4.1. Introduction

Cancer medically known as a malignant neoplasm, characterized by self-sufficiency in the absence of growth signals, their ability to evade apoptosis, resistance to anti-growth signals, sustained angiogenesis, uncontrolled proliferation, invasion and metastasis (Hanahan and Weinber, 2000). There are over 200 different known cancers that afflict humans. The chances of surviving the disease vary greatly by the type and location of the cancer and the extent of disease at the start of treatment. While cancer can affect people of all ages, and a few types of cancer are more common in children, the risk of developing cancer generally increases with age. In 2008, approximately 12.7 million cancers were diagnosed (excluding non-melanoma skin cancers and other non-invasive cancers) and 7.6 million people died of cancer worldwide (Jemal et al., 2011). Cancers as a group account for approximately 13% of all deaths each year with the most common being: lung cancer (1.4 million deaths), stomach cancer (740,000 deaths), liver cancer (700,000 deaths), colorectal cancer (610,000 deaths), and breast cancer (460,000 deaths). This makes invasive cancer the leading cause of death in the developed world and the second leading cause of death in the developing world (Jemal et al., 2011). The three most common childhood cancers are leukemia (34%), brain tumors (23%) and lymphoma (12%). Rates of childhood cancer have increased by 0.6% per year from 1975 to 2002 in the United States and by 1.1% per year between 1978 and 1997 in Europe.

Cancer therapy has been remarkably consistent with the last 50 years. Chemotherapy, radiotherapy and surgery have been the cornerstones of conventional treatment. Not surprisingly, the clinical success of these treatments has reached a plateau (Braverman, 1991). Current chemotherapeutic drugs act for the most part by killing cancer cells. Researchers began focusing on the studies of the agents that act by changing the biological properties of cancer cell so that they lose the ability to divide continuously. Hence treatment based on tumor differentiation and inductions of apoptosis are beneficial to prevent the uncontrolled growth of tumor cells (Sun et al., 2004). One of the major problems in the treatment of cancer is the acquisition/development of resistance and refractoriness to conventional therapeutics (Bonavida et al., 2006). The overall development of tumor cell resistance to therapeutics is, in large part, the result of the ability of tumor cells to develop specific mechanisms to overcome cell death or apoptosis. The possibility to interfere selectively in the regulation of the apoptotic signaling pathways may result in either the direct induction of cell death and/or sensitization of the cells to cytotoxic stimuli.
It is now widely recognized that the growth of any solid tumour depends on angiogenesis. The concept that tumor growth and metastasis are dependent on the development of new blood vessels was first formulated by Folkman (1971) and hence blocking angiogenesis could be a strategy to arrest tumour growth. This possibility stimulated an intensive search for pro- and anti-angiogenic molecules. Gullino (1978) showed that cells in pre-cancerous tissue acquire angiogenic capacity on their way to becoming cancerous. It is now widely accepted that the ‘angiogenic switch’ is ‘off’ when the effect of pro-angiogenic molecules is balanced by that of anti-angiogenic molecules, and is ‘on’ when the net balance is tipped in favour of angiogenesis (Bouck et al., 1996; Hanahan and Weinber, 2000).

Solid tumour starts as a dormant avascular nodule which could only grow and develop if it becomes vascularized. Neovascularization must occur to provide oxygen and nutrients to the tumour cells. Furthermore, the immature neovessels enhance tumor cell entry into the circulation and hence distant metastasis (Liotta and Stracke, 1988). It is now also recognized that neovascularization dependence goes beyond solid tumours; it also plays an important role in the development of hematological malignancies (Perez-Atayde et al., 1997). The prognostic role of neovascularization in lymphohaemopoietic malignancies remains contentious.

Metastatic spread of cancer continues to be the greatest barrier to cancer cure. Most cancer patients die as a result of distant tumor spread that is resistant to conventional anti-tumour therapies. Understanding the molecular mechanisms of metastasis is crucial for the design and effective clinical use of novel therapeutic strategies to combat metastasis. The selective process of tumor metastasis involves a series of interrelated rate-limiting steps. Tumour cell migration is considered as a major event in the metastatic cascade (Ali et al., 2003). The understanding of fundamental role of angiogenesis in cancer growth and metastasis has led to tremendous interest in research in its regulatory mechanisms and clinical implications in the management of cancer patients in the past few decades.

