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Research Article

Studies on the antitumor potentials of betulinic acid against murine ascites Dalton's lymphoma

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ABSTRACT

Background: Betulinic acid, a naturally occurring pentacyclic triterpene, exhibits a variety of biological activities including anticancer properties. Despite the wide importance of ethnobotanical studies on the anticancer therapeutic uses of betulinic acid, its exact role has not been fully elucidated. Therefore, the present studies were undertaken to evaluate the antitumor effect of betulinic acid in a murine malignant tumor model along with various biochemical changes in the tumor cells of the host.

Methods: Ascites Dalton's lymphoma (DL) tumor-transplanted Swiss albino mice were treated with betulinic acid (i.p., 10 mg/kg body weight) and the pattern of host's survival was analysed. The viability of DL cells was assessed using trypan blue exclusion test. The DL cells were also studied for the determination of apoptosis using fluorescence microscopy. Reduced glutathione and protein estimations were done in DL cells under different treatment conditions.

Results: Betulinic acid treatment caused a significant increase in life span (ILS ~150%) of the tumor-bearing hosts, which may indicate tumor regression/antitumor activity. Following betulinic acid treatment, decrease in DL cells viability and damaging changes in the cell membranes and a decrease in reduced glutathione content in DL cells were observed.

Conclusions: Present findings reveal the potent antitumor activity of betulinic acid against murine ascites Dalton's lymphoma. The cytotoxicity of betulinic acid to build up antitumor effects may involve induction of apoptosis as well as a decrease in glutathione level in tumor cells.

Keywords: Betulinic acid, Dalton's lymphoma, Antitumor activity, Apoptosis

INTRODUCTION

Cancer is one of the most serious health problems to human. According to World Cancer Report released by the World Health Organization (WHO) in 2008, by 2030 it could be expected that there could be 27 million incident cases of cancer worldwide, which is more than double the figures for cancer cases diagnosed in 2008.[1] Surgery, chemotherapy and radiotherapy are the main conventional methods of cancer treatment. In chemotherapy, hundreds of drugs of diverse chemical nature and implying different mechanisms of action have been used against a wide range of cancers.[2,3] However, the full use of these drugs has been limited due to development of various side effects in the hosts. Thus, in an attempt to overcome the side effects of chemotherapy, the development of new drugs, using drugs in combination and the use of number of plant as well as animal-derived natural products have been tried. Therefore, natural products have played a major role in the anticancer drug discovery. Over 60% of the anticancer drugs are obtained from natural sources. Secondary metabolites have wide range of biological properties which can be used for the treatment of various diseases.[4] Triterpenes represent a varied class of natural compounds. Pentacyclic lupane-type triterpenes possess various medicinal properties. On various *in vivo* cancer model systems the antitumor properties of lupane-derived triterpenoid plant extracts have been demonstrated.[5-9]

Betulinic acid (3b-hydroxy-lup-20(29)-en-28-oic acid), (Figure 1) is a naturally occurring pentacyclic lupane-

type triterpene widely distributed in the plant kingdom.[10] It was originally isolated from the bark of the white birch, *Betula pubescens*, from which it got its name.[11] Betulinic acid could also be isolated from various other sources which include ber or Indian plum *Ziziphus mauritiana* (Rhamnaceae), jamun or jambul, *Syzygium formosanum* (Myrtaceae), Diospyros spp. (Ebenaceae) and Paeonia spp. (Paeoniaceae).[12-20] Betulinic acid (BA) exhibits a number of biological properties. However, it is mainly recognized for its anti-HIV-1 activity and specific cytotoxity against a number of tumor cell lines.[21] The anticancer activity of betulinic acid was initially discovered against human melanoma, and was recognized as 'melanoma specific cytotoxic agent.[22] It has now been shown that betulinic acid exhibits anticancer activity against different types of human cancers like neuroblastoma, leukemia, colon, breast, hepatocellular, lung, prostate, renal cell, ovarian and cervix carcinomas.[23-26] In anticancer activity, betulinic acid appears to function by means of inducing apoptosis, triggering changes in the mitochondrial membrane potential, production of reactive oxygen species.[27] Because of its selective cytotoxicity against tumor cells and favourable therapeutic index, and even at doses up to 500 mg/kg body weight betulinic acid does not demonstrate side effects, thus, suggesting it to be an attractive and promising antitumor agent.
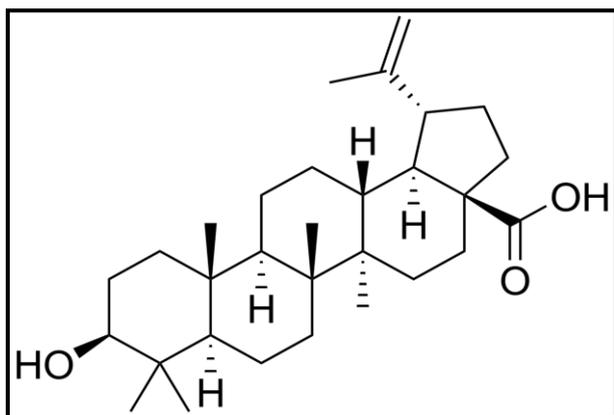

