4. DISCUSSION

Cancer has become a major healthcare challenge worldwide. It is projected that by 2030, globally there will be ~26 million new cancer cases and 17 million cancer deaths per year unless further preventive measures with adequate diagnosis and treatment are practiced (Thun et al., 2010). The main goals of cancer diagnosis and treatment programme are to cure or considerably prolong the life of patients and to ensure the best possible quality of life to cancer survivors. Chemotherapy, surgery and radiotherapy are the three main widely accepted treatments, for cancer. However, for better treatment results, combination of more than one type of cancer therapy or chemotherapeutic drugs is commonly used. More than 100 different drugs have been approved for their use in cancer chemotherapy. However, therapeutic efficacy of most of these drugs is limited due to the development of various side effects in the host and/or acquired drug resistance by cancer cells. Thus, attempts have also been made to overcome the drug-induced diverse side effects in the host by the development and use of new anticancer drug(s), or by using the drugs in combination with some modulating agents without compromising its therapeutic efficacy.

CDDP is one of the most effective drugs used in cancer chemotherapy against various types of cancers. The cytotoxic mode of action of CDDP is mediated by its interaction with DNA to form DNA adducts that lead to the death of cancer cells. However, its therapeutic application is limited because of its toxicity to normal tissues in the host and has, therefore, been frequently used in combination with one or more anticancer drugs/agents, with better results.

Ascorbic acid (AA) also known as vitamin C is a well known antioxidant, which can protect the body from damages caused by free radicals that can be generated during
normal metabolism as well as through exposure to toxins and carcinogens (Banerjee et al., 2009). AA at a nontoxic concentration, in combination with certain pharmacological agents produces a synergistic or additive effect on the growth inhibition of cancer cells. The pro-oxidant activity of AA is due to its ability to redox-cycle with transition metal ions, and thereby stimulates the formation of species such as superoxide, hydrogen peroxide and hydroxyl radicals. Many in vitro studies have shown that AA treatment enhances the cytotoxicity of various anticancer drugs including CDDP (Kurbacher et al., 1996) in different cancer cell lines. The improvement of antitumor activity of cyclophosphamide in vivo against murine ascites Dalton’s lymphoma has also been reported (Prasad et al., 2010). Sarna and Bhola (1993) used AA by intraperitoneal (i.p.) injection in vivo in combination with CDDP and also treated tumor cells in vitro with CDDP or AA plus CDDP and then transplanted these tumor cells to animals and found an improved therapeutic efficacy in combination treatment condition. Nonetheless, it has also been highlighted that the decrease in the activity of some agents may be the consequence of direct inactivation of the drug in vitro by AA, as nicely described in the case of bortezomib (Zou et al., 2006).

In general, the findings from various reports suggest that AA (vitamin C) together with chemotherapy drugs may overcome chemotherapy-dependent resistance in cancer cells by increasing the delivery of chemotherapy drugs into cancer cells, making the tumor cell membrane more permeable to have enhanced drug delivery, stabilize p53 genes and decrease Bcl-2 and telomerase activity. Although AA has been well documented to reduce the incidence of most malignancies with its antioxidant/protective function (Block, 1991; Kathleen, 1998), however, the definite mechanism of AA in producing anticancer effect still remains inconclusive (Verrax and Calderon, 2008).
In present study, antitumor efficacy of CDDP alone or in combination with AA against murine ascites Dalton’s lymphoma (DL) was evaluated. The treatment of tumor-bearing mice with combination of AA plus CDDP group revealed a very significant increase in the host survivability (ILS~142%) as compared to the group treated with either AA or CDDP alone (Table 1; Figure 7) suggesting additive/synergistic antitumor activity of AA and CDDP against murine ascites Dalton’s lymphoma. Earlier reports by Prasad et al. (1992) showed sequence-dependent synergistic antitumor effect of AA and sub-therapeutical dose of CDDP leading to tumor regression with significant increase in host survivability. The changes in total body weight of tumor-bearing mice under different experimental conditions may also be related with the changes in tumor growth and in survivability pattern. A steady increase in average body weight in the control group of tumor-bearing mice from ~25g on the day 1 of tumor transplantation to about 35g on the 20th day of tumor growth was observed suggesting regular DL tumor growth in the host (Figure 8). However, analysis of the changes in the body weights of tumor-bearing mice in different treatment groups showed that the combination of mice with AA plus CDDP caused utmost decrease in body weight of mice which may be correlated with reduced tumor growth/ tumor volume and an increase in survival time (Figure 7). The enhancement of CDDP-induced tumor inhibition may be due to modulation of permeability of tumor cell membrane by AA, causing an increase in the uptake of CDDP into tumor cells and making the DNA repair machinery less efficient due to more adduct formation in the DNA. Platinum uptake analysis of DL cells showed that under the condition of combination treatment platinum (CDDP) uptake is enhanced by the DL cells (Figure 22).

The microenvironment in cancer cells in both animals and humans are known to be acidic as compared with that in normal tissues because of elevated production of lactic acid as a result of anaerobic glycolysis. The pH of cellular environment greatly
influences the uptake and/or activity of various anticancer agents (Tannock and Rotin, 1989). The decrease in tumor pH noted especially after combination of AA plus CDDP treatment (Figure 9) might be due to changes in tumor metabolism influenced by AA which could also be an important step in sequence-dependent antitumor activity of AA and CDDP, whereby, the antitumor activity of CDDP is enhanced resulting in an increase in host survivability.

AA has various biomedical efficacies such as anti-inflammation, immune modulation and antioxidant in the immune system (Hartel et al., 2004). In cancer therapy, AA has a great potential on the reduction of the side effects of chemotherapeutic drugs as well as increasing of therapeutic efficacy. It has been reported that AA prevents proliferation and metastasis of cancer cells (Lin et al., 2006; Hahm et al., 2007; Kim et al., 2011). AA at a nontoxic concentration, in combination with certain pharmacological agents produces a synergistic or additive effect on the growth inhibition (Sarna and Bholia, 1993). However, the extent of modification of the effects of agents by AA depends upon the tumor cells and the type of pharmacological agents. In present study, the tumor-bearing mice have a lower serum AA concentration as compared to its normal counterpart (Table 2; Figure 20 & 21). Ghosh and Das (1984) also reported decreased level of serum AA in tumor-bearing hosts. The low levels of serum AA may be due to their increased utilization to scavenge lipid peroxides as well as their sequestration by tumor cells. As was expected, AA treatment increased its concentration in serum, DL cells and ascites supernatant (SN). Following CDDP treatment there was an increase in AA concentration, which was noted to further increase in serum, DL cells and SN after combined administration of AA plus CDDP (Table 2; Figure 20 & 21). Kurbacher et al. (1996) reported that vitamin C could increase the antineoplastic activity of CDDP and some other anticancer agents. The possible anticarcinogenic effects of AA may be accounted for by its ability to detoxify
carcinogens as well as its ability to block carcinogenic processes through its antioxidant activity. Cameron and his colleagues (1975) reported that administration of high doses of vitamin C to terminal cancer patients produced regression of tumor and prolonged their life expectancy. Cameron and Pauling (1976) proposed AA to have an inhibitory effect on the action of hyaluronidase, which is responsible for invasiveness of malignant tumors. In vitro studies have shown that AA at high concentrations enhances the cytotoxicity of 5-FU in a dose-dependent manner in mouse lymphoma (Nagy et al., 2003). AA can scavenge reactive oxygen- and nitrogen species and thereby prevent oxidative damage to important biological macromolecules such as DNA, lipids and proteins. AA can function as a strong pro-oxidant in presence of metal ions like Cu(II) and Fe(III) (Jansson et al., 2005). The pro-oxidant activity is due to its ability to redox-cycle with transition metal ions, and thereby stimulates the formation of ROS such as superoxide, hydrogen peroxide and hydroxyl radicals. It has been suggested that AA promotes oxidative metabolism by inhibiting use of pyruvate for anaerobic glycolysis (Ramp and Thornton, 1968). AA at high doses inhibits prostaglandins of the second series which have been correlated with increased cell proliferation. Clinical trials indicate that AA may confer protection on various normal tissues without attenuating antitumor response. The mechanism of protection is based on physiological differences between the tissue types and on differential uptake of AA. Pauling et al. (1985) reported that large quantities of dietary AA decreased the incidence of and delayed the first appearance of spontaneous mammary tumors in RIII/Imr in mice. Vitamin C can inhibit tumors produced by nitrosamine in animals through inhibition of nitrosamine formation (Chen et al., 1988). It is suggested that depletion of AA could be an early and critical event during drug induced toxicity. Epidemiologic studies have also linked lower dietary AA consumption with an increased risk for gastric cancer (Block, 1991; Cohen and Bhagavan, 1995). It has also been reported that AA may also be used as an adjuvant
in to enhance antitumor efficacy of CDDP in the host (Abdel-Hamid et al., 2011). Experiments conducted on mouse neuroblastoma cells revealed that combination of AA and H$_2$O$_2$ significantly caused cancer cell death (Hardaway et al., 2012). In present study, the observed increase in AA concentration after combination treatment of AA plus CDDP could be a better effective strategy to facilitate CDDP induced damage/ killing of DL cells (Table 2; Figure 20).

