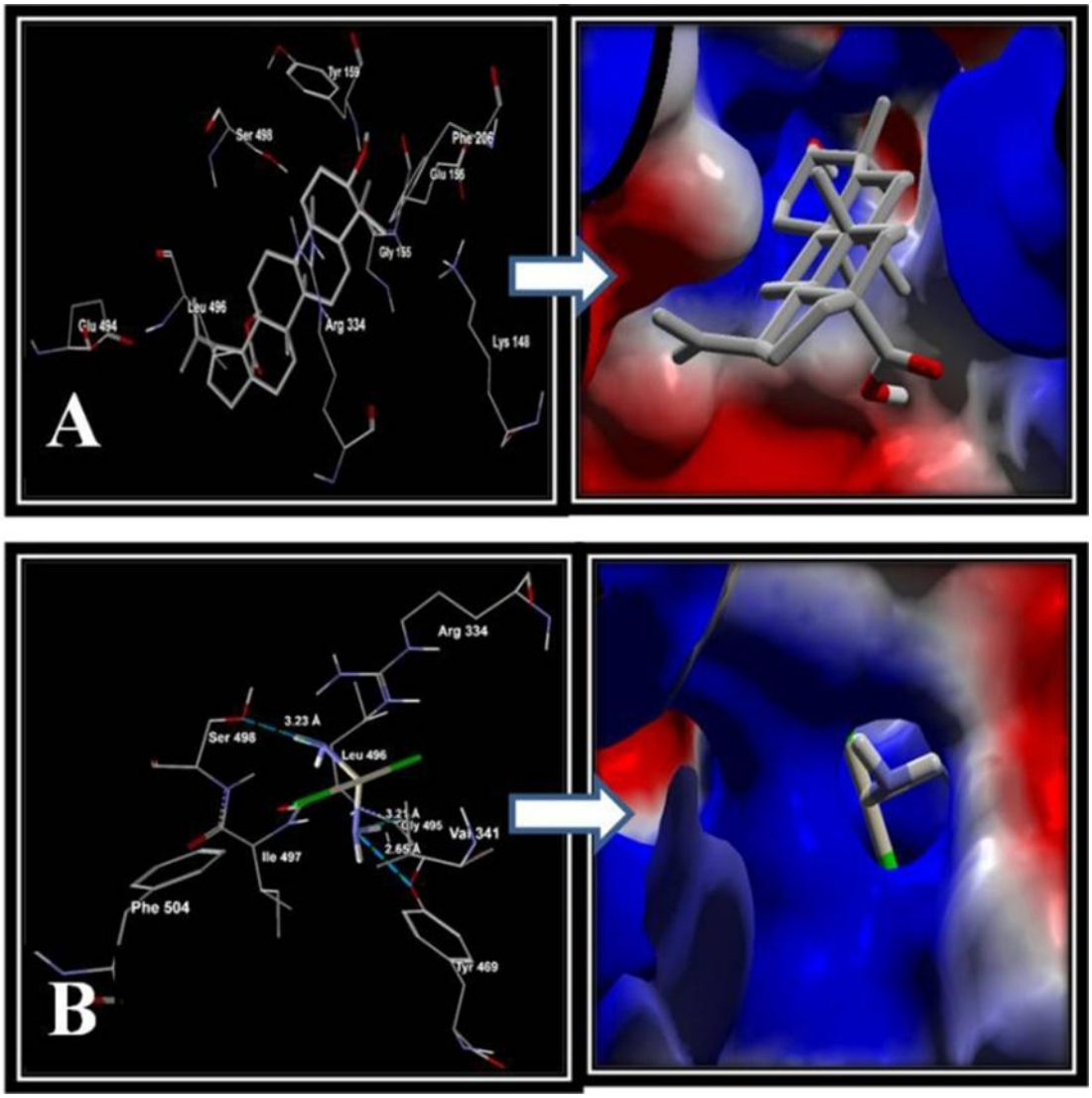

Figure 10: The binding modes and molecular interaction of betulinic acid and cisplatin with succinate dehydrogenase. (A) Betulinic acid and succinate dehydrogenase, (B) Cisplatin and succinate dehydrogenase.