Hematological malignancies are the types of cancer that affect blood, bone marrow, and lymph nodes. As the three are intimately connected through the immune system, a disease...
affecting one of the three will often affect the others as well. Although lymphoma is a disease of the lymph nodes, it often spreads to the bone marrow, affecting the blood. Hematological malignancies account for 9.5% of new cancer diagnoses in the United States. Within this category, lymphomas are more common than leukemias. Lymphoma’s have been broadly classified into 2 types: Hodgkins and Non-Hodgkins lymphoma.

The tumor, Dalton's lymphoma, was originated in the thymus gland of a DBA/2 mouse at the National Cancer Institute, Bethesda, US in 1947. Subsequently, an ascites form was developed by repeated intraperitoneal transplantation of tumor (Chakrabarti, et al., 1984). It is characterized by highly invasive and malignant nature, killing the host in a very short period of life span. Despite advances in cancer chemotherapy, the treatment of peritoneal carcinomas remains a significant failure. Unlike solid tumors, which can be removed by surgery, the peritoneal tumors result in inoperable accumulation of malignant ascites, and hence to date their treatment remains a challenge. Peritoneal concentrations of intravenously administered drugs remain low owing to poor drug penetration into the peritoneal cavity and therefore would be insufficient to eliminate such tumors. Intraperitoneal chemotherapy of peritoneal tumors would likely be a better method owing to high local concentration in tumor regions and low systemic toxicity (Sadzuka et al., 2000; Reddy et al., 2006). The effective chemotherapy of tumors depend on continuous exposure to anticancer agents for prolonged periods.

Liver metabolizes most of the drugs and kidney filters out all the unwanted exogenous substances. Therefore, these two organs are likely to be affected upto a greater extent by the drug treatment (Fraiser et al., 1991).

The purpose of the present study was to evaluate the anticancer efficacy of our compound 6-propionylpterin on Daltons lymphoma ascites (DLA) grown in the intraperitoneal cavity of swiss albino mice. The effect of the compound on the appearance, body weight and mean survival time (life span) of the animal, haematological parameters, liver and kidney functions and the activity of antioxidant enzymes were evaluated.
4.2 Materials and Methods

4.2.1. Animals

Healthy male swiss albino mice weighing 25±2 g were obtained from Venkateshwara Enterprises, Bangalore, India. The mice were grouped and housed in polypropylene cages and maintained under standard conditions 25±2 °C with 12 h dark/light cycle. The animals were fed with commercial standard animal pellet diet and water ad libitum. The animals were acclimatized to the laboratory for 2 weeks before the inception of the experiments. This part of work has been carried out in the Department of Environmental Biotechnology, Bharathidasan University, TamilNadu. All the animal experiments were duly approved by the Institutional Animal Ethics Committee (BDU/IAEC/2011/12/2932011) guidelines.

4.2.2. DLA cell line and induction of cancer

Dalton’s Lymphoma Ascites (DLA) cells were obtained from Amala Cancer Research Centre, Thrissur, Kerala, India. The cells were maintained in vivo in Swiss albino mice by intra-peritoneal transplantation. While transforming the tumor cells to the grouped animal the DLA cells were aspirated from peritoneal cavity of the mice using saline. The cell count was done and further dilutions were made, so that the concentration was maintained as 1X10^6 cells/mL/mouse. This volume was given intraperitoneally (ip) and the tumor was allowed to grow in the mice for a minimum of 72 h before starting the study (Gothoskar and Ranadive, 1971). Transplantation was carried out using a sterile disposable syringe under aseptic conditions.

4.2.3. Experimental groups

The animals were divided into 6 experimental groups each containing 6 animals. Treatment was given in sterile disposable syringe under aseptic conditions.

- Group 1: Normal mice (received 100 µL of sterile saline by ip)
- Group 2: Normal mice treated with low dose of 6-propionylpterin (250 µg/kg by ip)
- Group 3: Normal mice treated with high dose of 6-propionylpterin (500 µg/kg by ip)
- Group 4: DLA induced mice (received 100 µL of sterile saline by ip)
- Group 5: DLA induced mice treated with low dose 6-propionylpterin (250 µg/kg by ip)
- Group 6: DLA induced mice treated with high dose 6-propionylpterin (500 µg/kg by ip)
Total experimental period was 10 days and after administering the last dose, 3 animals in each group were fasted overnight and sacrificed. The remaining 3 animals were left to calculate the mean survival time.