Figure 1: Chemical structure of betulinic acid.

Most of the anticancer research on BA has been carried out using cancer cell lines *in vitro* and its effect *in vivo* needs to be further researched. Therefore, on the basis of possible anticancer therapeutic uses of betulinic acid, and as the antitumor activity of betulinic acid has not been assessed in murine ascites Dalton's lymphoma, this study was undertaken to evaluate its anticancer potential in vivo against murine ascites Dalton's lymphoma.

METHODS

Chemicals

Betulinic acid (≥98 % purity), reduced glutathione, and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from the Sigma Chemical Co., St. Louis, Mo, USA. Cisplatin solution (1 mg/ml of 0.9 % NaCl) was obtained from Biochem Pharmaceutical Industries, Mumbai, India. Ethylenediaminetetra-acetic acid (EDTA) and other chemicals used in the experiments were of analytical grade and purchased from SRL Pvt. Ltd., Mumbai, India.

Animals and tumor maintenance

Inbred Swiss albino mice colony was maintained under conventional laboratory conditions at room temperature (20±2ºC) with free access to food (Amrut Laboratory, New Delhi) and water *ad libitum*, keeping 5 to 6 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 intra peritoneal (i.p.) transplantation of viable tumor cells to the animals as per the established procedure in the lab.[28] Tumor-transplanted hosts usually survived 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 (Figure 2). The maintenance, use of these animals and the experimental protocol of the present study was approved by the institutional ethical committee, North-Eastern Hill University, Shillong.
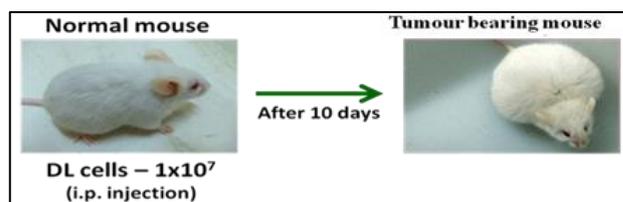

Figure 2: Tumor growth in a normal mouse after transplantation of DL cells.

Drug treatment schedule

Betulinic acid at a dose of 10 mg/ml was dissolved in DMSO (dimethyl sulfoxide) before use. It was diluted in PBS (Phosphate buffered saline) to get desired concentration and based on the earlier reports, 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.[29]

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 tumor-bearing control and received normal saline only. Group-II mice were injected intraperitonially with betulinic acid (10 mg/kg body weight) on the 6th, 8th and 10th day post-tumor transplantation. Group-III mice were injected intraperitonially with cisplatin (2 mg/kg body weight) on the 6th, 8th and 10th day post-tumor transplantation (Figure 3).
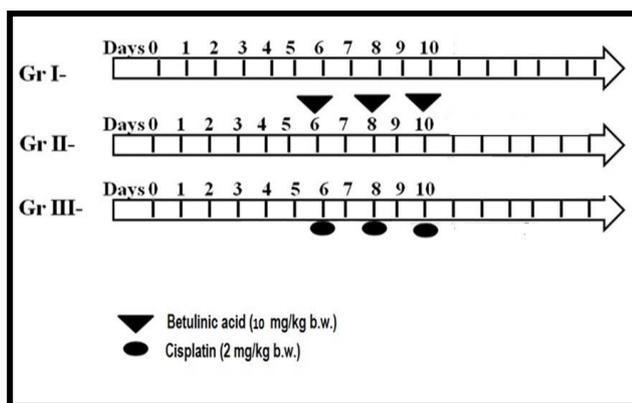
Figure 3: Schedule of drug treatment in tumor-bearing mice.

$$\%ILS = \frac{\text{Mean survival days for treated animals}}{\text{Mean survival days for control animals}} \times 100 - 100$$

Similarly for the biochemical analysis, 10 mice were kept in each group and two animals were sacrificed by cervical dislocation after 24, 48, 72 and 96 hours 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 separately. The experiments were repeated three times.

