4. DISCUSSION

Cancer is a serious risk to human health that affects the lives of millions of people. The main goal of cancer diagnosis and treatment is to cure or considerably prolong the life span of the patients. To fulfil this goal, the cancer patients are generally treated with three main conventional methods namely surgery, radiotherapy and chemotherapy which may be supplemented with other recent therapies. Although the efficacy of chemotherapy and other standard therapies for the majority of cancer types has been improved during the last decades, the treatment of most human malignancies is still facing problem. Moreover, toxic side-effects often developed due to the chemotherapeutical drugs hampers the efficacy of drug and affects the quality of life. Therefore, the development of novel, potent and non-toxic anti-cancer agents is a continuous effort of scientists all over the world. The use of natural resources has shown promise in this direction.

Natural products, which are a rich source of compounds with enormous structural diversity, have been extensively explored in the field of drug discovery and have led to remarkable successes. This is particularly evident in the field of cancer therapeutics, where over 50% of the approved drugs discovered in the last two decades of the 20th century were of natural origin (Cragg and Newmann, 2005). Many widely used anticancer agents such as vincristine, irinotecan, etoposide and paclitaxel which represent a range of structurally diverse anticancer drugs, are all naturally derived and play a dominant role in cancer chemotherapy. Twenty nine pure compounds (mostly phenolics) were reported to be isolated from anti-cancer plants and tested against different cancer cell lines (Tariq et al., 2017).
As the largest class of natural products, terpenoids consist of approximately 25,000 chemical structures thus far with potential applications in the fragrance and flavour industries to the pharmaceutical and chemical industries (Gershenzon and Dudareva, 2007). Betulinic acid (Figure 5) is one such naturally occurring pentacyclic triterpenoid that exhibits a variety of biological activities including potent antitumor properties. Betulinic acid (BA) is widely distributed in the plant kingdom throughout the world (Alakurtti et al., 2006). For example, considerable amount of BA is available in the outer bark of white-barked birch trees. Initially, betulinic acid was considered to be a melanoma specific cytotoxic agent (Pisha et al., 1995), but later studies have suggested its anticancer activity against a broad spectrum of cancers (Zuco et al., 2002). Betulinic acid was found to exhibit anticancer activity not by induction of apoptosis through the activation of caspases independent of the p53 gene status and CD95 activation (Fulda, 2008). Instead, betulinic acid-induced apoptosis was mediated via direct effects on mitochondria which involve cytochrome c release, activation of caspases and apoptotic DNA fragmentation (Fulda and Debatin, 2000). Since its rediscovery in the 1990s BA has attracted considerable attention as a potential antineoplastic drug that may lack toxic effects towards healthy tissues. In this study BA was identified as a potent cytotoxic compound against murine ascites Dalton’s lymphoma (DL).

In the present study, antitumor efficacy of betulinic acid (BA) in the tumor-bearing hosts was evaluated against murine ascites Dalton’s lymphoma, using cisplatin, a positive reference drug, which is considered one of the most effective anticancer chemotherapeutic agents used in clinical practices (Verma and Prasad, 2013). The anticancer activity of cisplatin has been attributed mainly due to its ability
to bind with cellular DNA involving intrastrand and interstrand crosslinks (Fuertes et al., 2003). The chemical nature and molecular mechanism of action of cisplatin is different from that of betulinic acid, but apoptosis has also been recognized as an important mechanism of cell death mediating the anticancer effect of cisplatin (Lee et al., 2001). Therefore, in the present study cisplatin was used as a positive reference drug to compare the therapeutic efficacy of BA in the host in relation to its ability to induce apoptosis.

The well-founded criteria for assessing the value of any anticancer drug is to examine the increase in life span of the hosts after the treatment. For the assessment of antitumor activity, ascites Dalton’s lymphoma (DL) has been commonly used as an important experimental murine malignant tumor model (Amenla et al., 2013). The results of the host’s 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. The mean survival time of betulinic acid treated mice was significantly increased to about 50 days with ILS of about 150 % which was quite comparable with that of cisplatin treatment showing ILS of about 177% (Figure 8).