The blood sample was withdrawn before sacrificing by retro orbital plexus method in sterile tube containing heparin (15-20 IU/mL). Haematological parameters like RBC count and WBC count was determined. For collecting serum, the blood was collected separately in unheparinized tubes, allowed to clot at room temperature (22 °C) and centrifuged at 2000 rpm for 15 min. The serum samples was used for the analysis for serum glutamate oxaloacetate transaminase (SGOT) (Reitman and Frankel, 1974), serum glutamate pyruvate transaminase (SGPT) (Reitman and Frankel, 1974), lactate dehydrogenase (LDH), urea (Natelson, 1957), uric acid (Caraway, 1963) and creatinine (Slot, 1965).

4.2.4. Tumor growth response

Antitumor effect of 6-propionylpterin was determined by monitoring the activity of mice with respect to body weight, ascitic tumor volume, viable and non-viable cell count, mean survival time (MST) and increase in life span (%ILS) and DNA fragmentation assay.

4.2.4.1. Body weight

All the mice were weighed from the day of transplantation till the end of treatment period. Average increase in the body weight was determined.

4.2.4.2. Life span

The MST of each group were monitored by recording the mortality daily for 6 weeks and %ILS was calculated using following equation (Gupta et al., 2004)

\[
\text{MST} = \frac{\text{Day of first death} + \text{Day of last death}}{2}
\]

\[
\% \text{ILS} = \left[ \frac{\text{T} - \text{C}}{\text{C}} \right] \times 100
\]

where, T = No. of days the treated animals survived
C = No. of days the control animals survived
4.2.4.2. Ascitic tumor volume

The tumor diameter was measured from the start of day of treatment and recorded up to 30 days by using vernier calipers. The tumor volume was calculated by using the formula \( V = \frac{4}{3}\pi r_1^2 r_2 \), where ‘\( r \)’ is the mean of \( r_1 \) and \( r_2 \) which are the two independent radii of the tumor mass (Natesan et al., 2007).

4.2.4.3. Trypan blue exclusion method

Trypan blue dye exclusion method was used to assess the cytotoxic effect of 6-propionylpterin on DLA cells. Ascitic fluid containing the tumor cells was with-drawn from the peritoneum of DLA induced groups and washed with phosphate buffered saline (PBS). The cells were suspended in PBS were mixed with equal volume of trypan blue dye and mixed well. The total number of dead and living cells was counted using a hemocytometer and the percentage viability/cytotoxicity was calculated.

\[
\% \text{ of cytotoxicity} = \frac{\left( T_{\text{dead}} - C_{\text{dead}} \right)}{T_{\text{total}}} \times 100
\]

where, \( T_{\text{dead}} \) is the number of dead cells in the 6-propionylpterin treated group

\( C_{\text{dead}} \) is the number of dead cells in the untreated induced group

\( T_{\text{total}} \) is the number of dead and live cells in the 6-propionylpterin treated group

4.2.4.4. Acridine orange/Ethidium bromide (AO/EB) staining method

Ethidium bromide intercalates and stains DNA, providing a fluorescent red-orange stain. Although it will not stain healthy cells, it can be used to identify cells that are in the final stages of apoptosis - such cells have much more permeable membranes. Consequently, ethidium bromide is often used as a marker for apoptosis in cells population and to locate bands of DNA in gel electrophoresis. The stain may also be used in conjunction with acridine orange (AO) in viable cell counting. This AO/EB combined stain causes live cells to fluorescence green whilst apoptotic cells retain the distinctive red-orange fluorescence. The DLA cells both treated and untreated were aspirated from mice. The cells suspended in PBS was mixed with equal ratio of AO/EB stain and observed under fluorescence microscope. The number of dead and viable cells was noted and the % cytotoxicity was calculated as said above (4.2.4.3)
4.2.4.5. DNA fragmentation assay

DNA was isolated from the DLA cells according to the method of Kuo et al. (1995). Briefly 1X10⁶ cells were lysed in 1 ml of lysis buffer (50 mmol/L Tris pH 8.0, 20 mmol/L EDTA and 2% SDS) at 37 °C for 1 h. To precipitate protein, 0.4 ml of saturated NaCl was added and tubes were left on ice for 5 min. The precipitate was centrifuged at 3000 rpm for 30 min. RNase was added to supernatant at a final concentration of 20 µg/mL and incubation was continued for 15 min at 37 °C. DNA was then precipitated by adding twice the volume of chilled phenol:chloroform: isoamyl alcohol (25:24:1) and incubated for 15 min at 4 °C and centrifuged at 10000 rpm for 20 min. To the supernatant, ice cold ethanol was added. Samples were frozen at -70 °C overnight. The precipitate of DNA was recovered by centrifuging at 10,000 rpm for 15 min at 4 °C. The DNA precipitate was dissolved in electrophoretic buffer and estimated spectrophotometrically.