Hosts survival and antitumor study

The survival patterns of animals in each group were monitored daily and deaths, if any, were recorded. The antitumor efficacy was expressed in percentage of average increase in life span (% ILS), and was calculated using the formula:

(T/C x 100)-100, where, T and C are the mean survival days of treated and control group of mice respectively.

Cell viability

Viability of DL cells as well as spleen cells (representative of corresponding normal cells) collected under different treatment conditions was checked by trypan blue exclusion test following the method of Talwar.[30] The DL cells and spleen were collected from mice at different time intervals (24, 48, 72 and 96 hours), washed with PBS and cell suspensions were prepared in PBS. Aliquot of the cell suspension was mixed with an equal volume of trypan blue (0.4% in PBS) and incubated for 10 minutes. Viable (unstained cells) and dead cells (stained cells) were analysed with a Neubauer haemocytometer under light microscope. The percentage of viability was calculated using the formula:

% viability = Average total number of viable cells from treated mice/Average total number of viable cells from control mice x 100

Apoptosis study

Flourescence based apoptosis was determined in DL cells collected from mice under different treatment conditions using acridine orange-ethidium bromide (AO/EB) staining method following the method of Shylesh et al.[31] as previously used in the lab. After treatment, the DL cells were collected from mice at different time intervals (24, 48, 72 and 96 hours). The cells were washed twice with PBS and treated with AO/EB (100µg/ml PBS of each dye) for 5 minutes and gently washed with PBS. The cells in different treatment groups were thoroughly examined under fluorescent microscope and photographed (A1000IS - canon). The viable cells nuclei stain green due to permeability of only acridine orange whereas, apoptotic cells appear red due to co-staining of both the stains. One thousand cells were analysed and percentage of apoptotic cells was counted from twenty selected view fields under microscope.

Scanning electron microscopy

The DL cells were collected from the animals under varying experimental conditions (as given in the treatment protocol), washed once with PBS and resuspended in PBS. A thin smear of these cells was prepared on a cover glass which was then fixed in 2.5% (v/v) glutaraldehyde at 4ºC. Fixed cells were rinsed in 0.1 M phosphate buffer and dehydrated in graded ethanol series of 30, 50, 70, 90 and 100% for 20 minutes each. Cover glass with cells was then cut in small size and was affixed to an aluminium stub with double stick tape and then critical point-dried in a critical point drier (CPD-030, BAL-TEC Co.) and coated with gold in an ionic sputter coater (SCD-005, BAL-TEC Co). They were viewed, examined thoroughly and photographed under the scanning electron microscope (JEOL JSM-6360).

Protein estimation

The protein estimation in DL cells was determined following the method of Bradford.[32]

Total reduced glutathione (GSH) estimation

Total reduced glutathione (GSH) level was determined in the DL cells following the method of Sedlak and Lindsay.[33] Briefly, 5% tissue homogenates were prepared in 0.02 M EDTA (pH 4.7) in a motor-driven teflon-pestle homogenizer. Total reduced glutathione was determined by adding the homogenate (0.1 ml) to 1 ml of 0.2 M Tris-EDTA buffer (pH 8.2) and 0.9 ml of 0.02 M Tris-EDTA buffer (pH 4.7) followed by 20 µl of Ellman's reagent (DTNB 0.01 M, in methanol). After 30 minutes of incubation at room temperature, the reaction mixture was centrifuged at 3000 xg for 20 minutes and the absorbency of the clear supernatant was read against a reagent blank at 412 nm in a Carey-100 spectrophotometer.