The interaction of metastatic cells with the host environment occurs, to a large extent, through the cell surface. Cell membrane/surface changes are an important factor that influences the structural and functional properties of malignant cells (Gallagher, 1985). Cell-cell adhesion regulates the pattern of growth and behaviour of malignancy in tumors (Yakota, 2000). Significant modifications of cell surface and disintegration of the plasma membrane of rat hepatoma cells have been reported after bacterially fermented mistletoe preparation (BFMP) treatment (Ribereau-Gayon et al., 1986). The plasma membrane disintegration and formation of cytoplasmic vacuoles on tumor cells observed during CDDP treatment could be an indication of tumor cell lysis, eventually leading to cell death (Figure 10). Aberrant release of microvesicles arises in cancerous state (Lee et al., 2011). The formation and shedding of membrane vesicles has been reported to occur during T-lymphocyte mediated cytolysis (Liepens et al., 1978). Blebs have also been reported to appear in unusual numbers on many transformed cell lines e.g. Adenovirus-type-5-transformed hamster embryo cells and human carcinoma A 549 and mouse embryo cells transformed chemically (Gonda et al., 1976). It is likely that blebs result from alterations of the cortical microfilament network. Ascites DL cells showed the presence of numerous surface membranous blebs all over the cells and shedding of membrane vesicles during treatment condition which according to the report by Cocucci et al., 2009 may serve to shuttle bioactive molecules between cells, which in turn may modulate the extracellular environment. The combination treatment
of mice with AA plus CDDP showed enhanced cellular membrane deformities on DL cells which may ultimately lead to cell death (Figure 10). The reorientation of cell surface blebs and membrane disintegration resulting from CDDP alone as well as combination of AA plus CDDP treatment appears to be an important step towards tumor cell lysis. Hence, taking into consideration the present data and the earlier reports, it is clear that the formation of membrane blebbing is an important event occurring during tumor cell lysis following CDDP treatment which might arise as a consequence of induced oxidative stress during drug metabolism (Lemasters et al., 1987) and/or immune response mediated killing (Liepins, 1983).

Micronuclei (MN) assay is a widely used cytogenetic method to assess in vivo and in vitro chromosomal damage of various alkylating agents (Maier and Schmid, 1976) drugs (Devi et al., 2010). MN are chromatin masses that arise from chromosome fragments of intact whole chromosomes lagging behind at the anaphase stage of the cell division (Czyzewska and Mazur, 1995). These displaced chromosomes or chromosome fragments are eventually enclosed by a nuclear membrane and, except for their smaller size, are morphologically similar to nuclei after conventional nuclear staining (Terradas et al., 2010; Fenech et al., 2011). Chemicals that cause CA are known to induce MN in a variety of proliferating cell systems, both in vivo and in vitro. Genotoxic agents have the potential to interact with DNA and may cause DNA damage. MN are indicators of chromosome instability, since the frequency of MN is higher in tumor cells which show a defective DNA damage repair system and disrupted cell cycle checkpoint machinery (Terradas et al., 2010). Here, single and multiple micronuclei were observed in the DL cells of mice in all treatment groups (Figure 11). Time-dependent decrease in the micronucleus incidence was observed during 24-96 h with maximum frequency of MN observed during 24 h in all treatment conditions (Figure 12). It is well known that apoptotic cell death is induced by DNA-damaging agents (Darzynkiewicz et al, 1997)
and micronucleus arises from disturbed genetic materials such acentric chromosomal fragments (Heddle and Carrano, 1977) or even whole chromosomes (Weissenborn and Streffer, 1991), the cells containing micronucleus may indicate for their potential cell death later. After exposure of cells to toxic agents, in contrast to the interphase apoptotic cell death, the post-mitotic apoptosis occurs as a result of irreparable damages. The post-mitotic apoptosis may represent ‘delayed apoptosis’, thus the cells containing MN may die by ‘delayed apoptosis’. CDDP significantly elevated the frequency of MN in the tumor cells while the frequency of micronucleated cells decreased significantly in the combined treated group (Figure 12). Moreover, the decrease in frequency of MN observed during 24-96 h of combination treatment reveal that AA could exert time-dependent antimutagenic effect indicating greater detoxification ability at later stages.

Uncontrolled proliferation and a defect in apoptosis constitute crucial elements in the development and progression of malignancy. Apoptosis is characterized by membrane blebbing, shrinking of cells and their organelles, DNA fragmentation, and finally cell disintegration (Elmore, 2007). Induction of apoptosis may potentially allow many chemotherapeutic drugs including CDDP to kill cancer cells (Gonzalez et al., 2001). The observation based on fluorescence microscopy (AO/EtBr staining), is a good reliable indicator for confirmation of apoptotic features (Okada and Mak, 2004). The analysis of apoptosis in DL cells based on AO/EtBr staining showed that as compared to respective treatment with CDDP alone, the number of apoptotic DL cells increased after combination treatment with AA plus CDDP (Figures 14). The treatment with AA alone also showed time dependent increase in appearance of apoptotic features in DL cells (Figure 14). The varied effect of AA on the chemotherapeutic drug-induced apoptosis in different cancer cell lines has been reported. The anticancer activity of arsenic trioxide against HL-60 cells was enhanced when co-treated with AA and arsenic trioxide and it involved induction of apoptosis in these cells (Yedjou et al., 2009).
Intracellular ascorbate enhanced apoptosis by increased use of etoposide in HL60 cells but had no effect on etoposide-induced apoptosis in Jurkat cells. In both cell types, melphalan-induced apoptosis was not altered by cellular ascorbate (Gokhale et al., 2006). The present findings of increase in apoptotic tumor cells after combination treatment with AA plus CDDP is in conformity with earlier reports, although the exact mechanism behind it may not be clearly evident at present. It has been reported that AA increases the apoptosis via up-regulation of p53 during CDDP treatment of human colon cancer cells (Bragado et al., 2007; An et al., 2011). In ascorbate-supplemented cells, increased CDDP- induced apoptosis was seen, involving activation of the MLH1/c-Abl/p73 signalling cascade. It has also been reported that the cellular response to DNA damage requires activation of MLH1 which may cooperate with the tumor-suppressor p53 gene to promote cell cycle arrest and cell death (Catani et al., 2002). It has been reported that AA (10 mM) induces apoptosis in B16 murine melanoma cells by a reduction in the mitochondrial membrane potential and release of cytochrome c (Kang et al., 2003).

To further corroborate fluorescence based result on CDDP-induced apoptosis, the characteristics of DL cells were studied after CDDP alone and in combination with AA treatment using SEM and TEM. Cell surface and ultrastructure studies following AA treatment reveal cytolysis, cell membrane disruption/disorganization, mitochondrial alteration, nuclear and nucleolar reduction, and increased apoptotic and phagocytic activity in cancer cells (Walingo, 2005). Surface morphological alterations are important signs of cell injury which may be considered as specific markers of apoptosis (Gyulkhandanyan et al., 2012). The SEM observations revealed a series of surface changes in DL cells following different treatment (Figures 15 & 16). CDDP alone or AA plus CDDP treatment for 24-96 h revealed membrane blebs, loss in microvilli, cell membrane fusion, shrinkage and deformities in comparison to the control DL cells.
where ruffles and membrane projections are evenly distributed over the cell surface. The formation of membrane blebs/vesicles in tumor cell observed after different treatment conditions (Figures 15 & 16) could be an indication of the appearance of apoptotic features in DL cells.