Loading buffer (10 mmol/L EDTA, 0.25% bromophenol blue and 50% glycerol) was added to each sample in the ratio 1:5. Approximately 10 µg DNA was loaded into each well of 1.8% agarose gel which contains 5 µg/mL ethidium bromide. The electrophoresis was carried out at 50V in TBE buffer (2 mmol/L EDTA, 89 mmol/L tris and 89 mmol/L boric acid, pH 8.6) for 2–3 h. After the electrophoresis, the gel was observed under UV lamp. Apoptotic effect was determined by fragmentation and laddering of DNA in the gel.

4.2.5. Preparation of tissue homogenate

The liver tissues of experimental animals were excised, rinsed in ice-cold normal saline, blotted dry and stored at -20 °C. The homogenate was prepared by grinding the liver tissue (0.25g) using 50mM homogenization buffer (NaH₂PO₄-0.780 g, Na₂HPO₄-0.709 g, EDTA-0.06 g, NaCl-0.876 g and a pinch of DTT, Distilled water-100 mL), pH7.2. A small volume of PMSF (10 µL) and triton-X 100 (5 µL) was added while homogenizing the tissue. The homogenate was centrifuged at 10000 rpm for 15 min at 4 °C and the supernatant was used for antioxidant assays. The protein content of the samples were determined by Bradford method (Bradford, 1976).
4.2.5.1. Superoxide dismutase (SOD) activity (Markland and Markland, 1974)

The reaction mixture consisted of 0.1mL tissue homogenate, 0.25mL absolute alcohol, 0.15 mL chloroform, 1.0 mL distilled water and centrifuged at 2500 rpm for 15 min. To the supernatant 2.0 mL of 0.1M Tris EDTA buffer was added. Pyrogallol (0.5 mL) was added at the time of taking reading. OD at 420nm was taken spectrophotometrically at different time interval (0,1,2 and 3 min). The activity of SOD has been expressed as U/mg of protein.

Formula:
\[
\frac{c}{50} \times \frac{\text{Assay volume}}{\text{volume of tissue extract}} \times \text{dilution factor} \times \frac{1}{\text{mg of protein}}
\]

Calculation:
\[
x = \frac{0 \text{ min } O.D - 1 \text{ min } O.D}{1}
\]
\[
y = \frac{0 \text{ min } O.D - 2 \text{ min } O.D}{2}
\]
\[
z = \frac{0 \text{ min } O.D - 3 \text{ min } O.D}{3}
\]

Control = a

\[
B = \frac{x + y - z}{3}
\]
\[
C = \frac{b}{a} \times 100\% \text{ of antioxidant}
\]

4.2.5.2. Glutathione peroxidase activity (Rotruck et al., 1973)

The reaction mixture consisting of 0.1 mL of 30% H\textsubscript{2}O\textsubscript{2}, 0.2mL of 0.8mM EDTA, 0.1 mL of 10mM sodium azide, 0.4 ml of phosphate buffer (0.4M, pH 7.0), 0.1 ml of sample (homogenized) was incubated at 37\degree C for 10 min. The reaction was arrested by the addition of 0.5ml of 10 % TCA and the tubes were centrifuged at 3000 rpm for 3 min. To 0.5 ml of supernant, 3.0 mL of Na\textsubscript{3}HPO\textsubscript{4} and 1.0 mL DTNB were added and the colour developed was read at 420 nm immediately.

The activity of GPx was expressed as U/mg protein.
Formula:
\[
\frac{Test\ O.D}{0.015} \times 1 \times \frac{1}{volume\ of\ sample} \times \frac{1}{mg\ of\ protein}
\]

4.2.5.3. Catalase activity (Sinha, 1972)

To 0.1 mL of tissue homogenate, 1.0 mL of phosphate buffer (0.01M; pH 7.0), 0.5 mL H₂O₂ was added. The reaction was stopped by adding 0.25 mL of K₂Cr₂O₇ after 30 sec/60 sec. The mixture was incubated in boiling water bath for 10 min/20 min. It was cooled to room temperature and the green color developed at 570 nm spectrophotometrically. The activity has been expressed as U/mg of protein.

Formula:
\[
\frac{Test\ O.D}{0 sec} \times \frac{Std\ O.D}{\frac{1000}{34}} \times \frac{1}{volume\ of\ the\ extract} \times \frac{1}{mg\ of\ protein}
\]

\[Test\ O.D = \frac{2x+y}{2} \quad \frac{x – 0\ sec – 30\ sec}{y – 0\ sec – 60\ sec}\]

4.2.6. Semi-quantitative Reverse Transcription-PCR (RT-PCR)

The genes involved in the apoptotic pathway namely iNOS, p53, caspase – 3 and mdm2 were detected by semi-quantitative RT-PCR, where housekeeping β-actin gene was used as internal control.