Statistical analysis

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

RESULTS

Hosts survivability

Control tumor bearing mice (group 1) survived for about 19-21 days. The mean survival time of betulinic acid-treated mice were significantly increased to about 50 days with the ILS of about 150 % which was quite comparable with that of cisplatin treatment showing ILS of about 170% (Figure 4).
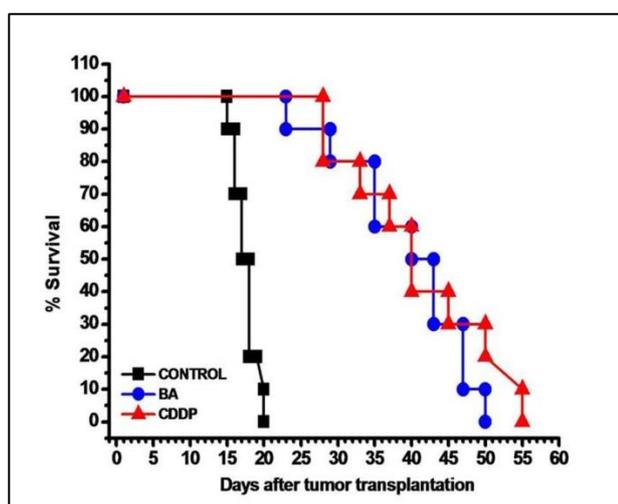

Figure 4: The survival pattern of tumor-bearing mice in control and different treatment groups. BA-betulinic acid; CDDP- cisplatin.
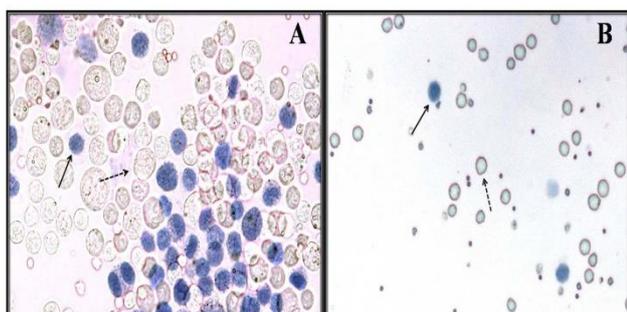

Figure 5: Representative microphotograph to show the cell viability; (A) DL cells after treatment with betulinic acid; (B) Spleen cells after treatment with betulinic acid. Viable cells (dotted arrow) are colourless whereas dead cells (regular arrow) stain blue.

Cell viability

The viability of DL cells and spleen cells under different treatment conditions *in vivo* was checked using trypan blue exclusion test (Figure 5 A and B). BA treatment showed a significant decrease in the viability of DL cells in a time dependent manner. However, as compared to DL cells, spleen cells showed much higher viability at the corresponding time of BA treatment. It shows that betulinic acid was more cytotoxic to DL cells as compared to normal cells in the host (Figure 6).
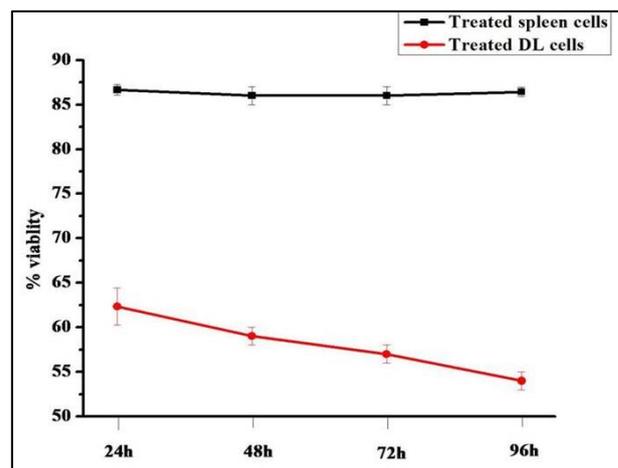

Figure 6: Percent viability of DL cells and spleen cells as determined by trypan blue exclusion test after treatment of tumor-bearing mice with betulinic acid. Results are expressed as mean±SD, n=3.
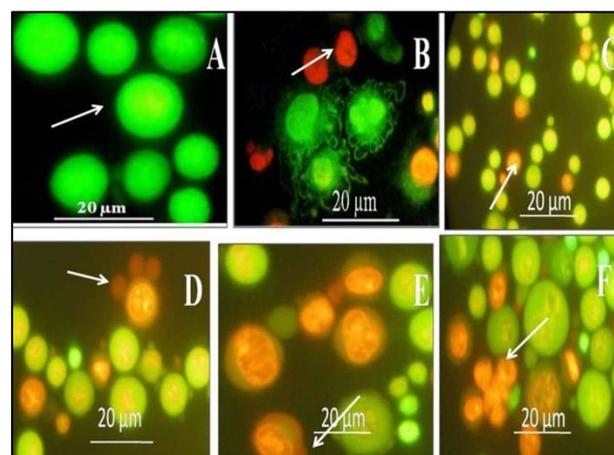
Apoptosis

Figure 7: Flourescence based determination of apoptosis in DL cells using AO/EB staining method; (A) control; (B) cisplatin; (C-F) betulinic acid treatment for 24-96 hours. Cells stained green represent viable cells, whereas orange/red stained cells represent apoptotic cells.