Mitochondria are the sites of fatty acids β-oxidation, Krebs’ cycle, electron transport, and oxidative phosphorylation. Mitochondria undergo various structural changes concomitant with changes in their functions under a variety of pathological conditions. Various recent findings have indicated that mitochondria may be involved in the maintenance of malignant phenotype, mutagenesis and control of apoptosis (Murphy and Smith, 2000; Modica-Napolitano and Singh, 2004; Liang and Grootveld, 2011). Alterations in mitochondrial structure and function occur early during apoptosis before nuclear or chromatin structures are affected suggesting that mitochondria may play a pivotal role in the process (Modica-Napolitano and Singh, 2002; Mammucari and Rizzuto, 2010). Ultrastructural examination of DL cells treated with CDDP alone or in combination of AA plus CDDP showed mitochondrial structural abnormalities in DL cells. In present study, control DL cells showed distinct normal mitochondrial features with regular cristae (Figure 17a-b). Mitochondrial membrane disruption, disappearance of membrane processes, elongation of mitochondria with thickened and reduced number of mitochondrial cristae and formation of vacuoles are prominent features observed during the later stages of CDDP treatment (48-96 h) in vivo (Figure 17e-g). Combination treatment with AA plus CDDP showed enhanced irregularities in the structure of mitochondria acquiring a roundish shape, more thickened and irregular arrangement of cristae, and mitochondrial membrane disruption with more pronounced vacuoles, during the later periods of treatment (Figure 17i-k). Prasad et al. (2010b) reported that CDDP treatment brings about various ultrastructural deformities in mitochondria with the appearance of vacuoles, formation of elongated shape and
thickened cristae with mitochondrial swelling which may be an important factor in the development of biochemical injury in mitochondria affecting the overall metabolism in the cells. Arismendi-Morillo (2009) suggested that mitochondrial alterations in cancer cells are heterogeneous and not specific to any neoplasm. The mitochondria in cancer, independently of histogenesis, predominantly are seen with lucent-swelling matrix associated with disarrangement and distortion of cristae and partial or total cristolysis. It was reported that inhibition of Hsp90 chaperone roles using I7AAG induces mitochondrial swelling, change in membrane potential, mitochondrial elongation and vacuolization (Vishal et al., 2011). Swelling of mitochondria bring about a decrease in membrane potential leading to decreased rates of oxygen consumption and decreased phosphorylating ability of mitochondria. Electron microscopic studies revealing fewer and structurally altered mitochondria may also infer the respiratory impairment in cancer cells (White et al., 1974; Rasmussen et al., 2003). CDDP treatment has been reported to cause a decrease in mitochondrial respiration (Souid et al., 2003). Besides different substances, inhibition of mitochondrial respiration was shown to lead to apoptosis induction (Wolvetang et al., 1994). Abnormalities in the mitochondrial structure and function of growing tumors revealed the involvement of a hypoxia-inducible factor (HIF-1α) in activating the glycolytic enzymes under various oncogenic stimulations (Kim and Dang, 2006). Additionally, HIF-1α also prevents entry of pyruvate to Krebs’ cycle by inhibiting pyruvate dehydrogenase complex resulting in attenuation of mitochondrial function thereby allowing pyruvate to enhance production of lactate by lactate dehydrogenase which becomes a factor in sustaining cancer cell survival (Koukourakis et al., 2006). The observation of the appearance of membrane vesicles and vacuoles in tumor cells in the present study following CDDP or combination treatment of mice with AA plus CDDP could be an indication of tumor cell lysis which may eventually lead to cell death.
Succinate dehydrogenase (SDH), a component of complex-II of the respiratory chain has a key role in Krebs’ cycle for energy conservation and utilization. It has also been observed that a complete lack of SDH expression hampers electron flow, ultimately resulting in major oxidative stress known to promote tumor formation (Lemarie et al., 2011). Moreover, if normal SDH activity resumed, there could be a concurrent dissolution of the tumor. Thus, the relative expression of SDH can be utilised as a marker of oxidative damage with increased expression corresponding with a decrease in ROS accumulation. Prasad and Giri (1999) reported that CDDP treatment caused a fall in oxygen consumption rate by DL cells and selective increase of Ca\(^{2+}\) particularly in kidney and DL cells. In present study, CDDP treatment of tumor-bearing mice caused a decrease in the SDH activity in DL cells (Figure 18) which may indicate depressed oxidative metabolism and mitochondrial dysfunction. Kusao et al. (2008) suggested that low expression of SDH in cultured cells treated with higher concentration of chemotherapeutic agent exhibit more oxidative damage. As SDH is an important mitochondrial membrane bound enzyme involved in energy metabolism through the TCA cycle and mitochondrial complex-II electron transport, a decrease in this enzyme activity would conciliate the mitochondrial energy metabolism leading to accumulation of succinate and abnormal functioning of mitochondria (Selak et al., 2005). Thus, further decrease in the SDH activity in DL cells during 24-48 h of AA plus CDDP combined treatment (Figure 18) may aid in the development of some anoxic condition or mitochondrial dysfunction contributing to tumor cell lysis.

Sialic acids are a group of neuraminic acid (5-amido- 3,5-dideoxy-D-glycero-D-galacto-nonulosonic acid), widely distributed as terminal sugars on oligosaccharides attached to protein or lipid moieties and plays an important role in the antigenic characterization of cells. The unique structural features of the molecule, which includes a negative charge owing to a carboxyl group, enables it to play a role in cellular
functions, such as binding and transport of positively charged molecules (e.g. \( \text{Ca}^{2+} \)), cell-to-cell adhesion and repulsion, influencing conformation of glycoproteins on cell membranes, and even masking specific cellular recognition sites which is of particular importance in the reaction of the environment to foreign cells including cancer cells (Schaeur, 1985). It has been documented that the terminal sialic acid is involved in cellular adhesion to selectin and lectins (Kelm and Schauer, 1997; Schauer, 2000). Thus, changes in sialic acid content could be an important factor for the characteristic changes in transformed or cancer cells. A number of workers have reported sialic acid as an important marker of high sensitivity and specificity in diagnosis and response to treatment of cancer (Narayanan, 1994; Joshi and Patil, 2010; Durgawale et al., 2011; Taqi, 2012). The alterations in glycoproteins start at an early stage of tumorigenesis and increased levels of sialic acid in cancer patients can be explained by spontaneous release (shedding) of aberrant sialic acid rich glycoproteins and glycolipids (Dall’Olio and Trere, 1993; Durgawale et al., 2011). It has been suggested that tumor cells have ability to change their surface properties and alter the sialo-glycoconjugates expressed on their plasma membranes which may affect their behaviour and invasion ability (Passanniti and Hart, 1988). Sialylation is one of the most common and versatile type of terminal glycosylation. An aberration in sialylation patterns is a recurrent characteristic of malignant cells which can be detected by lectins or lectin-like proteins (Fragkiadakis and Stratakis, 1997). Lectins are proteins or glycoproteins of non-immune origin with specific binding affinities for the carbohydrate moieties of glycoconjugates and have gained considerable popularity as histological reagents in many areas of diagnostic investigation, especially those related to changes in the expression of cell membrane glycoconjugates (Gabius et al., 1991; Miyoshi et al., 2001). The agglutination behaviour of normal and malignant cells depends upon the sialic acid moieties present at the cell surface (Prasad, 1986). Prasad and Sodhi (1982) reported that CDDP has an effect on
the surface of the cells, and brings about definite changes in cell lectin agglutinability and in the topographical pattern of lectin-binding sites on the cell surface. Evidences from both human and experimental tumor models demonstrate that altered sialylation of tumor cell surfaces are associated with a metastatic tumor phenotype (Miyagi et al., 2004; Varki and Varki, 2007). Increased concentrations of sialic acid at the cell surface of malignant or transformed cells have been associated with the metastatic potential and changes in immunogenicity (Wongkham et al., 2003; Babal et al., 2006). An important feature noted in the present study is the increase in sialic acid concentrations in the DL cells (Table 4; Figure 25 & 26) with tumor growth in mice. This increase may be due to the enhanced activity of enzymes involved in sialic acid synthesis and/or transfer. Sialic acid contents are reported to be elevated during malignancy (Raval et al., 2004; Trivedi et al., 2012; Lazescu et al., 2013). The observation of increased sialic acid content in the tissues particularly in liver, kidney and spleen of tumor-bearing mice as compared to the normal counterpart (Table 4; Figure 25 & 26) may also be helpful for DL cells in the host as sialic acid has been known to be important in the transport of proteins, amino acids and ions to cancer cells (McDonagh and Nathan, 1990). Effect of CDDP and combination of AA plus CDDP treatment in tumor-bearing mice showed a decrease on the quantitative changes in sialic acid of DL cells and tissues during 24-96 h (Table 4; Figure 25 & 26). About 70% of the total sialic acids of eukaryotic cells are found on the cell surface and the observed decrease of sialic acid in DL cells under different treatment conditions may be associated with an enhancement of the immune response of the hosts. Simmons and Rios (1974) suggested that the loss of sialic acid from surface membrane could lower the negative surface charge which may lead to increased cell deformability, inhibition of cell aggregation and enhanced phagocytosis. The observed decrease of sialic acid after CDDP or combination treatment may also suggest the possible exposure of certain antigenic sites on the tumor cell surfaces as sialic acid are
known to involve in masking the expression of surface antigens (Sarna et al., 1988). Along with decreased sialic acid level in DL cells, CDDP-mediated decrease of sialic acid in tissues of tumor-bearing mice should also bring about restoration of the functional activity of the tissues to normal in the hosts, thereby facilitating tumor regression.