4.2.6.1. RNA isolation

The tumour cells aspirated from the peritoneum region was centrifuged and the supernatant containing ascitic fluid was discarded. To the pellet TRIzol reagent (Medox Biotech, India) was added and homogenized. The homogenate was centrifuged at 10000 rpm at 4 °C for 10 min. The supernatant containing the RNA was transferred to another tube to which equal volume of chloroform was added. It was mixed well for 30 sec and incubated at room temperature for 10 min. It was then centrifuged at 12000 rpm at 4 °C for 15 min. The aqueous
phase containing the RNA was then transferred into a fresh tube. Isopropanol (0.5 mL) was added and incubated at room temperature for 10 min. It was then centrifuged at 12000 rpm at 4 °C for 15 min. The RNA pellets were pooled and 70% chilled ethanol was added and centrifuged at 12000 at 4 °C for 15 min. The supernatant was discarded and the pellet was air-dried and dissolved in minimum amount of deionized water and stored at -20 °C.

4.2.6.2. cDNA synthesis

cDNA was generated from total RNA by reverse transcription reaction. The reaction was performed at 37 °C for 50 min using Verso™ cDNA kit (Thermo Scientific) and the procedure was followed as given in the kit. The total volume of reaction mixture is 20 µL.

4.2.6.3. RT-PCR

Two microlitres from the product was used for PCR using gene specific upstream and downstream primers (Table 4.1) (Gopinath et al., 2010). Initial denaturation at 95 °C for 2 min was followed by a PCR cycle of denaturation at 95 °C for 15 sec, annealing temperature varies with each gene for 30 sec, extension at 72 °C for 1min with a final extension at 68 °C for 7 min. Finally, the products were analyzed on a 1.2% agarose gel.

4.3. Results and Discussion

4.3.1. Tumour response

The main objective of the present study was to evaluate the anticancer efficacy of 6-propionylpterin on DLA induced tumor bearing mice. On visual observation toxic symptoms were not observed externally in animals in 6- propionyl pterin treated groups, in terms of their general appearance in respect of their skin and hair texture and in their behavioral pattern in respect of food and water intake and activity. The internal organs like the heart, spleen, liver and kidneys retained their usual distinctive colors and appearances in the 6- propionyl pterin treated groups compared with those of normal untreated group mice.

Development of Dalton’s lymphoma is determined by the increments in body weight and volume of the ascitic fluid (Koiri et al., 2009). In the present study, the tumor development in
DLA induced mice has been visually observed based on the enlargement of the peritoneal region (Fig. 4.1). The observation revealed that the 6-propionylpterin significantly reduced the tumor volume and also showed an inhibition in tumor cell proliferation. The body weight of the DLA induced mice significantly increased. The body weight increased to 8.45 g in DLA induced mice as against 2.15 g in normal mice (Table 4.2). The increase in body weight might be due to the increased volumes of ascites by actively proliferating peritoneal cells. In low and high dose of 6-propionyl pterin treated mice there was a reduction in body weight increase and was observed to be 4.18 g and 3.84 g respectively which was nearer to normal range. Rajesh et al. (2011) reported 8.5±0.20 g increase in body weight of tumour induced mice as against 1.32±0.03 in normal control. This is accordance with our result. But in the groups treated with the methanolic extract of Aerva lanata (MEAL) and aqueous extracts of Aerva lanata (AEAL) it was found to be 7.20±0.26 g and 7.26±0.28 g respectively. The treatment of Withania somnifera has been reported to reduce the tumour weight by ~50% (Christina et al., 2004). Present study showed that our compound has more potency in controlling the accumulation of ascitic fluid and arresting the proliferation of actively dividing cells.