Cytotoxicity of a chemical compound is tumor cells killing ability independent from the mechanism of death (Graham-Evans et al., 2003). Assessment of a compound’s cytotoxicity may be made using different in vitro cytotoxicity tests. MTT assay is a sensitive, quantitative and reliable assay that measures cell viability and cell proliferation. The MTT assay converts tetrazolium salt into a purple formazan product via mitochondrial dehydrogenases. The metabolic activity of cells is proportional to the color density formed. Considering that only active mitochondria contain these enzymes, this reaction only occurs in viable cells. The
IC$_{50}$ value which is the drug concentration that kills 50% of the cells, was determined at 24, 48, 72 and 96 h of BA treatment on DL cells and spleen cells (normal cells) by MTT assay. BA caused an increase in the cytotoxicity of DL cells in both time as well as dose dependent manner. In vivo 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 after BA treatment. This indicates that spleen cells (normal cells) were less sensitive to BA as compared to DL cells (Figure 9 A and B).

The analysis of the viability of DL cells using trypan blue exclusion test under different treatment conditions also revealed that the dead cells were increased significantly in mice after treatment with betulinic acid in a time dependent manner as compared to control 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. It signifies that betulinic acid was more cytotoxic to DL cells as compared to normal cells in the host (Figure 10, 11).

Uncontrolled proliferation and a defect in the process of apoptosis constitute crucial elements in the development and progression of malignant tumors (Bryan et al., 2011). Apoptosis may be characterized by definite morphological changes in the cells (Kerr et al., 1972). The observations based on transmission electron microscopy, scanning electron microscopy and fluorescence microscopy (AO/EB staining) are good reliable indicators for confirmation of apoptotic features (Hitoshi and Tak, 2004). 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 (Higuchi, 2003; Pelicano et al., 2004). 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 (Gerster, 1995). Apoptosis is a well-described mechanism of cell death induced by a variety of substances (Kroemer and Reed, 2000). The assay based on AO/EB staining is a good reliable indicator for the authentication of apoptotic features. The assay based on AO/EB staining showed that after betulinic acid treatment DL cells undergo chromatin condensation, and marginalization followed by their fragmentation, cell’s shrinkage and membrane blebbing (Figure 12). Finally, the cells produce apoptotic bodies varying in size and structure. 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 higher apoptotic index in DL cells in a time dependent manner was observed after treatment with betulinic acid (Figure 13).

Cell cycle analysis by flow cytometry is commonly performed to test whether the compound induced apoptotic cell death is associated with arrest of any of cell cycle phases. 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. The effect of a given antiproliferative agent on cell cycle progression appears to depend on the concentration of the compound and also on the duration of the treatment (Surh et al., 1999). DNA histogram analysis reveals the relative amount of cells distributed in different phases of cell cycle. In general cells that suffer apoptosis can be detected as a subdiploid peak (SubG1) by flow cytometry. SubG1 peak corresponds to cells with fragmented DNA, a feature of the
apoptotic cell death. The induction of apoptosis was also confirmed by flow cytometric analysis of cell cycle. Cell cycle arrest is one of the targets of many anticancer drugs, such as doxorubicin, cisplatin, 5-fluorouracil and paclitaxel. 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 (Dubrez et al., 1995). The flow cytometric study confirmed BA-induced apoptosis in DL cells by an increase in percentage of cells in S phase (Figure 14, 15). BA has also been reported to induce cell cycle arrest at S phase in K562 cells (Qiuling et al., 2010). BA has been shown to exhibit 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.

Light microscopy revealed that the treatment of DL cells with BA for 24-96 h induced marked changes in cell morphology. The cells treated with BA showed nuclear fragmentation, membrane rupture, membrane blebbing, appearance of vacuoles inside the cytoplasm and formation of apoptotic bodies (Figure 16). The remarkable phenotypic alterations in apoptotic cells may be caused by the destruction of the normal nuclear architecture (Collins et al., 1997; Martin and Diego, 1997).

To further corroborate the light microscopy and fluorescence based results on betulinic acid-mediated apoptosis, transmission electron microscopy (TEM) study was done to observe the ultrastructural characteristics of DL cells. TEM has been commonly used to study the ultrastructure of cells (Guha et al., 2007). The application of TEM is also used in the detection of associated cellular micro-lesions in apoptosis and cell cycle arrest (Zhou and Zhu, 2003; Mills et al., 2004). In addition, the ultrastructural features in DL cells showed that the control cells had
large nuclei, clear nucleoli, uniform chromatin, abundant euchromatin, normal organelle structure, and a smooth plasma membrane structure. Cisplatin treatment at 72 h showed the appearance of chromatin condensation, vacuolization of cytoplasm and disintegration/distortion of the plasma membrane. BA treatment of mice for 24 h showed the appearance of chromatin condensation and cytoplasmic vacuoles in DL cells. Membrane disorganization, fragmented nuclei and vacuolization in the cytoplasm were observed during 72 h of BA treatment which finally leads to lysis of the tumor cells (Figure 17).