LPO, the oxidative breakdown of polyunsaturated fatty acids, is a major contributor to the loss of cell function under oxidative stress conditions. The end product of LPO, malondialdehyde (MDA), due to its high cytotoxicity and inhibitory action on protective enzymes, is suggested to act as a tumor promoter and a co-carcinogenic agent (Otamiri and Sjodahl, 1989). Low concentration of oxygen free radicals have been reported to stimulate cell proliferation, whereas high levels induce mutagenecity, cytotoxicity and cell death (Ray and Husain, 2002). All cellular components are susceptible to attack by ROS, particularly by hydroxyl radicals. Disturbance in the balance between the production of ROS and the efficiency of the antioxidant defense results in oxidative stress (Kang et al., 2002) which may lead to cellular damage (Guo and Chen, 2012). Cancer cells generate large amounts of hydrogen peroxide, which may contribute to their ability to mutate and damage normal tissues and moreover, facilitate tumor growth and invasion (Szatrowski and Nathan, 1991). In other words, development of cancer produces oxidative stress, which may in turn promote cancer progression (Tesarova et al., 2007). Mitochondrial oxidative phosphorylation is regarded as the main source of free radicals (Naoi et al., 2009). Mitochondrial function in the cells is particularly susceptible to oxidative damage (Vladimirov et al., 1980). The poly-unsaturated fatty acids located in mitochondrial membranes are targets for peroxidation. Peroxides are formed naturally during mitochondrial respiration and a decline in mitochondrial respiratory function along with an insufficient supply of energy can significantly increase mitochondrial free radical
production (Van Houten et al., 2006). Hence, the uncontrollable production of ROS directly impairs mitochondrial structure and function. CDDP treatment is associated with induction of oxidative stress by generation of free radicals and reactive oxygen species (Martins et al., 2008; Mitazaki et al., 2013). In present study, the increased LPO level in the DL cells and tissues after CDDP treatment (Table 9; Figure 35 & 36) may be attributed to antitumor activity in DL cells and toxicity in tissues. Also the increase in mt-LPO in DL cells following different treatment conditions in present study (Figure 19) may support the view that CDDP-mediated increase in oxidative stress in mitochondria may lead to development of subsequent mitochondrial structural and functional abnormalities. Mitochondrial damage or dysfunction by CDDP has been considered as a prominent mediator of CDDP-induced toxicities. LPO can alter vital membrane protein structure and function. Attack on proteins can lead to the modification of amino acids, oxidation of sulfhydryl groups leading to conformational changes, altered enzymatic activity, crosslinking, peptides bond cleavage as well as carbohydrate modification in glycoproteins, loss of metal in metalloproteins, altered antigenicity, and increased proteolytic susceptibility (Stadtman, 1992). All cells maintain antioxidant defenses because of the damaging effects of ROS. AA is known to dispose, scavenge, and suppress the formation of free radicals or oppose their action and increase with the severity of the disease. The potential role of dietary AA to reduce the activity of free radical-induced reactions has drawn increasing attention (McCall and Frei, 1999). High antioxidant intake has been shown to reduce cancer risk and may also mitigate the effects of oxidative DNA damage, which is hypothesized to be causally linked to carcinogenesis. Co-administration of AA in CDDP treated tumor-bearing mice significantly decreased the level of LPO in tissues (except spleen) (Table 9; Figure 35 & 36). The results from present study show that AA may ameliorate CDDP-induced oxidative stress by decreasing LPO, thus demonstrating protection from CDDP-induced
tissue toxicities in the host. The decrease in LPO also implies the free radical scavenging property of AA. Thus, increase in mt-LPO could be a critical factor in CDDP-induced cytotoxicity in the tumor cells.