Acridine orange is a vital dye that stains both live and dead cells, whereas, ethidium bromide stains only those

cells that have lost membrane integrity. Cells stained green represent viable cells, whereas orange/red stained cells represent apoptotic cells. Control DL cells were round in shape with uniform green fluorescence while cisplatin treatment after 96 hours showed many apoptotic nuclei with membrane blebbing and fragmented nuclei. In betulinic acid treated mice, DL cells illustrated the appearance of membrane blebbing and folding at 24 hours. Chromatin condensation and cell membrane abnormality with fragmented nuclei were observed at 48 hours of BA treatment. After 72 hours of the treatment, cells with severe membrane rupture with many fragmented nuclei and cytoplasmic vacuoles were noted. BA treatment of mice for 96 hours showed the damage in plasma membrane with scattered fragmented nuclei and apoptotic bodies outside the cells. The results suggest that BA was able to induce marked apoptosis in DL cells (Figures 7, 8).
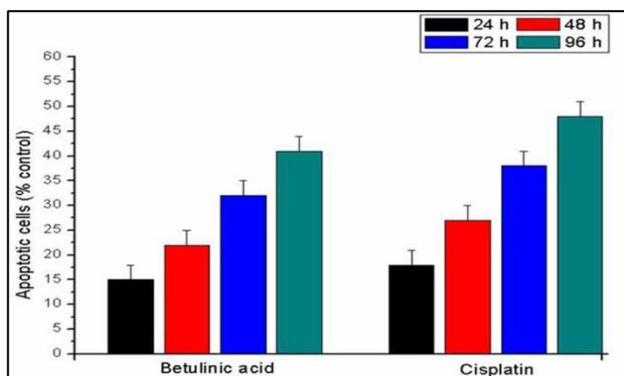

Figure 8: Apoptotic index in DL cells after treatment with betulinic acid and cisplatin respectively.
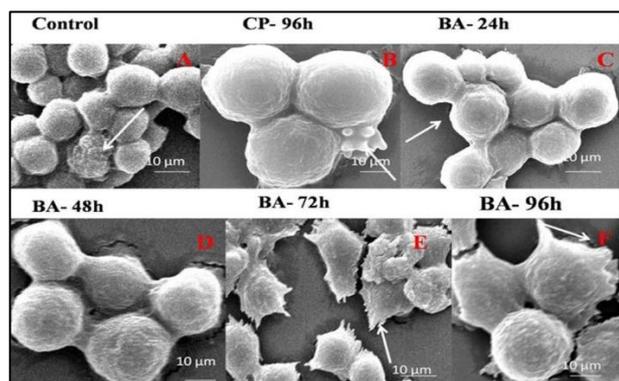
Scanning electron microscopy

Figure 9: Scanning electron micrograph of DL cells; (A) control DL cells are round in shape with few membrane projections and ruffles distributed evenly over the cell surface; (B) cisplatin treatment showed apoptotic features including loss in membrane ruffles and appearance of membrane folding/blebbing; (C-F) betulinic acid treatment for 24-96 hours showed membrane blebbing/ folding and apoptotic bodies. CP: cisplatin; BA: betulinic acid.

To further investigate apoptotic features and the surface morphological changes, DL cells were also processed for SEM. DL cells in control group were round in shape with fine membrane projections and ruffles distributed evenly over the surface. Cisplatin treatment (96 hours) showed membrane folding and shrinkage. At 24 hours of betulinic acid treatment, cells exhibited few membrane projections over the cells surface and a reduction in its length. The surface of DL cells were relatively smooth with no obvious microvilli. Severe cell membranes folding and shrinkage were also evident after 48 hours of treatment. After 72 hours of betulinic acid treatment some irregular blebs and apoptotic bodies were observed and few phagocytic cells were also visible attaching to tumor cells. BA treatment of mice at 96 hours resulted in severe morphological alterations, with a complete loss in fine membrane projections. Cell shrinkage, membrane blebs and severe membrane folding were seen which are typical characteristics features of apoptosis (Figure 9).