DISCUSSION

Triterpenoids have been studied extensively for their potential use as anticancer agents,[28] and betulinic acid (BA) (Fig. 1) is one of the most promising compound in this category.Our earlier studies have established the potential antitumor activity of BA against murine ascites Dalton's lymphoma (DL).[12,13] Present studies further demonstrate that BA treatment causes changes in mitochondrial function and cells cycle arrest in S phase in DL cells which could be associated with its anticancer activity.Cytotoxicity of a chemical compound is tumor cells killing ability independent from the mechanism of death.[27] Cytotoxicity study showed that BA treatment for 96 h showed cell death in DL cells and spleen cells. The *in vitro* IC_{50} value of BA in DL cells and spleen cells were found to be 72.26 µg/ml and 211.4 µg/ml respectively. This indicates that spleen cells (normal cells) were less sensitive to BA as compared to DL cells (Figure 3 A and B).

Cell cycle analysis by flow cytometry has played significant role in the studies of cancer, anticancer agent screening, mechanism illustration, and cytokinetic research.[28] In addition to determining the relative cellular DNA content, flow cytometry also enables the identification of the cell distribution during the various phases of the cell cycle.[29] There is compelling evidence that apoptotic death induced by chemopreventive or chemotherapeutic agents is closely linked to perturbation of a specific phase of the cell cycle.Four distinct phases could be recognized in a proliferating cell population: the G1, S (DNA synthesis phase), G2 and M phase (mitosis). However, G2 and M phase, which both have an identical DNA content, could not be discriminated based on their differences in DNA content.[29,30] In general, cells that undergo apoptosis can be detected as a sub-diploid peak (SubG1) by flow cytometry. SubG1 peak corresponds to cells with fragmented DNA, a feature of the apoptotic cell death. It has been shown that the ability of cells to arrest cell cycle in G1/G0 or S phase was related to their drug sensitivity and increased with cell resistance.[31] The

flow cytometric study revealed BA-induced apoptosis in DL cells by an increase in percentage of cells in S phase. BA has also been reported to induce cell cycle arrest at S phase in K562 cells.[32] BA treatment exhibited a decrease in the G2/M phase at 24 h and 48 h of treatment with a time dependent increase of cells in S phase, indicating an apoptotic phenomenon as obtained from cell cycle analysis (Figure 4, 5).

It has been indicated that mitochondria may serve as direct targets for betulinic acid and they are implicated frequently in programmed cell death because the release of mitochondrial proteins into the cytosol triggers several relevant pathways.[33] At least 3 mitochondrial specific events have been well defined in cells undergoing apoptosis, namely, loss of mitochondrial transmembrane potential ($\Delta\Psi_m$), induction of mitochondrial permeability transition (MPT), and cytosolic translocation of apoptogenic factors, such as cytochrome C (Cyt C), and apoptosis-inducing factor.[34] To release such proteins, the mitochondrial membrane must undergo specific changes that allow the passage of proapoptotic proteins.[34] Such alterations can be detected using various dyes, based on the principle that intact and disrupted mitochondria exhibit differential patterns of dye uptake. Mitochondrial membrane potential ($\Delta\Psi$m) is the product of stored energy for the mitochondrial respiratory chain maintained by a balance of ions such as sodium and potassium within the mitochondrion. This potential difference normally exists at -180 to -200 mV and is necessary for transport of precursor proteins into the mitochondrion.[35] The $\Delta\Psi_m$ reduction is a general feature of cell death and defines an early stage of apoptosis preceding other manifestations of this process such as DNA fragmentation, ROS production and the late increase in membrane permeability.[36,37] Rhodamine 123 is a fluorescent lipophilic cationic dye that accumulates in the mitochondria of living cells and has been used as an optical probe for monitoring changes in membrane potentials in cells.[38,39]

In the present study BA-mediated changes in mitochondrial membrane potential ($\Delta\Psi_m$) was studied using rhodamine-123 dye by confocal laser scanning microscopy and flow cytometry. Mitochondrial membrane potential decreased after BA treatment as visible from reduced rhodamine123 fluorescence intensity (Figure 6, 7). A left shift of the peak from the mean value of control indicate enhanced cytochrome c release, opening of mitochondrial permeability transition pore with subsequent loss in mitochondrial membrane potential (Figure 8). Mitochondrial dysfunction and activation of apoptosis via caspases activation involving mitochondrial pathways after BA treatment has also been reported in other cell lines.[33] Thus, the mitochondrial membrane potential is indeed compromised as the cells undergo programmed cell death.