Inhibition of protein synthesis can alter cellular responsiveness to the anticancer drugs. Tumor-bearing mice in present studies showed a decrease in the protein concentration in all the tissues as compared to that in normal mice (Table 3; Figure 23). CDDP treatment causes definite biochemical injury which could be involved in resulting toxicity/cytotoxicity. As compared to control, a decrease of protein in the tissues (except testes) was noted after CDDP alone and combination of AA plus CDDP treatment as well (Table 3; Figure 23 & 24). These changes may involve some alterations in the rate of protein synthesis or decreased uptake of protein in these tissues. The inhibitory effect of CDDP on DNA, RNA and protein synthesis has been demonstrated earlier in vitro in mammal cells (Harder and Rosenberg, 1970). The observation of CDDP-mediated decrease in protein content may be important in contributing to the toxicity/cytotoxicity of CDDP when drug dosages are increased as tumor resistance to the drug occurs. Heminger et al. (1997) indicated that CDDP inhibited protein synthesis by causing an inhibition of elongation and suggested that this may contribute to the cytotoxic effects of CDDP during chemotherapy. On the other hand, the decreased protein concentration noted in the DL and ascites SN, may involve inhibited protein synthesis and/or proteolysis by peptidases within the cell. CDDP binding affects the activity of receptors, enzymes, and other proteins, and the resulting protein damage contributes to cytotoxicity. However, in comparison to CDDP alone, combination treatment of AA plus CDDP showed improvement in the protein concentration with a significant increase in liver, kidney, spleen and SN and significant decrease in DL cells (Table 3; Figure 23 & 24).
Glutathione (GSH) (L-γ-glutamyl-cysteinylglycine) has been the focus of interest in cancer chemotherapy (Arrick and Nathan, 1984; Kidd, 1997; Khynriam and Prasad, 2003). It is the most abundant cytoprotective tripeptide thiol that maintains redox environment of the cell, and thus helps in the vitality of the body cells (Wu et al., 2004; Vokuvic et al., 2001; Balendiram et al., 2004). Under physiological conditions more than 98% of intracellular glutathione is maintained in reduced form, GSH (Wang and Ballatori, 1998). GSH neutralizes oxygen molecules before they cause damage to cells. GSH mostly exists in its reduced form (GSH), but it can be oxidized by many factors including free radicals and during intoxication reactions. Reduced GSH plays an important role in cell defense mechanisms by acting as an antioxidant through detoxification of ROS. It can react with many toxic agents to form conjugates which are eliminated from cells (DeLeve and Kaplowitz, 1991; Wang and Ballatori, 1998). Intracellular GSH is a requisite for normal lymphocyte activation as well as T cell and NK cytotoxicity (Multhoff et al., 1995). The ratio of reduced to oxidized glutathione (GSH/GSSG) in the cell is a good indicator of the level of oxidative stress which may be initiated by a decline in the antioxidative defense system or factors that may decrease the concentrations of antioxidants. When GSH/GSSG ratio shifts toward the oxidizing state, it results in the activation of several signaling pathways, thereby reducing cell proliferation and increasing apoptosis (Sen, 2000). GSH controls the onset of tumor cell proliferation by regulating protein kinase C activity and intracellular pH (Terradez et al., 1993). The resistance of many cells to oxidative stress is also associated with high intracellular levels of GSH. Elevation of GSH levels has been shown to increase the antioxidant capacity and resistance of cancer cells to CDDP, while a depletion of GSH levels could potentiate the cytotoxicity of a variety of antitumor agents by decreasing the rate of cellular antioxidant capacity (Abdalla, 2011; Traverso et al., 2013). Present findings showed variations in GSH concentrations in different tissues of normal and
tumor-bearing mice and also at different stages of tumor growth. Earlier reports on the
determination of GSH in mice showed that total GSH level in DL tumor cells increased
significantly after 5th day of tumor growth reaching maximum level on the 10th day and
a slight decrease thereafter over the next 4-5 days when tumor growth was declining
(Prasad et al., 2010c), thus, suggesting its involvement in facilitating proliferation and
metabolism of tumor cells. During the growth of Ehrlich ascites tumor an increase in
GSH level in tumor cells has also been reported (Estrela et al., 1992). Present study
showed that total GSH level in tissues and DL cells decreased during 11th-14th day of
tumor growth (Table 5; Figure 27 & 28). Significant decrease in GSH level in DL cells
of tumor-bearing mice observed after CDDP alone or combination treatment of AA plus
CDDP may increase CDDP-induced cytotoxic effect, thus, increasing tumor cells’
susceptibility to cell death (Table 5; Figure 27 & 28). The decrease in total GSH level
by CDDP seems to play a significant role in the antitumor activity of CDDP against
Dalton’s lymphoma. CDDP has been shown to be sufficiently electrophilic to react with
GSH directly (Jones and Basinger, 1989) and the resulting GSH–platinum complexes of
both endogenous and exogenous sources is actively eliminated from cells by the GSH
S-conjugate export pump (Ishikawa and Ali-Osman, 1993). Depletion of cellular GSH
by buthionine sulfoximine (BSO) has been shown to sensitize tumor cells to oxidative
stress, irradiation and certain chemotherapeutic agents in vitro (Navarro et al., 1999;
Schnelldorfer et al., 2000), however, administration of GSH ester to BSO-treated mice
is protective against CDDP toxicity (Anderson et al., 1990). The harmful effect of free
radicals in various tissues of the body is controlled by antioxidant defense system of the
cells, of which the most important free radical chain breaking molecule is GSH (Erat et
al., 2007), thus, decrease in GSH concentration may lead to alteration in this antioxidant
defense mechanisms. It has been known that elevation of intracellular GSH in tumor
cells is associated with mitogenic stimulation (Shaw and Chou, 1986). The decreased
concentration of GSH increases the sensitivity of organs to oxidative and chemical injury. Therefore, the observed CDDP- or AA plus CDDP mediated decrease in GSH level in the tissues particularly in liver and kidney (Table 5; Figure 27 & 28) may have a role in altering cellular antioxidant machinery and in lessening the protective mechanism of tissues which may be involved to develop toxicity in the host. The reduced renal GSH can markedly increase the toxicity of CDDP. The depletion of GSH is also believed to be related to perturbation of intracellular calcium homeostasis by the increased cytosolic calcium (Zhang and Lindup, 1996) which can lead to oxidative stress and cell injury (Olafsdottir et al., 1988). Prasad and Giri (1999) have shown that CDDP treatment of tumor-bearing mice caused an increase in the concentration of calcium in kidney and DL cells. Mitochondria plays an important role in maintaining calcium homeostasis (Smaili et al., 2000; Murphy and Smith, 2000) and the change/rise in calcium could be related to mitochondrial injury. Elevation of GSH in cellular resistance to platinum agents has been reported in several human and murine tumor cell lines (Kartalou and Essigmann, 2001). Although combination treatment caused a decrease in the GSH level in the tissues during 24-72 h, GSH level was restored to approximately control levels at 96 h (Table 5; Figure 27 & 28). As GSH is known to play a role in detoxifying many reactive metabolites, the increased levels in total GSH in the tissues after combination treatment of AA plus CDDP as compared to CDDP alone treatment of tumor-bearing mice, could also represent a protective mechanism in response to various toxic radicals thereby, suggesting the possible involvement of cellular GSH as a mechanistic step in AA-mediated protection against CDDP-induced toxicity/cytotoxicity. A protective role of AA in 2,4-dichlorophenol induced teratogenic/carcinogenic toxicity along with significantly increased liver AA and GSH levels has also been reported (Nagyova and Ginter, 1995). The protective role of AA against CDDP-induced nephrotoxic effects has been noted with possible cooperative
involvement of GSH in its protective function (Prasad et al., 2006). Combination treatment enhanced the CDDP-mediated decrease of total GSH in DL cells, which might occur through the potentiating effect of AA on CDDP or its metabolite to enhance its effect in lowering GSH levels. Depletion of GSH activity has been reported to increase the sensitivity of the cells to cytotoxicity of CDDP. Hence, the observed CDDP-mediated decrease in GSH in DL cells may weaken their antioxidant defense and facilitate their killing but surviving cells may try to strengthen their antioxidant ability by recovering/accumulating more GSH. The decrease in GSH levels in tumor cells has been suggested as a means of enhancing the cytotoxic/genotoxic effects of chemotherapeutic agents (Komiya et al., 1998) and it may also constitute early possible signalling events in the apoptotic cell death (Zunino et al., 1989).

GSH and its associated enzymes have been implicated in resistance to platinum compounds (Hospers et al., 1988). GSH-related antioxidant enzymes have been reported to be involved in the detoxification of peroxides, xenobiotics, hydroperoxides and drugs (Meyer et al., 1998), and some of the excess oxygen radicals such as hydrogen peroxide, superoxides, hydroperoxy and hydroxyl radicals (OH) are shown to be implicated in a variety of disorders including cardiovascular disease, reperfusion injury, rheumatoid arthritis, immune injury and cancer (Ross, 1988). ROS such as hydrogen peroxide, superoxide anions, and OH are generated under normal cellular conditions and are immediately detoxified by major scavenger enzymes (glutathione based enzymes such as GST, GR, CAT, etc.). However, excessive ROS production by CDDP causes antioxidant imbalance and leads to LPO and antioxidant depletion (Wiejl et al, 1998).

Glutathione-S-transferases (GST) are a family of phase II detoxification system involved in conjugation of a wide range of xenobiotics to the endogenous nucleophilic GSH (Townsend and Tew, 2003). GST represents an integral part of the detoxification system and protects cells against oxidative and chemical-induced toxicity and stress by
catalyzing the conjugation of GSH to electrophilic compounds of toxic substrates (Chien et al., 1994; Hayes and Pulford, 1995). GST is one of the several factors that are proposed to affect tumor sensitivity to anticancer drugs, including CDDP (Kodera et al., 1994), and over expression of GSTs can increase susceptibility to carcinogenesis and inflammatory disease (Townsend and Tew, 2003; Balendiran et al., 2004; Hayes et al., 2005). In present study, CDDP treatment caused a significant decrease in GST activity in liver (Table 6; Figure 29 & 30), which may be connected with CDDP-induced increase in free radical generation causing tissue toxicity, and the observed decrease in DL cells may also have a role in the decrease/failure of cellular defence mechanism which may also support the view of CDDP-induced mitochondrial damage and DL cytotoxicity as observed in present study. However, the increase in GST activity in kidney, spleen, testes (Table 6; Figure 29 & 30) after combination of AA plus CDDP treatment may indicate the utilization of GSH in detoxification reactions and reflecting the role of AA in scavenging free radicals. Elevated levels of GSTs have been associated with the development of resistance towards chemotherapeutic agents (Blair et al., 1997; Cullen et al., 2003). Increase in GST activity in liver and spleen has been noted in combination treatment of AA plus CDDP as compared to CDDP alone treatment, thus implying GSTs to have peroxidase and isomerase activities which are involved in protection of cells against hydrogen peroxide-induced cell death (Sheehan et al., 2001). Therefore, the observed increase in GST activity in the tissues may have a role in cellular defense.

Glutathione reductase (GR) also plays an important role in cellular defence against oxidative stress. GR catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) in the presence of NADPH (Sweet and Blanchard, 1991). By generating GSH, GR indirectly participates in the protection of cells against oxidative stress and cytotoxic compounds and is deeply involved in the maintenance of
the redox status of cells. CDDP treatment caused a significant decrease in GR activity in liver and kidney, while combination treatment of AA plus CDDP caused an increase in the enzyme activity in kidney and decrease in liver, spleen and testes (Table 7; Figure 31 & 32). The observed increase in GR activity in kidney could be in an attempt to maintain the intracellular ratio of reduced and oxidized GSH. GR plays an essential role in the cellular defense against oxidative stress and a controlled decrease in GR level in human fibroblasts was reported to reduce cell viability (Chavkova et al., 2001; Li et al., 2000). When GR activity is impaired, the ability of the cell to reduce GSSG to GSH may be devastated, leading to GSSG accumulation within the cytosol. GR activity in DL cells was noted to be significantly decreased during 24-96 h of CDDP alone and combination treatment conditions (Table 7; Figure 31 & 32), which may hamper cellular ability to convert GSSG to GSH. Therefore, CDDP-mediated decrease in GR activity in DL cells could be one of the other possible steps involved to decrease the GSH level, thus, affecting cellular antioxidant machinery and reduced cell viability facilitating antitumor activity.