Protein estimation

Proteins are responsible for structure, function and various enzymatic changes in the cell. Protein concentration in DL cells revealed that there is a significant decrease in protein content from 24-96 hours after betulinic acid and cisplatin treatment respectively as compared to that of control (Figure 10).
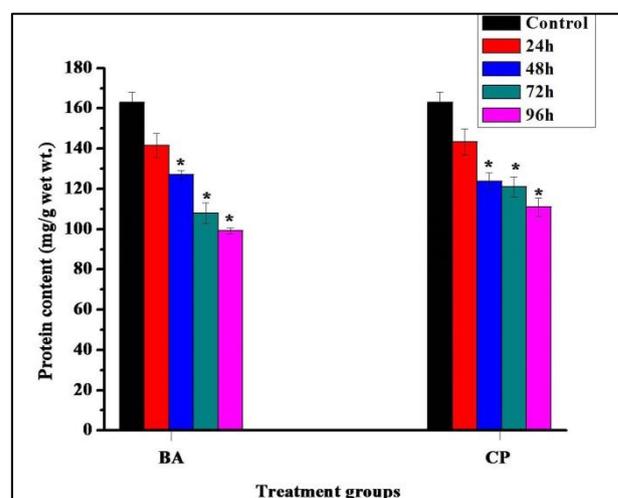

Figure 10: Protein content in DL cells of tumor-bearing mice after treatment with betulinic acid and cisplatin respectively. The results are expressed as mean±SD, n=3. BA- betulinic acid treatment, CP-cisplatin treatment.

Total reduced glutathione (GSH)

GSH is an antioxidant that helps to protect cells against ROS (reactive oxygen species). DL cells showed prominent, time dependent decrease in GSH after BA treatment. In case of reference drug i.e. cisplatin, GSH level also decreased in DL cells (Figure 11).
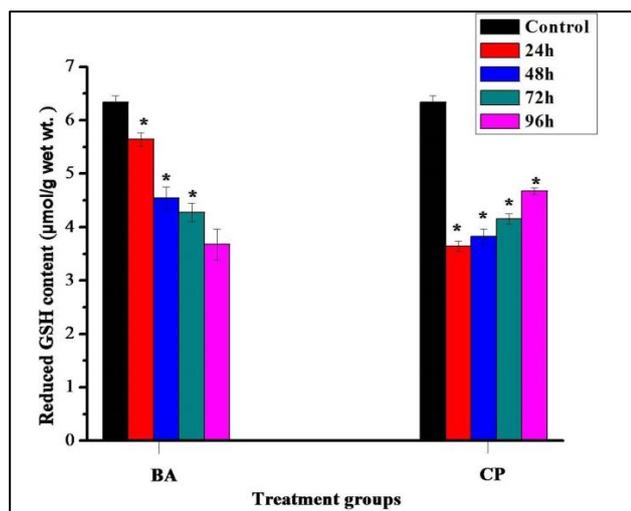
Figure 11: Reduced GSH content in DL cells of tumor-bearing mice after treatment with betulinic acid and cisplatin respectively. The results are expressed as mean±SD, n=3. BA-betulinic acid treatment, CP- cisplatin treatment.

DISCUSSION

Although the efficacy of chemotherapy and other standard therapies for different cancer types has been improved during the last decades, the treatment of most human malignancies is still facing high mortality rates. Moreover, toxic side-effects of the chemotherapeutical drugs often limit their full uses. Therefore, the development of novel potent anti-cancer agents is always a primary requirement in cancer research. Since its rediscovery in the 1990s BA has attracted considerable attention as a potential anti-neoplastic drug that lacks toxic effects towards healthy tissues.

In the present study antitumor efficacy of betulinic acid against the ascites Dalton's lymphoma tumor-bearing hosts was evaluated and compared with that of cisplatin as a positive reference drug which is one of the most effective cancer chemotherapeutic agents in clinical practice. To evaluate the antitumor activity of different drugs, ascites Dalton's lymphoma has been commonly used as an important murine experimental tumor model.[34] The results of the hosts survival data showed that betulinic acid is quite effective against DL, showing a significant increase in life span of the hosts as compared to that of control (Figure 4). The analysis of cell viability of DL cells under different treatment conditions revealed that the number of dead cells was increased significantly in mice after treatment with betulinic acid in a time dependent manner as compared to control (Figure 5 A, B and Figure 6) which may result the increased survivability of the hosts. Further, as compared to DL cells, the spleen cells showed much higher viability at the corresponding time of BA treatment (Figure 6). It signifies that betulinic acid was more cytotoxic to DL cells as compared to normal cells in the host.