Alterations in metabolism have been implicated in cancer, with the main focus on the Warburg effect, a phenomenon in which cancer cells upregulate glycolysis and lactate production while decreasing glucose contribution to the citric acid (TCA) cycle in the mitochondria, even in the presence of sufficient oxygen.[40] Succinate dehydrogenase (SDH; EC 1.3.5.1) (succinate-coenzyme Q reductase, respiratory Complex II) catalyzes the oxidation of succinate to fumarate with the reduction of ubiquinone to ubiquinol. Through the coupling of these two reactions in the inner mitochondrial membrane, SDH links glucose oxidation in the TCA cycle with ATP production in the mitochondria.[39] BA treatment showed time dependent decrease in SDH activity in DL cells (Figure 9). The BA-mediated decrease in SDH activity in DL cells may assist the development of anoxic conditions or mitochondrial dysfunction contributing to cytotoxicity. Thus, targeting mitochondria as a cancer therapeutic strategy has gained momentum in the recent years.

In silico molecular docking is an application, wherein molecular modelling techniques are used to predict how a protein interacts with small molecules (ligand).[41] The concept of docking is important in the study of various properties associated with protein-ligand interactions such as binding energy, geometry complementarity, electron distribution, hydrogen bond donor acceptor properties, hydrophobicity and polarizability.[42] AutoDock4.2 was used for the molecular docking studies of SDH with BA and CDDP.[22] Docking results indicate that BA and cisplatin strongly bind to the active site of SDH with binding energies of -1.74 and -3.63 (ΔG) [kcal/mol] respectively (Table 2). The docking results of BA-SDH interaction showed an almost similar interaction in the active site of SDH as that of CDDP, a known inhibitor of SDH, which caused inhibition in SDH activity (Figure 10). This interaction betweenBA and SDH should cause an inhibition in SDH activity in DL cells, thus contributing to cytotoxicity in DL cells.

CONCLUSION

The findings show that betulinic acid treatment causes DL cells arrest in S phase of cell cycle and a decrease in mitochondrial membrane potential which may play a vital role to inhibit the proliferation of DL cells. Further, enzyme assay and molecular docking studies showed that there is decrease in SDH activity and strong interaction between SDH and BA, which might cause an inhibition in SDH activity thus, contributing to cytotoxicity in DL cells. It is suggested that betulinic acid may prove to become a potent anticancer agent with high efficacy and low toxicity in the treatment of different types of cancers and murine ascites Dalton's lymphoma in particular.