Catalase (CAT) is an endogenous antioxidant enzyme that neutralizes ROS by converting H$_2$O$_2$ into H$_2$O and O$_2$ and thus protects cells from oxidative damage (Hunt et al., 1998). A common catalase-262 C/T polymorphism has been identified in the promoter region of human CAT, and the variant of this gene affects transcriptional activity and CAT levels in RBCs (Forsberg et al. 2001). Because of the importance of CAT enzyme in regulating ROS levels in human body and the clear role of ROS in tumorigenesis, genetic polymorphisms of this gene are believed to play a role in ROS-induced carcinogenesis. Several evidences revealed that CAT activity is reduced/suppressed in cancerous tissues (Durak et al., 1994; Arruda et al., 1996). Low levels may be due to treatment by anticancer drugs which reduces antioxidants and induces oxidative stress which increases with disease progression (Borek, 2004). The
CDDP treatment of tumor-bearing mice caused a decrease in CAT activity in all the tissues studied (Table 8; Figure 33 & 34). The decreased levels of CAT activity may result in accumulation of large amounts of hydrogen peroxide that produces large amount of hydroxyl radicals resulting in oxidative stress, which may also support the view of enhanced LPO as observed in the tissues of CDDP alone treated mice in present study. Negahdar et al. (2005) reported that low levels of CAT activity in breast cancer resulted in higher production of ROS due to inadequate enzyme activity to detoxify high levels of hydrogen peroxide thereby, leading to formation of hydroxyl radicals. Decreased CAT levels in patients with lung cancer have also been reported (Cobanoglu et al., 2010). Moreover, Corrocher et al. (1986) reported decreased CAT activity in human hepatoma and suggested that the antioxidant defense system of hepatocellular carcinoma cells was severely impaired. It has also been reported that the low levels of CAT are due to inactivation of the enzyme by the superoxide anions (Kono and Fridovich, 1982; Baskaran and Singh, 2011). CAT is implicated in destruction of harmful H$_2$O$_2$ generated in excess by different sub-cellular processes, and by biotic and abiotic stresses. Induction of CAT activity provides protection against oxidant attacks created by CDDP and/or its metabolites. The observed increase in CAT activities in the tissues after combination treatment may be a compensatory regulation in response to the CDDP-induced oxidative stress and reflects the antioxidant role of AA administration in the initial stages of tumor formation which may be useful as secondary therapy to prevent the oxidative damage. Thus, the enhanced CAT activity in the tissues of tumor-bearing mice may also serve to prevent LPO. These results imply that the increased activities of GR and CAT, as well as increased concentration of reduced GSH in the tissues after combination treatment of AA plus CDDP are sufficient for elimination of excess hydrogen peroxide production and suppression of oxidative stress induced by CDDP in the host.
Cell uptake is the first step of drug action in tumor cells. Intracellular platinum content is considered to play an important role in cytotoxicity and drug resistance. The cytotoxic activity of CDDP correlates with the amount of platinum bound to DNA (Lindauer and Holler, 1996). Although Gately and Howell (1993) described that the uptake of CDDP is directly proportional to its concentration, is not inhibited by structural analogues, and not saturable, however recent studies show that CDDP uptake can be specifically stimulated or inhibited by pharmacological agents and the activation of signal transduction pathways. For example, in cultured cells uptake of CDDP is mediated by the copper transporter Ctr1 which selectively enhances the killing of cultured cancer cells platinum-based anticancer drug CDDP (Ishida et al., 2010). Hyperthermia, as well as radiation or chemical stimuli, cause higher uptake rate probably due to an increased permeability of the cell membrane (Ohtsubo et al., 1997). The present study shows that uptake of platinum by DL cells was more at the initial hour of treatment and decreased gradually reaching a state of little or no change at the later stage in both CDDP alone and combination treated group (Figure 22). The gradual decrease in intracellular platinum uptake during 48-96 h of different treatment conditions may also be correlated with the recovery of GSH levels during the later period. Earlier study in freshly isolated peripheral blood mononuclear cells also showed that the increased intracellular GSH concentration is correlated with decreased platinum-DNA binding (Sadowitz et al., 2002). This change has been suggested to occur due to the damage that blocks membrane functionality as a result of extensive platination (Reile et al., 1992). Decreased intracellular concentration due to decreased drug uptake, or increased reflux can cause resistance to CDDP (Misawa et al., 1995; Johnson et al., 1996; Chen et al., 1998). The enhanced drug uptake by DL cells noted after combination treatment of AA plus CDDP (Figure 22) could be an important contributory factor in the cytotoxic effects of CDDP against murine ascites Dalton’s
lymphoma leading to tumor regression under reduced GSH levels and GST activity in tumor cells as observed in present study. This study is supported by the results reported by Khynriam and Prasad (2003) indicating that depletion in GSH level enhanced platinum uptake in DL cells due to a decrease in the formation of platinum GSH conjugates. It has been reported that tumor cells are deficient in the ability to repair DNA after reaction with CDDP (Szymkowski et al., 1992). Thus, the availability of more drugs during initial stage of treatment may be thought to give rise to various metabolic dysfunctions directly or indirectly related to CDDP cytotoxicity which may be partially repaired or retained within the DL cells, leading to tumor regression.

Depletion in RBC leads to iron deficiency, anaemia (Balinger, 2007) which is a frequent complication of cancer diseases. Patients with malignant tumours often develop anaemia during some period of their disease. This symptom has been attributed to chronic blood loss or deficiencies in dietary intake, but mostly to an incompletely compensated haemolysis. The association between altered immunity and the occurrence of cancer has been reported in a variety of murine species (Burns and Leventhal, 2000). In addition to oxygen transport, RBCs also function as conveyors of nutrients, and serve as targets for drugs, pathological factors and environmental xenobiotics (Pickula et al., 1996). In present study, during tumor growth progression, the haematological parameters i.e. Hb, RBC and PCV significantly decreased while WBCs increased (Table 10; Figure 37 & 38). Depletion in the number of RBCs count along with the Hb concentration was detected in mice treated with CCl₄ (Mandal et al., 1998). Acute dose-dependent depression in leukocytes (leukopenia), erythrocytes (anaemia), and platelets (thrombocytopenia) has been reported after treatment with CDDP in Fischer 344 rats (Ohno et al., 1993). Tung et al. (1975) mentioned that, the reduction in the values of blood parameters (PCV, RBC and Hb) may be attributed to the hyperactivity of bone marrow, which leads to production of RBCs with impaired integrity that are easily
destroyed in the circulation. The decrease in Hb content and RBC counts after CDDP treatment recorded in the present study may be correlated to cause decreased blood antioxidant capacity leading to anaemic condition, disturbed hematopoiesis, destruction of erythrocytes, and reduction in the rate of their formation and/or their enhanced removal from circulation. This suggestion of decreased antioxidant capacity and increased fragility in RBC is supported by the observation of various types of CDDP-induced morphological abnormalities in erythrocytes (Figure 37) in the present study. CDDP treatment has been reported to cause anaemia (Wood and Hrushesky, 1995), decrease in RBC, hematocrit (Hct), Hb concentration and erythropoietin (EPO) production (Unami et al., 1996). However, an increase in the Hb values, RBC counts and PCV noted after combination treatment of AA plus CDDP (Table 10; Figure 37 & 38), and significant decrease in the frequency of abnormal RBCs as compared to CDDP alone treatment (Table 11) may suggest a protective role of AA against CDDP-induced hematotoxicity.

Solid tumors, both primary lesions and metastases, are infiltrated by large numbers of tumor-associated leukocytes which are a heterogeneous population of cells consisting of various (and variable) subsets of T cells (helper, suppressor and cytotoxic), B cells, natural killer (NK) cells, and macrophages (van-Ravenswaay-Claasen et al., 1992). Leukocytosis is a pathological condition often encountered in a clinical setting, usually caused by an increase in the number of neutrophils, affecting the WBC that frequently rises as a reaction to infection, chronic inflammation and cancer (Ruka et al., 2001). Our findings on neutrophils count also support this submission because as compared to normal mice without any malignancy, an increase in neutrophil count was observed in the mice bearing ascites Dalton’s lymphoma (Table 12; Figure 40). Although there is a decrease in basophils and neutrophils after combination treatment, the overall increase in the number of different types of WBC (Table 12) may
suggest its significant effect on tumor growth. T lymphocytes play a key role in maintaining antitumor immunity providing an important opportunity for the immunotherapy of cancer (Ben-Efraim, 1996). Thus, combination treatment of tumor-bearing mice with AA plus CDDP could be helpful in developing suitable condition in the hosts such as improved hematological values, which may be involved in decreasing CDDP-induced hematotoxicity, strengthening hosts’ immunity thereby potentiating CDDP antitumor efficacy and host survivability. The consistent increase in WBC, particularly lymphocytes, may also play a role in the antitumor activity by enhancing antitumor immunity in the host.