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 h 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 18).

It has been indicated that mitochondria may serve as direct targets for betulinic acid (Fulda et al, 1999). These organelles are implicated frequently in programmed cell death because the release of mitochondrial proteins into the cytosol triggers several relevant pathways (Kluck et al., 1999). 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 (Zamzami et al., 1995; Decaudin et al., 1997). To release such proteins, the mitochondrial membrane must undergo specific changes that allow the passage of proapoptotic proteins (Zamzami et al., 1995). Such alterations can be detected using various dyes, based on the principle that intact and disrupted mitochondria exhibit differential patterns of dye uptake. The ΔΨm reduction is a general feature of cell death. Decrease in the ΔΨm 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 (Kroemer et al., 1997). In the present study BA-mediated changes in mitochondrial membrane potential (ΔΨm) 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 rhodamine 123 fluorescence intensity (Figure 19, 20). 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 21). Mitochondrial dysfunction and activation of apoptosis via caspases activation involving mitochondrial pathways after BA treatment has also been reported in other cell lines (Fulda et al., 1999). Thus, the mitochondrial membrane potential is indeed compromised as the cells undergo programmed cell death.

Protein is an indicator of biological entity or activity. And hence in any biological reaction or estimation or bioprocess, protein analysis and quantification is done to determine the state of biological reaction or process. Inhibition of protein
synthesis can alter cellular responsiveness to the anticancer drugs. In the present findings, the highest concentration of protein was found in liver, followed by kidney and DL cells in the tissues of tumor-bearing mice. As compared to the tumor-bearing control, there was a significant decrease in the protein contents in liver, kidney and DL cells after BA treatment during 24-96 h (Figure 22 A) which may be due to inhibition of protein synthesis. In case of cisplatin treatment, protein content was also found to decrease significantly in liver, kidney and DL cells from 24-96 h of treatment as compared to that of control (Figure 22 B). These changes may also involve some alterations in the rate of protein synthesis in these tissues.

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 (Warburg, 1956; Lunt and Vander Heiden, 2011). 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 (Cardaci and Ciriolo, 2012). Assay of SDH activity showed that as compared to the corresponding control, BA treatment showed time dependent decrease in SDH activity in DL cells and liver (Figure 23 A and B). Alterations in mitochondrial structure and functions have long been observed in cancer cells. Thus, targeting mitochondria as a cancer therapeutic strategy has gained momentum in the recent years.
Lactate dehydrogenase (LDH; EC 1.1.1.27) plays an important role in tumor cells and it is popularly known as Warburg effect which suggests that cancer cells dependence on glycolytic energy progressively increases as malignant transformation occurs (Xu et al., 2005). The reduction in the glycolytic capacity of tumor cells would restrict their ability to proliferate, invade adjacent tissues, and migrate to distant organs. This suggests that the attenuation of glycolysis in tumor cells may represent a useful strategy for preventing or stopping the development of cancer (Lopez-lazaro, 2007). LDH is located in the cytosol, and is released into the surrounding culture medium upon cell damage or lysis. Recent observations showed that attenuation of LDH reduced the glycolytic metabolism of cancer cells and produced antitumor effects in animals (Bui, 2006; Fantin et al., 2006). The inhibition of LDH activity may represent a relatively nontoxic approach to interfere with tumor growth (Fantin et al., 2006). In the present study LDH activity was studied in the different tissues, DL cells, ascites fluid and blood serum of tumor-bearing mice. BA treatment caused a significant decrease in LDH activity in liver, kidney and DL cells (Figure 24 A, B, and C). However, in case of DL cells the decrease was more pronounced. Cisplatin (CDDP) treatment also resulted in a similar time dependent decrease in LDH activity in liver, kidney and DL cells (24-96 h) (Figure 24 A, B, and C). In case of ascites fluid and blood serum, BA treatment resulted in a significant time dependent increase in LDH activity from 24-96 h of treatment as compared to the corresponding control, thus, indicating release/leakage of LDH from DL cells due to membrane damage (Figure 24 D and E). This inhibitory effect on LDH activity may indicate inhibition/decreased glycolysis in DL cells. Multiple studies on various cancer cell lines have shown that attenuation of LDH in tumor cells increases
apoptosis (Fantin et al., 2006; Le et al., 2010) and reduces migration and invasion ability (Xie et al., 2009; Sheng et al., 2012) demonstrating its use as a potential therapeutic target. Therefore, it is suggested that LDH inhibition could be a well-tolerated therapy that will impede tumor growth and metastasis.