In DLA tumor bearing mice, a regular rapid increase in ascitic tumor volume was observed. Ascitic fluid is the direct nutritional source for tumor cells and a rapid increase in ascitic fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells (Prasad and Giri, 1994). The tumor volume of DLA induced treated group was 87.06 cm$^3$ (250 µg/kg-low dose treated) and 77.91 cm$^3$ (500 µg/kg-high dose treated), while the tumor volume in DLA induced group was 195.33 cm$^3$ (Table 4.3). A significant decrease in ascitic volume was observed with 55.4% decrease in low dose of 6-propionyl pterin treated and 60.11% decrease in high dose of 6-propionyl pterin treated mice which implied that the compound was able to suppress DL development in mice. The tumour volume reduction observed in the present study is comparable with the reduction (~2 times) effected by the extract of macrofungus Phellinus rinuosus (Berk) (Ajith and Janardhanan, 2003). Similarly ~50% decrease in ascitic volume by Ruthenium II-4-carboxy-N-ethyl benzamide has also been reported by Koiri and his coworkers (Koiri et al., 2009).
Prolongation of life span is a reliable criterion for judging the anticancer efficacy of the compound (Hogland, 1982). An enhancement of life span by 20% or more was considered as an effective response (Gupta et al., 2000). In our study, there was a significant increase in life span of DLA induced 6-propionyl pterin treated mice. The Mean Survival Time (MST) was 17.6 days for DLA induced mice as against 39.75 days for normal mice. The treatment with our compound has increased the MST significantly to 30.32 and 32.56 days in low dose treated (250 µg/kg) and in high dose (500 µg/kg) treated mice respectively (Table 4.2). The percentage increase in life span was found to be 71.49% and 84.16% in low and high dose of 6-propionyl pterin treated respectively. Earlier there was 27.2% and 61.8% increase in life span of 4mg/kg (low dose) and 16 (mg/kg) lovastatin treated groups (Ajith et al., 2006). There was 34.37% increase in life span in DLA treated with Lawsonia inermis extract and 59.37% increase in vincristine treated group (Priya et al., 2011). The % ILS of MEAL and AEAL treated DLA induced mice was 72 and 76 (Rajesh et al., 2011). Compared to these reports the present findings are encouraging.

The percentage of cytotoxicity based on tryphan blue dye exclusion method and AO/EB (Fig. 4.2) staining in low (250 µg/kg) and high (500 µg/kg) dose of 6-propionylpterin treated DLA induced mice were found to be 10.91% and 11.20% respectively. In general, the percentage of cytotoxicity was much less in the treated groups and also there was not much between the low dose and high dose treated groups. In DNA fragmentation assay, there was absence of marked pattern of laddering of DNA of tumor cells in all the three groups (induced; low dose treated; high dose treated) (Fig.4.3). Ucker and Colleagues (1992) have noted an absence of the oligonucleosomal ladder in different cell types. Apoptotic chromatin condensation was observed without any DNA fragmentation in Fas-expressing murine WR 19L cells wherein apoptosis had been induced by Fas ligand (Sakahira et al., 1999).

In the present study, it is proposed that the 6-propionyl pterin similar to sepiapterin induces the iNOS activity. Sepiapterin, supplied BH4 via salvage pathway, which in turn enhances the activation of iNOS enzyme (Nakai et al., 2003). Xu and coworkers (2002) have recommended that microencapsulated NO generating cells cause tumour suppression in mice.
In view of this, the present study utilizes the essential cofactor 6-propionyl pterin to enhance the iNOS activity leading to induced NO generation. In agreement with that similar compound methyl pterin has been reported to be able to catalyze the NOS enzyme activity (Gorren et al., 2001).

High concentration of NO (due to induced iNOS activation) mediated cancer cell apoptosis and the inhibition of cancer growth. The mechanism of tumour killing by increased concentration of NO may be associated with concomitant up-regulation of Fas/Fas ligand proteins (Xu et al., 2002). Similarly, in the present study 6-propionyl pterin induced apoptosis without DNA fragmentation may be due to Fas/Fas ligand proteins.

The total number of cells in low dose treated group was half of DLA induced group and it was even lesser in the high dose treated group. The tumor volume indicating the volume of ascites fluid was lesser. When the animals were sacrificed and observed, there were visual clumps of tumor cells in the intraperitoneal region (Fig. 4.4). The tumor cells in the ascites fluid had formed clumps due to increased adhesion. Earlier the research group of Van Haastert (Van Haastert et al., 1982) has observed that a derivative of pterin is responsible for the aggregation of cells in Dictyostelium discoideum, a model system for eukaryotes. Recently Kim and his colleagues (2011) have studied the role of sepiapterin in the regulation of angiogenesis using Human umbilical vein endothelial cells (HUVEC) as model. They have demonstrated that sepiapterin, a precursor of BH₄ inhibits VEGF-A-induced HUVEC proliferation and adhesion through down-regulation of VEGFR-2 downstream signaling pathways, independently of NO synthesis. Also they have proved that sepiapterin treatment significantly increased cell adhesion. Our results are in agreement with these findings wherein the cells showed increased adhesion with very few free cells.