The development of chromosomal aberration (CA) and sperm abnormality (SA) has been used as sensitive biological indicators in the mutagenic bioassays of a drug (Giri et al., 1998a, b; Garcia-Sagredo, 2008). Damage to DNA in the form of gene mutations, large scale chromosomal damage, recombination and numerical chromosome changes are generally considered to be markers in mutagenecity tests. The therapeutic efficacy of CDDP has been limited by its dose dependent side effects (Krakoff, 1979) and its mutagenic potentials have also been reported in bacteria as well as in mammalian cells (Overbeck et al., 1996; Giri et al., 1998a, b; Attia, 2010). In the present study, development of all these mutagenic parameters were seen after CDDP treatment of tumor-bearing mice in vivo and supports earlier findings of its genotoxic properties (Pillaire et al., 1994; Brozovic et al., 2011; Mazumdar et al., 2012).

Chromosomal alterations are typical features of neoplastic cells, and for certain cancers specific chromosome abnormalities are commonly present (Yunis, 1983; Mitelman, 2000). CA are the microscopically visible part of a wide spectrum of DNA changes and are usually considered to derive from unrepaired or misrepaired DNA lesions induced by exogenous or endogenous exposure to DNA damaging agents. An increase in CA could also be due to genetic or acquired conditions conferring a higher
susceptibility to genetic damage. CA is induced by several agents/drugs that damage chromosomal DNA (Natarajan, 1976). Exposure to carbosulfan, a potent genotoxic agent induced CA in a dose-dependent manner (Giri et al., 2002).

Since blood cells originate in the bone marrow, the incidence of CA was analyzed in the bone marrow cells under different treatment conditions. Bone marrow cells are susceptible to oxidative damage and sensitive to clastogenic chemicals (Umegaki et al., 1997). In present study, various types of aberrations such as chromatid breaks, isochromatid breaks, chromosomal fragments, exchanges and sister chromatid union were observed (Figure 41) in both CDDP alone and in combination treatment some of which were also observed in AA alone treatment. CDDP treatment revealed maximum increase in the frequency of CA than the other treated groups, with chromatid and isochromatid breaks occurring more frequently (Figure 42), and supports the earlier findings of the clastogenic properties of CDDP (Kliesch and Adler, 1987; Adler and el-Tarras, 1989; Giri et al., 1998b). The clastogenicity of CDDP in bone marrow cells was well investigated by Edelweiss et al. (1995) and Choudhury et al. (2000), who observed that the most impressive effect of a single dose of CDDP was an increase in the frequency of CA and in the number of abnormal metaphases obtained after CDDP treatment. Under different treatment conditions the total frequency of aberrant metaphases as well as CA were noticed to be maximum at 24 h of treatment, which gradually decreased during the later phase (Figure 42). This result is supported by the earlier reports that antitumor agents produce a high frequency of aberrations in rodents 24 h after a single dose (Hayashi et al., 1984; Rosselli et al., 1990). These elevated levels may also be seen as an indicator of an early phase of carcinogenesis, where various genetic alterations are also generated in different tissues. The decrease in the frequency of CA in the later hours of treatment could be due to death of damaged cells, clearance of mutagen from the body, and post-replication repair process which might be
operating for recovery from the CDDP-induced damage to DNA. In fact, an involvement of post-replication repair process in CDDP-induced DNA damage has been established (Sorenson and Eastman, 1988). Hydroquinone, a well known chemical agent induced CA by an oxidative process giving rise to ROS that can interact with proteins involved in microtubule assembly, spindle formation and human topoisomerase II, and these are properties which are potentially associated with its clastogenic effects (Obe et al., 2002). The increase LPO noted in present study may cause oxidative stress within the cells and may subsequently be involved in the formation of CA in bone marrow cells. A comprehensive review of genetic rearrangements consequent to CA and their role in the pathogenesis of solid and hematologic cancers has been reported (Mitelman et al., 2004). Rosenberg (1985) proposed that CDDP lesions on O6 of guanine in normal cells are repaired before replication, while in cancer cells, the lesions are not removed, because of a deficiency in this repair process, hence mutation rate increases beyond the limits of survivability. This might thereby explain the fairly high frequency of aberrations observed in the tumor cells following 24-96 h CDDP treatments. AA (vitamin C) is the major non-enzymatic antioxidant which has synergetic action in scavenging oxygen-derived free radicals (Renugadevi & Prabu, 2010), and protects DNA against damage induced by ROS (Duthie et al., 1996). In the present study, total CA were decreased appreciably in AA plus CDDP treated group at corresponding time points (Figure 42), showing that AA exhibited reduction in the CA induced by CDDP. This results reveal that the combined effect of AA plus CDDP was stronger than the sum of the effect of CDDP alone treatment, so a synergistic induction of DNA damage by CDDP and AA can be assumed (Blasiak et al., 2000), and also strongly suggests that AA effectively ameliorates the mutagenic effects induced by CDDP. Animals treated with curcumin plus a single dose of CDDP, at 18, 24 or 72 h following treatment, presented a statistically significant reduction in the total amount of chromosomal
damage and in the number of abnormal metaphases (Antunes et al., 2000b). The ability of AA to minimize the incidence of CA induced by cadmium chloride in cultured mouse spleen cells has been reported by Fahmy & Aly (2000). The protective effect of AA on CDDP-induced mutagenic effects in murine system has also been observed by Giri et al. (1998a). In mouse bone marrow cells treated with mitomycin or cyclophosphamide, AA decreased the frequency of sister-chromatid exchanges induced by these drugs (Krishna et al., 1986). Similar protection was obtained in rat bone marrow cells treated with AA or olive oil in combination with the antitumoral drug doxorubicin (Antunes and Takahashi, 1998). El-Refaiy and Eissa (2012) also reported the protective effect of AA against cadmium-induced cytotoxicity in rat bone marrow cells. Based on these findings, it may be suggested that the decrease in the frequency of CA after combination treatment with AA is due to the protective effect of AA in the abatement of CDDP-induced mutagens.

Sperm abnormality (SA) test is considered feasible and reliable endpoints to identify chemicals that induce spermatogenic dysfunction. The abnormalities in sperm morphology have been suggested to be a consequence of chromosomal aberrations (Bruce et al., 1974; Ushijima et al., 2000). In vivo cytogenetic assays involving mammalian germ cells have also been used extensively for genetic toxicological studies and are of great importance since some of the effects produced by exposure to hazardous chemicals may be transmitted to next generation through gametes (Chamorro et al., 2003). SA due to chemical mutagens are well documented (Wyrobek and Bruce, 1978). Chemically-induced increase in sperm abnormality is highly correlated to known germ cell mutational activity. CDDP been considered as a potent mutagen causing formation of abnormal male germ cells population at higher doses. CDDP has been reported to cause sertoli cells dysfunction (Pogach et al., 1989; Aydiner et al., 1997) and embryotoxicity (Giavini et al., 1990) in rats. In present study, sperm abnormality
analysis in mice showed CDDP induced various types of sperm abnormalities in tumor-bearing male mice for 10 days when treated alone and in combination with AA (Figure 43). Among different treatment conditions, CDDP caused maximum abnormalities in the sperm with banana head shape occurring more frequently suggesting that CDDP could reach the germ line cells and indicates its potentiality as germ cell mutagen also. Several studies indicate that various species of ROS generated through metal catalysis potentially interact with gene strands causing mutations, thereby inducing changes in sperm morphology (Roy and Rossman, 1992; Hsu et al., 1998; and Bench et al., 1999). Therefore, the present mechanism of abnormal production of sperm may be an oxidative stress dependent phenomenon induced by platinum catalysis. However, combination with AA showed a significant decrease in the abnormality which also depicts the similar protective role of AA as observed for CA. It has been reported that dietary AA protects human sperm from endogenous oxidative DNA damage that could affect sperm quality (Fraga et al., 1991).