Cancer cells have increased rates of glucose metabolism compared to normal cells. A variety of mechanisms have been proposed for the accelerated glucose seen in growing tumors and in transformed and malignant cells (Wolf et al., 2011; Wang 2014). Among the several enzymes of glycolysis, hexokinase/glucokinase (HK; EC 2.7.1.1) is regarded as one of the most important enzymes for glucose metabolism in cancer cells (Patra et al., 2013). Furthermore, increased concentrations of hexokinase with decreased rates of glucose-6-phosphatase have been reported to accelerate glucose phosphorylation, which results in increased glucose consumption (Gershon et al., 2013). Hexokinase catalyses the ATP-dependent conversion of aldo- and keto-hexose sugars to the hexose-6-phosphate (H6P) (Chambers et al., 2008). In tumor-bearing control, there is a time dependent increase in HK activity. BA treatment caused a significant decrease in HK activity in liver (Figure 25 A), kidney (Figure 25 B), and DL cells (Figure 25 C) from 24-96 h of treatment. Cisplatin (CDDP) treatment also caused a significant time dependent decrease in HK activity in liver (Figure 25 A), kidney (Figure 25 B), and DL cells (Figure 25 C) (24-96 h). Hexokinase is believed to be an integrator of metabolism and mitochondria bioenergetics, facilitating glucose utilization and inhibiting cell death. Hexokinase is a promising cancer drug target as it links cancer cell glycolysis with mitochondrial-mediated apoptosis. Because both glycolysis and respiration are energy producing processes, inhibiting one or both pathways using selectively targeted drugs
potentially would serve as an anticancer mechanism. These features of hexokinase are being exploited to develop selective strategies to neutralize the enzyme by either promoting its release from mitochondria or by blocking its activity.

Pyruvate kinase (PK; 2.7.1.40) catalyzes the irreversible transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, producing one molecule of pyruvate and one molecule of ATP. Because the pyruvate kinase reaction is essentially irreversible under intracellular conditions it is also an important site of regulation. Tumor cells use more glucose than normal cells through insufficient energy metabolism by producing lactate instead of using oxidative phosphorylation (Pelicano et al., 2006; Chen et al., 2007). As tumor cells with uncontrolled cell growth need more energy, the essential enzyme pyruvate kinase is overexpressed to maintain high glycolytic activity (Vander Heiden et al., 2010). In control group, pyruvate kinase activity increases in liver, kidney and DL cells from 24-96 h. BA treatment results in a significant decrease in pyruvate kinase activity in liver (Figure 26 A), kidney (Figure 26 B), and DL cells (Figure 26 C). Inhibition of the enzyme pyruvate kinase which catalyzes the third irreversible reaction of glycolysis, represents another strategy for inhibiting glycolysis; this enzyme seems to play an important role in tumor growth and invasion, and its inhibition may produce antitumor effects (Mazurek et al., 2005).

Glutathione (GSH) and GSH-related enzymes such as glutathione S-transferase (GST), glutathione reductase (GR) and glutathione peroxidase (GPx) have been reported to be involved in intracellular defence mechanisms in the detoxification of peroxides, xenobiotics, hydroperoxides and drugs (Chasseaud, 1979; Meyer et al., 1998). GSH and its associated enzymes have been implicated in
resistance to alkylating agents (Kramer et al., 1988). They may also play a role in resistance to natural product drugs which are a part of the multidrug resistance phenotype (Kramer et al., 1988). In the present study, changes in GSH and GSH-related enzymes (GST, GR and GPx) in liver, kidney, and DL cells of tumor-bearing mice were evaluated under different treatment conditions.