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REFERENCES

1. Khazir A, Mir BA, Pilcher L, Riley DL. Role of plants in anticancer drug discovery. Phytochem Lett, 2014; 7: 173-183.
2. Cragg GM, Newman DJ. Plants as source of anticancer agents. J Ethnopharmacol, 2005; 100: 72-79.
3. Amin AR, Kucuk O, Khuri FR, Shin DM. Perspectives for cancer prevention Withnatural compounds. J Clin Oncol, 2009; 27: 2712–2725.
4. Liby KT, Yore MM, Sporn MB.Triterpenoids and rexinoids as multifunctional agentsfor the prevention and treatment of cancer. Nat Rev Cancer, 2007; 7: 357–369.
5. Patlolla JMR, Chinthalapally VR.Triterpenoids for cancer prevention and treatment: current status and future prospects. Curr Pharm Biotechno, 2012; 13: 147-155.
6. Vlietinck AJ, De Bruyne T, Apers S, Pieters LA. Plant-derived leading compounds for chemotherapy of human immunodeficiency virus (HIV) infection. Planta Med, 1998; 64(2): 97–109.
7. Pisha E, Chai H, Lee IS, Chagwedera TE, Farnsworth NR, Cordell GA, Beecher CWW, Fong HHS, Kinghorn AD, Brown DM, Wani MC, Wall ME, Hieken TJ,Das Gupta TK, Pezzuto JM. Discovery of betulinic acid as a selective inhibitor of humanmelanoma that functions by induction of apoptosis. Nat Med, 1995; 1: 1046–1051.
8. Schmidt ML, Kuzmanoff KL, Ling-Indeck L, Pezzuto JM.Betulinic acid Inducesapoptosis in human neuroblastoma cell lines. Eur J Cancer, 1997; 33: 2007–2010.
9. Thurnher D,Turhani D, Pelzmann M, Wannemacher B, Knerer B, Formanek M, Wacheck V, Selzer E. Betulinic acid: a new cytotoxic compound against malignant head and neck cancer cells. Head Neck, 2003; 25: 732–740.
10. Fulda S. Betulinic acid: a natural product with anticancer activity. MolNutrFood Res, 2009; 53(1): 140-146.
11. Zuco V, Rhigetti SC, Cleris E, Marchesi L, Passserini CG, Formelli F. Selectivecytotoxicity of betulinic acid on tumor cell lines but not on normal cells. Cancer Lett, 2002; 175(1): 17–25.
12. Bhaumik A, Prasad SB. Studies on the antitumor potentials of betulinic acid Against murine ascites Dalton's lymphoma. Int J Basic Clin Pharmacol, 2016; 5(4): 1664-1671.
13. Bhaumik A, Gurung AB, Bhattacharjee A, Prasad SB.Betulinic acid-mediated induction in apoptosis and inhibition in lactate dehydrogenase activity in murine ascites Dalton's lymphoma. Eur J Pharm Med Res, 2017; 4: 494-503.
14. Prasad SB, Giri A, Khynriam D. Studies on chromosomes of murine Dalton‟s lymphoma cells and the effect of cisplatin on chromosomes of these cells and bone marrow cells*in vivo*. Cytologia, 1998; 63: 405-413.
15. Verma AK, Prasad SB.Antitumor effect of blister beetles: An ethno-medicinal Practicein Karbi community and its experimental evaluation against a murine malignant tumormodel. J Ethnopharmacol, 2013; 148: 869-879.
16. Verma AK, Prasad SB. Changes in glutathione, oxidative stress and mitochondrial membrane potential in apoptosis involving the anticancer activity of cantharidin isolated from redheaded blister beetles, *Epicautahirticornis*. Anticancer Agents Med Chem, 2013; 13(7): 1096-1114.
17. Alitheen NB, Mashitoh AR, Yeap SK, Shuhaimi M, Abdul Manaf A, Nordin L .Cytotoxic effect of damnacanthal, nordamnacanthal, zerumbone and betulinic acid isolated from Malaysian plant sources.Int Food Res J, 2010; 17: 711-719.
18. Graham-Evans B, Tchounwou PB, Cohly HH. Cytotoxicity and proliferation studies with arsenic in established human cell lines: keratinocytes, melanocytes, dendriticcells, dermal fibroblasts, microvascular endothelial cells, monocytes and T-cells. Int J MolSci, 2003; 4: 13-21.
19. Zhu Y, Li M, Wang X, Jin H, Liu S, Xu J, Chen Q. Caspase cleavage of Cytochrome C1 disrupts mitochondrial function and enhances cytochrome C release. Cell Res, 2011; 22: 127-141.
20. King A, Selak MA, Gottlieb E. Succinate dehydrogenase and fumarate hydratase:linking mitochondrial dysfunction and cancer. Oncogene, 2006; 25: 4675-4682.
21. Halgren TA. Merck molecular force field. I. Basis, form, scope, parameterization and performance of MMFF94. J Comput Chem, 1996; 17: 490-519.
22. Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, Olson AJ. AutoDock4 and AutoDockTools4: Automated docking with selective receptorflexibility. J ComputChem, 2009; 30: 2785-2791.
23. Autodock Forum ADL. Parameters for docking with metal ions in receptor, 2009 (accessed November 2016), http://autodock.1369657.n2.nabble.com/ADL-Parameters-for-docking-with-metal-ions-in-receptor-d2505649.html.
24. Arce ER, Mosquillo MF, Pérez-Díaz L, Echeverría GA, Piro OE, Merlino A, Coitiño EL, Ribeiro CM, Leite CQ, Pavan FR, Otero L. Aromatic amine N-oxideorganometallic compounds: searching for prospective agents against infectious diseases. Dalton Trans, 2015; 44(32): 14453-14464.
25. Molegro Molecular Viewer, version 2.5. Molegro-a CLC bio company; Aarhus, Denmark, 2012.
26. Guedes IA, de Magalhães CS, Dardenne LE. Receptor-ligand molecular docking. Biophysical Reviews, 2014; 6: 75-87.