Consistent with the earlier report (Kim et al., 2011) that sepiapterin inhibited VEGF-A, there was well-marked inhibition of angiogenesis in the DLA induced 6-propionyl pterin treated groups when compared to the DLA induced groups (Fig. 4.5). The blood vessel pattern of DLA induced treated groups were similar to that of normal (uninduced) groups. A few studies have
evaluated the relationship between tumor angiogenesis and tumor response to chemotherapy and/or radiotherapy. Tumor growth depends on angiogenesis; the rate of tumor cell proliferation is related to angiogenic activity. The observation that angiogenesis occurs around tumor was made nearly 100 years ago (A3). In 1971, Folkman proposed that tumor growth and metastasis are angiogenesis-dependent, and hence, blocking angiogenesis could be a strategy to arrest tumor growth (Goldman, 1907; Ide et al., 1939; Algire and Chalkley, 1945). There are a very few studies of angiogenesis in cases with lymphoma. Vacca et al. (1997) and Ribatti et al. (1996) reported increased capillary proliferation in the lymph node biopsies of high-grade non-Hodgkin’s lymphoma (NHL). Thus, in our present study, inhibition of angiogenesis by 6-propionyl pterin inhibited the tumor growth and metastatic spread of tumor cells. The viable cell count also considerably decreased.

The pteridine are known for its capability to suppress cell proliferation and as a potent antitumor compound. 6-Formylpterin (6-FP) is known as a potent xanthine oxidase inhibitor with an inhibition constant (Ki) of approximately 0.6nM (Lowry et al., 1949; Kalckar et al., 1950). Although 6-FP does not usually occur in vivo, it is produced from folic acid in vivo in some pathological conditions, such as carcinoma (Halpern et al., 1977). Arai et al. (2001) showed that 6-FP reacted with reducing agents in cells, such as NAD(P)H, and intracellularly generated hydrogen peroxide, which induced apoptosis in human promyelocytic leukemia HL-60 cells and suppressed cell proliferation in human pancreatic adenocarcinoma cell line PanC-1 cells. These findings indicated the possibility of 6-FP as an anti-cancer drug (Yamada et al., 2005). Enzinger et al. (2002) investigated and reported the potential of pteridines to destroy the redox balance and induce apoptosis of rat pheochromocytoma cells (PC12). Cytotoxic effects of the combined use of edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a radical scavenger and an approved medicine for acute brain infarction in Japan, with a pterin derivative, were examined in vitro (Arai et al., 2007). When pancreatic cancer cell line Panc-1 cells were incubated with 50 to 400 µM of a pterin derivative, 2-(N,N-dimethylamino methyleneamino)-6-formyl-3-pivaloylpteridine-4-one (DFP), and the equivalent dose of edaravone, reactive oxygen species (ROS), were generated, and cell death was induced.
4.3.2. Haematological parameters

Haematological parameters of DLA tumor induced and treated groups were found to be significantly altered from normal groups. There was a significant decrease in the RBC in the tumor induced groups and was restored to normal condition in the treated groups (Fig. 4.6a). The WBC count was high in cancer induced group compared to the normal group (Fig. 4.6b). Treatment with 6-propionyl pterin decreased the WBC count and brought back the level closer to the normal group. This indicated that the compound have a protective effect on the haemopoietic system. The tumor bearing mice showed reduced number of RBC and low level of haemoglobin but total WBC count was increased in DLA bearing mice. Myelosuppression and anaemia have been found frequently in cancer patients (Price and Greenfield, 1958). Anaemia (reduced haemoglobin) encountered in ascites carcinoma and lymphoma mainly occurs due to iron deficiency, either by hemolytic or myelopathic conditions which finally lead to reduced RBC number. The haemoglobin content, RBC, platelets and differential count of WBC had been reversed by the LPS-CP-2 treatment (Haiping et al., 2008). Similar results have been obtained by Priya et al. (2011) where the treatment of DLA bearing mice with the root extracts of Lineris almost brought the levels of RBC, total WBC count and platelets closer to the normal level. Kumar et al. (2011) reported that the extract of Indigofera cassioides showed protective effect on haemopoietic system on DLA and EAC (Ehrlich’s Ascites Carcinoma) bearing mice.