Glutathione (γ-L-Glutamyl-L-cysteinyl-glycine) is a sulfhydryl (-SH) antioxidant enzyme cofactor that serves essential functions within the cells (Sies, 1999). Glutathione, an important cellular antioxidant, is involved in protection against different free radicals, cellular peroxides and toxic compounds in cellular systems (Gerster, 1995; Dash et al., 2013). GSH, acts on multiple levels of the defence system. The thiol group of GSH participates in the protection against deleterious effects of reactive oxygen species (ROS) evolved during biological imbalance as well as cancerous conditions. GSH is an important constituent of intracellular protective mechanisms against various noxious stimuli including oxidative stress. However, reduced GSH as the main component of endogenous non-protein sulfhydryl pool is known to be a major low molecular weight scavenger of free radicals in the cytoplasm. Because of their exposed sulfhydryl groups, non-protein sulfhydryls bind a variety of electrophilic radicals and metabolites that may be damaging to cells. 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. Cellular GSH levels can be decreased through ROS-induced GSH oxidation or GSH export from the cells. Reduced GSH concentrations further enhance ROS production during
oxidative challenge (D’Alessio et al., 2003). GSH metabolism has a complicated role in both cancer and antineoplastic therapy. While GSH is important in the detoxification of carcinogens, its elevated state in many types of tumours may also increase resistance to chemo and radiotherapy (Midander et al., 1982). The findings from the present study showed variations in GSH concentrations in different tissues of tumor-bearing mice and also in DL cells at different stages of tumor growth. The highest concentration of GSH was found in liver, followed by kidney and DL cells in both betulinic acid and cisplatin treatment groups (Figure 27 A, B and C). GSH concentration did not change much in liver and kidney but decreased significantly in DL cells after betulinic acid treatment during 24-96 h (Figure 27 A). Cancer cells can generate large amounts of hydrogen peroxide which may contribute to their ability to mutate and damage normal tissues, and moreover, facilitate tumor growth and progression (Szatrowski and Nathan, 1991). It has been suggested that persistent oxidative stress in tumor cells could partly explain some important characteristics of cancer, such as activated proto-oncogenes, genomic instability, drug resistance, invasion and metastasis (Toyokuni et al., 1995) and the resistance of many cells against oxidative stress is often associated with high intracellular levels of GSH (Estrela et al., 1995). A decrease in the rate of cancer cell proliferation has also been correlated with a decrease in GSH level in tumor cells (Estrela et al., 1992). Thus, the variations in GSH concentration in the Dalton’s lymphoma cells and other tissues with tumor growth in the host may reflect alterations in the antioxidant machinery accompanied by changes in the rate of proliferation of Dalton’s lymphoma cells in the host. A depletion of GSH levels could increase the cytotoxicity of a variety of antitumor agents (Arrick and Nathan, 1984; Khynriam and Prasad, 2003) which in
turn could induce apoptotic cell death (Kane et al., 1993). GSH depletion can be seen to enhance the antitumour cytotoxicity of various drugs without increasing toxicity to normal tissues (Chen et al., 1998). The observed time dependent decrease in GSH level in DL cells after BA treatment (Figure 27A) may be a noteworthy step in the antitumor activity of BA against Dalton’s lymphoma. Therefore, it can be suggested that BA mediated decrease in the level of GSH in DL cells may have a role in the antitumor activity by increasing DL cell’s susceptibility to oxidative stress, thereby, increasing host’s survivability.

Glutathione S-transferases (GST; EC 2.5.1.18) are major phase II detoxification enzymes found mainly in the cytosol. They play an important role in protecting cells from damages caused by endogenous and exogenous compounds by conjugating reactive intermediates with glutathione to produce less reactive water-soluble compounds. GST is an important enzyme, which neutralizes the active sites of electrophiles and initiates the detoxification process (Kumar et al., 2014). GST binds with the –SH group of glutathione. These conjugates are then converted into water soluble entities such as mercapturic acid (final product) by the cleavage of glutamate and glycine residues from the conjugates and acetylation of free amino groups of cysteiny l residues (Habig et al., 1974; Sheweita, 2000). They have peroxidase and isomerase activities and are involved in the protection of cells against H₂O₂-induced cell death (Sheehan et al., 2001). GST dependent conjugation of GSH and multidrug resistance protein (MRP) dependent conjugate efflux act in synergy to confer resistance to specific anticancer drugs, carcinogens and other cellular toxins (Riddick et al., 2005). GSTs are involved in the biotransformation of exogenous substances, including mutagens, carcinogens, and other poisonous chemicals, and
play a crucial role in the detoxification process, thereby protecting cells from these compounds (Strange et al., 2001). Over expression of GSTs may increase susceptibility to carcinogenesis and inflammatory disease (Townsend and Tew, 2003; Balendiran et al., 2004). In the present study, betulinic acid treatment of tumor-bearing mice caused a significant decrease in GST activity in liver, kidney and DL cells during 24-96 h of treatment (Figure 28 A, B, and C). Cisplatin treatment also resulted in a significant decrease in GST activity in liver, kidney, and DL cells during 24-96 h of treatment (Figure 28 A, B, and C). Increased activity of GST has been reported in several human tumors (Saydam et al., 1997). The decrease in GST activity in DL cells after betulinic acid treatment (Figure 28 C) may also have a role in the decrease/failure of cellular defence mechanism which may also support the view of betulinic acid-mediated mitochondrial damage and DL cell cytotoxicity.