27. Lin KW, Huang AM, Lin CC, Hour TC, Pu YS, Lin CN. Anti-cancer effects of ursanetriterpenoid as a single agent and in combination with cisplatin in bladder cancer.Eur J Pharmacol, 2014; 740: 742–751.
28. Evan GI,Vousden KH. Proliferation, cell cycle and apoptosis in cancer. Nature, 2001; 411: 342–348.
29. Nunez R. DNA measurement and cell cycle analysis by Flow cytometry. Curr Issues Mol Biol, 2001; 3: 67-70.
30. Surh YJ, Hurh YJ, Kang JY, Lee E, Kong G, Lee SJ.Resveratrol, an antioxidant present in red wine, induces apoptosis in humanpromyelocyticleukemia (HL-60) cells. Cancer Lett, 1999; 140: 1-10.
31. Dubrez L, Goldwasser F, Genne P, Pommier Y, Solary E. The role of cell cycleregulation and apoptosis triggering in determining the sensitivity of leukemic cells to topoisomerase I and II inhibitors. Leukemia, 1995; 9: 1013-1024.
32. Qiuling WU, Jing HE, Jun F, Mei H. Antitumor effect of betulinic acid on humanacute leukemia K562 cells *in vitro*. J Huazhong UnivSci Technol [Med Sci], 2010; 30: 453-457.
33. Fulda S, Jeremias I, Steiner HH, Pietsch T, Debatin KM. Betulinic acid: a Newcytotoxic agent against malignant brain-tumor cells. Int J Cancer, 1999; 82: 435–441.
34. Zamzami JN, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B, Kroemer G. Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J Exp Med, 1995; 182(2): 367-377.
35. Martin J, Mahlke K,Pfanner N. Role of an energized inner membrane in Mitochondrialprotein import. Delta psi drives the movement of presequences. J Biol Chem, 1991; 266: 18051-18057.
36. Kroemer G, Zamzami N, Susin SA. Mitochondrial control of apoptosis. Immunol Today, 1997; 18: 44-51.
37. Modica-Napolitano JS, Singh KK. Mitochondrial dysfunction in cancer. Mitochondrion, 2004; 4: 755–762.
38. Cossarizza A, Cooper E, Quaglino D, Salvioli S, Kalachnikova G, Franceschi C. Mitochondrial mass and membrane potential in coelomocytes from the earthworm *Eiseniafoetida*: studies with fluorescent probes in single intact cells. Biochem Biophys Res Commun, 1995; 214: 503- 510.
39. Cardaci S,Ciriolo MR. TCA cycle defects and cancer: when metabolism tunes redox state. Int J Cell Biol, 2012; 2012: 1-9.
40. Warburg O. On the origin of cancer cells. Science, 1956; 123: 309-314.
41. Roy K, Paul S. Docking and QSAR studies of protoporphyrinogen oxidase inhibitor 3H-pyrazolo [3,4-d][1,2,3]triazin-4-one derivatives. J Mol Model, 2010; 16: 137-153.
42. Girija CR, Karunakar P, Poojari CS, Begum NS, Syed AA. Molecular docking studies of curcumin derivatives with multiple protein targets for procarcinogen activating enzyme inhibition. J Proteomics Bioinform, 2010; 3: 200-203.