Apoptosis was carried out in DL cells using AO/EB method. Apoptosis and/or necrosis are among the key mechanisms by which most compounds exert their cytotoxic effects, especially anticancer agents. Overload of intracellular ROS has been known to induce apoptosis or necrosis or the combination of both.[35,36] Many of the well-known cytotoxic/anticancer agents belonging to anthracyclins, alkylating agents, epipodophyllotoxins and camptothecins are known to induce apoptosis through oxidative stress-mediated mechanisms.[37] Apoptosis is a well-described mechanism of cell death induced by a variety of substances.[38] The assay based on AO/EB staining showed that after betulinic acid treatment DL cells undergo nuclei/ chromatin condensation, and marginalization followed by their fragmentation, cell's shrinkage and membrane blebbing. Finally, the cells produce apoptotic bodies varying in size and structure (Figure 7). The increase in the number of cells stained red with or without fragmented DNA at higher doses of betulinic acid suggests the possibility of late apoptotic cell death. The assay based on AO/EB staining is a good reliable indicator for the authentication of apoptotic features. The results of AO/EB showed higher apoptotic index in DL cells in a time dependent manner after treatment with betulinic acid (Figure 8).

To further substantiate the fluorescence based results on betulinic acid-mediated apoptosis, SEM studies were done to observe the morphological characteristics of DL cells. The use of SEM in the analysis of apoptosis is mainly referred to the study of cell surface alterations such as membrane blebbing, shrinking etc. These are important signs of cell injury and may be considered as specific markers of apoptosis. A series of surface changes in DL cells were observed following betulinic acid treatment as observed under a scanning electron microscope. Control DL cells showed evenly distributed membrane projections and ruffles over the cell surface. After 24-96 hours of betulinic acid treatment, cell membrane folding and shrinkage, irregular blebs microvilli, and certain deformities were noted. The formation of membrane blebs/vesicles in tumor cells observed after betulinic acid treatment also support the appearance of apoptotic features (Figure 9).

Protein is an indicator of biological entity or activity. And hence in any biological reaction or estimation or bio-process, protein analysis and quantification is done to determine the quantity, quality of protein and there by the state of biological reaction or process. In protein estimation a decrease in the protein content was observed in BA treated DL cells as compared to that of control. The protein content in BA treated DL cells was significantly found to decrease from 48-96 hours of treatment as compared to that of control (Figure 10). In cisplatin treatment, protein content was also found to decrease significantly from 48-96 hours as compared to that of control. These changes may involve some alterations in the rate of protein synthesis or decreased uptake of protein in DL cells.

Glutathione (GSH) is an important antioxidant found widely in plants and animals. Glutathione (γ-L-Glutamyl-L-cysteinyl-glycine) helps to prevent damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides and heavy metals.[39] The role of glutathione (GSH) in cancer has been a matter of interest. Oxidative stress as well as depletion of GSH triggers apoptosis which takes place through mitochondrial intrinsic pathway possibly by inducing loss of mitochondrial transmembrane potential. This event leads to activation of caspase-3 which plays an important role in carrying out the process of apoptosis. During tumor growth GSH level in DL cells increases. The increase in GSH level in tumor cells could be involved in proliferation and metabolism of tumor cells in the host. Betulinic acid treatment in tumor bearing hosts showed a significant decrease in total GSH levels in DL cells (Figure 11) which may alter the cellular redox balance and facilitate these cells towards the oxidative damage. The decrease in GSH level in DL cells after betulinic acid treatment may be a noteworthy step in the anticancer activity of betulinic acid against Dalton's lymphoma.

CONCLUSION

In conclusion, it may be suggested that betulinic acid treatment could enhance the therapeutic efficacy by induction of apoptosis in DL cells. Interestingly, betulinic acid has not been reported to develop any side effects in the hosts, thus, it could be a promising potent anti-cancer agent against different types of cancers and Dalton's lymphoma in particular.

ACKNOWLEDGEMENTS

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