Glutathione reductase (GR; EC 1.6.4.2) is associated with cellular defence mechanism against oxidative stress. GR catalyzes the NADPH-dependent reduction of glutathione disulfide (GSSG) to GSH (Sweet and Blanchard, 1991) and maintains more than 98% of intracellular glutathione in reduced form (Wang and Ballatori, 1998). It is essential for the glutathione redox cycle that maintains adequate levels of reduced cellular GSH (Dolphin et al., 1989). This reduction reaction is essentially irreversible, and accounts for the very high GSH/GSSG ratios found in cells. By generating GSH, GR indirectly participates in the protection of cells against oxidative stress and is deeply involved in the maintenance of the redox status of cells. In the present study, BA treatment caused a significant decrease in GR activity in liver, kidney and DL cells during 24-96 h of treatment (Figure 29 A, B, and C). Cisplatin treatment of mice resulted in a significant increase in GR activity in liver
during 24-96 h (Figure 29 A), in kidney a significant increase was observed during
24-48 h of treatment and then it was found to decrease at 72-96 h (Figure 29 B), but a
significant decrease was noted in DL cells from 24-96 h of treatment (Figure 29 C).
When GR activity is impaired, the ability of the cells to reduce GSSG to GSH may
be devastated, leading to GSSG accumulation within the cytosol. GR activity in DL
cells decreases (Figure 29 C), which may affect the conversion of GSSG to GSH,
thus, leading to the decrease in cellular GSH levels. The BA mediated decrease in
GR activity in DL cells (Figure 29 C) could be one of the other possible steps
involved to decrease the GSH level, thus, affecting cellular antioxidant machinery
resulting in antitumor activity.

Glutathione peroxidase (GPx; EC 1.11.1.9) is a scavenger enzyme that
catalyzes the oxidation of GSH to GSSG and the concentration of GSH and GSSG
are generally maintained by the coordinated activity of GR and GPx (Wang and
Ballatori, 1998). It also protects the cells or organisms from oxidative stress. The
GPx system is important for the protection of cells against oxidizing species such as
H₂O₂ and hydroperoxides of fatty acid and phospholipids formed because of ROS
(reactive oxygen species) (Li et al., 2000). In studies using hepatocytes (Jones et al.,
1981), lung epithelial cells (Suttorp and Simon, 1986) and tumor cells (Nathan et al.,
1981), the GPx/GSSG-reductase system has been shown to be important in
protection against H₂O₂ induced cytotoxicity. Thus, GPx is remarkably effective in
preventing cell death from peroxide-mediated oxidation. Increase in GPx associated
with various forms of carcinogens has been documented widely and the scavengers
of GPX are known to play an important role in cancer prevention. During
carcinogenesis, the GPx activity significantly decreases in mice (De and Das,
The present study showed a significant time dependent decrease in GPx activity in liver, kidney and DL cells after BA treatment (Figure 30 A, B, and C) reflecting that the system of defence mechanism against free radicals may have been severely damaged in the tissues. Therefore, it may be suggested that the observed decrease in GPx activity in DL cells during 24-96 h of BA treatment (Figure 30 C) may help accumulation of free radicals inside the cells leading to tumor cell death caused by oxidative stress, thereby increasing host’s survivability.