Thus, the results of the mutagenic parameters in present studies showing the significant reduction in CDDP-induced genotoxic damage in presence of AA clearly suggest the protective role of AA on CDDP’s mutagenic potentials thereby enhancing CDDP-mediated therapeutic efficacy. Holloway and Peterson (1984) described the ability of AA to confer marked protection to animals against many toxic chemical agents and heavy metals. AA mediated inhibition of bacterial Salmonella typhimurium mutagenicity induced by N-methyl-N'-nitro-N-nitrosoguanidine nitrosourea (Tyrsina et al., 1994) and rat mutagenicity induced by the alkylating agent, N-ethyl-N-nitrosourea (Aidoo et al., 1994) has been reported and may suggest that similar modulation mechanism might be involved as CDDP also behaves like an alkylating agent.

Histopathological evaluations are commonly used for detecting organ-specific effects related to chemical exposure (Travlos et al., 1996; Crissman et al., 2004). In
present study, histopathology of kidney, liver and testes were carried out in experimental mice on 5\textsuperscript{th} day of CDDP treatment to evaluate toxicity/damage in these tissues.

Nephrotoxicity is an inherent adverse side effect of the anticancer drugs for solid and hematologic malignancy (Kintzel, 2001). Renal tubular damage is a well-known renal complication induced by anticancer drugs (Kakihara et al., 2003). Nephrotoxicity of CDDP has been recognized as the most important dose-limiting factor (Mora Lde et al., 2003) caused probably due to apoptosis, inflammatory mechanism and generation of ROS (Sleijfer et al., 1985). CDDP is known to accumulate in mitochondria of renal epithelial cells (Santos et al., 2008), and several investigators have demonstrated that CDDP induces ROS in renal epithelial cells primarily by decreasing the activity of antioxidant enzymes and by depleting intracellular concentrations of GSH (Huang et al., 2001). CDDP treatment caused destruction of the renal tubular cells represented by glomerular atrophy, infiltration of cells and tubular congestions destruction of the renal tubular cells (Figure 44c). The decrease in GR and CAT activities and reduced GSH concentration and enhanced oxidative stress in kidneys after CDDP treatment as observed in present study may have implicated in the pathogenesis of CDDP-induced renal injury. However, the involvement of oxidative stress in CDDP-induced toxicity is further supported by the fact that many antioxidants prevent CDDP-induced nephrotoxicity (Ajith et al., 2002; Lee et al., 2007). Agents such as superoxide dismutase, dimethylthiourea and GSH have been shown to reduce the degree of renal failure and tubular cell damage when administered simultaneously with CDDP in rats (Tsuji et al., 2009). In present study, co-administration of AA with CDDP reduced destruction of renal tubular cells and regained glomerular attachment (Figure 44d) thereby showing nephroprotectivity with high antitumor activity and suggests that
antioxidants and free radical scavengers might provide nephroprotection in CDDP-induced renal injury.

CDDP is thought to kill cells primarily by forming DNA adducts, causing G2 arrest in the cell cycle, triggering apoptosis (Kishimoto et al., 2000). Treatment with CDDP showed sinusoidal distortion and remarkable locational hepatocytes damage (Figure 45c). These changes are mostly due to inflammation. Some studies have suggested that oxidative stress plays an important role in CDDP-induced liver damage (Lu and Cederbaum, 2006; Iraz et al., 2006; Pratibha et al., 2006) resulting in enhanced production of ROS, reduction in the mitochondrial membrane potential (Saad et al., 2004) and decrease in antioxidant enzymes (Mora et al., 2003). Therefore, antioxidants administration before CDDP treatment is essential to act against its toxicity (Lee et al., 2007; Kalender et al., 2010). In present study AA administration prior to CDDP injection showed amelioration in the histopathological changes showing mild to moderate damage of hepatic cell indicating recovery of the altered tissue (Figure 45d). This run in agreement with Atasayar et al. (2009) who demonstrated that combined treatment of vitamin C and E with single acute dose (toxic dose) of CDDP is able to normalize the histopathological alteration induced by CDDP on kidney when compared with CDDP alone treated group. The mechanisms by which AA decreases the hepatotoxicity induced by CDDP, is embodied in the fact that AA might ameliorate the oxidative damage by decreasing LPO and altering antioxidant defense system (El-Gendy et al., 2010). In addition, ascorbate has also shown to prevent hepatic GSH depletion in chemical-induced hepatotoxicity in mice, in which GSH acted as intracellular free- radical scavengers and protected cells against radical mediated LPO (Cuddihy et al., 2008).

CDDP-dependent nephrotoxicity is recognized as a very complex multifactorial process. Reno-protective approaches are being discovered, but the protective effects are
mostly partial, suggesting the need for combinatorial strategies (El-Sayed et al., 2008). CDDP has been reported to increase serum urea and creatinine level in nephrotoxicity (El-Sayed et al., 2008; Pabla and Dong, 2008). In present study, results obtained show that treatment with CDDP induced biochemical signs of nephrotoxicity manifested by increased serum creatinine and urea levels with increased lipid peroxides, depletion of GSH and inhibition of CAT activities. The increased serum creatinine and urea levels may be due to decreased glomerular filtration rate or increased ROS. Noori and Mahboobc (2010) reported that administration of CDDP to rats caused a reduction in glomerular filtration rate, which was correlated with increased creatinine and urea in plasma. However, combination treatment with AA plus CDDP decreased the urea and creatinine level in kidney (Table 13; Figure 47), indicating that AA has a protective role against CDDP-induced nephrotoxicity and its antioxidant property to scavenge CDDP-mediated free radicals generation may be involved in it. A study on normal rats co-treated with CDDP and AA depicted reduced CDDP-induced renal toxicity and oxidative DNA damage (De Martinis and Bianchi, 2001).

Liver function tests (LFT) involving the assay of some marker enzymes such as ALT, AST and ALP in serum have been commonly used to detect hepatic dysfunction (Thapa and Walia, 2007) where in ALT and AST are the most frequently utilized test of hepatocellular injury and represent as hepatocellular markers. In present study, CDDP induced marked elevation in ALP, ALT and AST levels (Table 14; Figure 48) showing an indication of CDDP-induced hepatotoxicity. However, these elevations were significantly attenuated by AA pre-treatment in combination group thereby suggesting hepatoprotective effect of AA. As an antioxidant agent, AA may have scavenged the reactive free radicals before reaching their hepatic targets. Both animal (Odigie et al, 2007) and human (Dogun and Ajala, 2005) studies have shown AA to be a potent antioxidant which mediates its antioxidant effect by scavenging free reactive oxygen
species (ROS). Other studies have equally shown the protection of AA and other vitamins in hepatic oxidative damage (Barja et al, 1994; Appenroth, 1997). Thus, results of the present study suggests AA’s ameliorating effects to be likely mediated via inhibition of free radicals generation and/or free radical scavenging activity.

The analysis of RFT and LFT also confirm the recovery in GSH level in liver and kidney after combination treatment with AA plus CDDP, thus showing the protective ability of AA against CDDP-induced liver and kidney damage.

Sperm formation in mammals results from the activity of spermatogenic cell lines. Some xenobiotics can cause degenerations in these lines. There are several chemicals that affect on reproductive system. Spermatogenesis occurs in several stages and destruction in any stage of it can affect the whole process. Exposure to chemicals affects sertoli cells leading to tubular atrophy (Hess and Nakai, 2000; Institoris et al., 2001). In present study, degenerations in seminiferous tubules, intercellular disassociation of germ cells, vacuolization in sertoli cells and reduction in leydig cells were seen following CDDP treatment (Figure 46c), thus supporting the view that oxidative stress induced by CDDP affected gonadal function. These observations suggest that CDDP may perturb gene expression related to spermatogenesis, causing severe seminiferous tubule degeneration which might result in infertility of the host. Combination treatment of mice with AA plus CDDP revealed reduced vacuolated tubules and less deranged spermatogonial mass (Figure 46d). AA has long been known to participate in spermatogenesis process of rodents and ameliorate oxidative stress related to testicular impairments in animal tissues (Steinberger and Steinberger, 1966). Moreover, dietary AA was found to protect against endogenous oxidative DNA damage in human sperms (Fraga et al., 1991). Thus, AA may be thought to enhance the defense system during stress to combat tissue destruction and safeguard cellular structure and function thereby, ameliorating CDDP-induced testicular toxicity in the male mice.
Based on the findings from present study, it may be suggested that combination treatment could potentially reduce CDDP-induced toxicity signifying its protective role, thus suggesting differential effects of the combined treatment on the cancer cells and normal tissues of the host. Although the precise mechanism and kinetics through which AA mediates its protective effects remains unclear presently, the finding that AA protects cells from CDDP toxicity may indicate its ability to detoxify carcinogens by exerting its cytoprotective profile.