Docking is frequently used to predict the binding orientations of small molecule drug candidates to protein/enzyme targets in order to in turn predict the affinity and activity of the small molecule. The receiving molecule that primarily binds to a small molecule or another protein or a nucleic acid is called receptor. A molecule that forms the complementary partner in the docking process is called ligand. AutoDock4.2 was used for the molecular docking studies of GPx, GST, GR, LDH, SDH, HK and PK with BA and CDDP (Morris et al., 2009). Docking results indicate that BA and CDDP strongly bind to the active sites of the above mentioned enzymes with binding energies of -6.22 and -3.55 for GPx, -8.46 and -3.29 for GST, -6.60 and -2.83 for GR, -8.42 and -3.48 for LDH, -1.74 and -3.63 for SDH, -7.93 and -3.50 for HK, -6.40 and -3.77 for PK (ΔG) [kcal/mol] respectively (Table 3) and this could be a possible reason for inhibition of these enzymes activities as noticed in enzymes assays. The docking results of BA and the above mentioned enzymes interaction showed an almost similar interaction in the active site of the enzymes as that of cisplatin, a known inhibitor, which causes inhibition in enzyme activity (Figure 31 A, B, C, D, E, F, G, H, I, J, K, L, M, and N).
The role of immune system on disease progression has been investigated previously and the prognostic importance of some hematological parameters including leukocyte and platelet counts has been shown in various malignancies (Zahorec, 2001; Yamanaka et al., 2007). In addition, the neutrophil-lymphocyte ratio (NLR) has been reported as a simple marker of systemic inflammatory response in cancer patients (Cho et al., 2009; Smith et al., 2009). The evaluation of hematological parameters is easy and cost-effective in determination of prognosis and tumor response to therapy. In this study haemoglobin (Hb), red blood cells (RBC), white blood cells (WBC), packed cells volume (PCV), differential leukocytes count (DLC) were investigated which has prognostic importance in predicting the survival of mice with murine ascites Dalton’s lymphoma. Cancer is frequently associated with erythrocyte abnormalities. Low lymphocyte counts (<1500 mm) indicate cell-mediated immunodeficiency which is a common feature in cancer physiology, but also that has relevant prognostic role for survival (Bruckner et al., 2000). The effect of betulinic acid administration increased the amount of haemoglobin (Figure 32 A), PCV (Figure 32 D), RBC counts (Figure 32 B), neutrophils (Figure 33 C), lymphocytes (Figure 33 A), while WBC counts (Figure 32 C) were decreased. Leukocytes formed in the bone marrow enter the blood for defence mechanism. There is an increase in haemoglobin, RBC, monocytes, lymphocytes when compared to tumor-bearing control group and a decrease in WBC and neutrophils (Table 4; Table 5; Figure 32, 33). One of the reliable criteria for judging the value of any anti-cancer drug is the decrease of WBC from blood. The results of present study clearly demonstrate that BA brought back haematological parameters to more or less to normal levels. Usually in cancer chemotherapy, the
major problems that are being encountered are of myelosupression and anaemia (Price and Greenfield, 1958) but results clearly shows that BA brought back haemoglobin close to normal. These results clearly demonstrated the antitumor effects of BA on tumor-bearing mice and also shows that BA does not have any hematotoxicity.

Histopathology is the microscopical examination of tissues from the body to spot characteristics of disease. Histology of liver from mice in control group showed normal hepatic architecture (Figure 34 A). The histopathological examination of the kidney from the control mice also showed normal renal cortex and medulla with normal histological features i.e. normal structure of glomeruli and the Bowman’s capsule with normal space between the glomeruli and Bowman’s capsule (Figure 35 A). In the present studies, liver and kidney sections from BA treated group did not show change in hepatic and renal architecture and the features were similar to that of control (Figure 34 B, 35 B). The parietal layer of its renal capsule is composed of simple squamous epithelium. The renal corpuscles of glomeruli are surrounded by proximal and distal convoluted tubules. Bundles of parallel tubules can be identified running into the cortex. All of these pathological findings indicate that BA protects liver and kidney structure and does not show any toxicity. The significant restoration of all of the above biochemical and histopathological parameters towards normal values upon BA treatment indicates the protection of vital organs such as liver and kidney from damage induced by DL. Hence, the present study confirms the potent hepatoprotective and antioxidant nature of BA.

Kidney is a complex and dynamic organ. Although excretion of wastes is the primary function, it also plays a significant role in regulation of total homeostasis.
Regulation of extracellular volume and control of electrolyte and acid base balance are also important functions of kidney. A toxicological insult to the kidney could affect any one or all of these functions. The toxic effects of affected kidney will be reflected as decreased elimination of wastes, an increase in blood urea nitrogen, and an increase in plasma creatinine. These parameters are clinical indices of nephrotoxicity. BA treatment did not show much change in serum creatinine level as compared to control. CDDP treatment results in a significant increase in plasma creatinine level as compared to that of control, thus indicating nephrotoxicity (Table 6; Figure 36).

Liver is one of the prime target organs of any disease. Cancer results in hepatocytes damage, liver injury, and inflammation that lead to increased cell permeability and leakage of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Hence, elevated levels of AST and ALT in the plasma are hallmarks of hepatic damage (Jain et al., 2012; Raghu et al., 2012). In BA-treated group, ALT and AST activities did not change much as compared to control. In comparison, CDDP treatment resulted in a significant increase in AST and ALT activities, thus, indicating hepatotoxicity (Table 7; Figure 37). These results were in agreement with previous findings showing that mice treated with BA have significantly reduced serum levels of ALT and AST elevated by d-galactosamine/lipopolysaccharide (Zheng et al., 2011), indicating that the hepatocytes sustained minimal injury and their membranes were stabilized. It can thus be inferred from this study that BA does not induce any significant hepatotoxicity.

Cholesterol is a neutral lipid that plays an essential role in the maintenance of the integrity of biological membranes and serves as a precursor in the synthesis of
many endocrine mediators. It serves as a precursor for the synthesis of steroid hormones, bile acids, and vitamin D (Russel and Setchell, 1992). It is also synthesized in mammalian cells via the mevalonate pathway. It has been reported that an increased cholesterol level participates in cancer cell malignancy, and dysfunction of cholesterol metabolism may also influence cancer progression (Li et al., 2003; Cruz et al., 2013). As compared to tumor-bearing control mice, serum cholesterol levels were significantly decreased in BA treated mice. Similarly, CDDP treatment also resulted in a significant decrease in cholesterol level was observed as compared to control (Table 8; Figure 38).

Thus, findings from the present studies establish the antitumor potential of BA against murine ascites Dalton’s lymphoma which induced various apoptotic features, significantly decreased mitochondrial membrane potential, GSH level, SDH and LDH activity in DL cells.

Based on the findings from various aspects of studies undertaken, following important conclusions may be derived:

- Betulinic acid is quite effective against murine ascites Dalton’s lymphoma showing a significant increase in life span of the hosts. Further, betulinic acid is found to be more cytotoxic to DL cells than normal cells i.e. spleen cells.

- Various apoptotic features observed in tumor cells after betulinic acid treatment could be an important step in developing the antitumor activity in the host. Cell cycle analysis revealed that betulinic acid treatment caused an increase in the cells in the S phase which may suggest that it prevents DL cells from replicating further, which may induce tumor cell death and favouring towards hosts survivability.
Betulinic acid treatment caused a time-dependent decrease in mitochondrial membrane potential which may also add to its antitumor effects against DL cells. The decrease in SDH activity in DL cells after betulinic acid treatment may also play a role in the development of mitochondrial dysfunction contributing to tumor cells death.

Betulinic acid treatment caused a decrease in the GSH levels in DL cells which may lead to a decrease in the protective ability of the cells thereby may become more prone to oxidative stress and cell injury.

Betulinic acid treatment caused an inhibition in the glycolytic enzymes activities such as hexokinase and pyruvate kinase thus, resulting decreased glycolysis in DL cells, leading to antitumor effects. Molecular docking findings indicate that betulinic acid binds strongly to the above mentioned enzymes, which could be one of the possible reasons for inhibition of these enzymes activities.

The decrease in LDH activity in DL cells and a simultaneous increase in the blood serum and ascites fluid after betulinic acid treatment may indicate release of LDH from DL cells due to membrane damage.

The observed betterment or no changes in the different haematological parameters after betulinic acid treatment may suggest that it shows no hematotoxicity in the hosts. Betulinic acid treatment also showed no/minimal toxicity in liver and kidney, which was further confirmed by assaying the different liver function and renal function tests. Serum cholesterol levels were also found to decrease after betulinic acid treatment.
Given the fact that no serious adverse side effects were observed following BA treatment, BA emerges to be an attractive cytotoxic agent for the treatment of various types of cancer and murine ascites Dalton’s lymphoma in particular.