4.3.3. Hepatoprotectivity

Most of the drugs given through systemic routes undergo their final metabolism in liver, and therefore, liver is likely to be affected adversely during chemotherapeutic treatments. Increased level of serum LDH indicates for vital tissue damage and the levels of SGOT and SGPT are used as blood based markers of liver damage. In our study, the level of SGOT in DLA tumor induced mice (193.77 U/L) was slightly higher than that of the normal mice (139.76 U/L) (Fig.4.7a). Among the normal groups the level of SGOT was slightly high in the treated groups than the untreated normal. In low dose of 6-propionylpterin treated normal group it was 161.88 U/L and in high dose of 6-propionylpterin treated normal group it was 163.48 U/L. Among the DLA induced groups, in the low dose of 6-propionylpterin treated it was 170.33 U/L and in high dose of 6-propionylpterin treated it was found to be 222.6 U/L. Like SGOT, the level of SGPT
was higher in induced group (32.9 U/L) than in normal group (24.34 U/L). Among the DLA induced group, the level of SGPT in low dose treated had been decreased and closer to normal group (28.34 U/L). But, the level of SGPT in DLA induced high dose treated had shot up to 56.22 U/L. This indicated the toxic effect of high dose (500 µg/kg).

In fast growing cancer cells, glycolysis proceeds at much higher rate than it is required by the citric acid cycle. Thus more pyruvate is produced than can be metabolized aerobically. Under hypoxic conditions of cancer, regeneration of NAD$^+$ from NADH during glycolysis is impaired. Under these circumstances, NADH is oxidized by coupling to the reduction of pyruvate to lactate, and NAD$^+$ so formed allows glycolysis to proceed. LDH is the enzyme that catalyzes this reversible reaction. Lactate has been found to be present in tumors at much higher levels than in the corresponding normal tissues (Hurd and Freeman, 1989; Schupp et al., 1993; He et al., 1995). In our study also, the level of LDH in normal was found to be 652.3 U/L and in tumor induced groups it was higher 1489.8 U/L. The level LDH got reduced in low dose treated tumor mice and was found to be 910.56 U/L and in high dose treated it was 955.67 U/L. The treatment with 6-propionylpterin brought the level similar to that of normal mice. The quantitative studies of biopsies from human cancers have indicated a positive correlation between tumor lactate concentration and incidence of metastasis (Brizel et al., 2001). Analysis of LDH isoenzymes was the first routing isozyme technique to be widely adopted by clinical laboratories. Many reports emphasize on the increase in A4 subunits in tumors of all origins (Bachur et al., 1977; Li et al., 1983). Therefore, LDH serves as useful tumor marker. Verma and Vinayak (2008) reported that the increased activity of LDH in DLA control mice decreases with treatment with doxorubicin. 30 µg of doxorubicin was found to be the most effective dose. LDH activity increases with 45 µg of doxorubicin dose. This may be due to higher toxic side effects of doxorubicin. This is in accordance to our result, where the level of LDH slightly increased in the normal groups treated with low and high dose of our compound as well as marked increase in the high dose treated induced group. This shows the slight toxicity of high dose.
4.3.4. Nephroprotectivity

Kidney is one of the most important organs, which is involved in the filtration of blood borne factors continuously. Lymphoma patients can develop renal failure in several ways. The lymphoma can directly obstruct, infiltrate, or cause rupture of various portions of the urinary tract. It can interfere indirectly with renal function by causing hypercalcemia or paraproteinemia, or it can lead through an immunologic route to amyloidosis or glomerulonephritis. Finally, the kidneys can be damaged as a result of treatment directed to lymphoma. In our study, the level of urea (Fig. 4.9a), uric acid (Fig. 4.9b) and creatinine (Fig. 4.9c) was higher in tumour induced mice rather than the normal group. The low dose of 6-propionyl pterin treated group possessed the value similar to that of normal groups. The DLA induced group treated with high dose showed slightly higher value than the normal which indicates its toxicity. Renal damage in a patient with lymphoma often results not from the disease but from the therapy administered.

Increased concentration of serum urea and creatinine are considered for investigating drug induced nephrotoxicity in animals and man (Rai et al., 2006). Elevation of urea and creatinine levels in the serum was taken as the index of nephrotoxicity (Anwar et al., 1999, Bennit et al., 1982, Ali et al., 2001). Serum urea content has been shown to closely parallel the glomerular function (Provoost and Molenaar, 1980). The serum urea accumulates because the rate of serum urea production exceeds the rate of clearance (Mayne et al., 1994). Creatinine, on the other hand, is mostly derived from endogenous sources by tissue creatinine breakdown (Mayne et al., 1994). An increase in creatinine reflects the pathological alteration in biliary flow (Ravikumar et al., 2005). Uric acid, the end of the pathway, is the substance excreted in the urine in humans. When patients with lymphoma are treated with radiation or with cytotoxic agents, the rapid breakdown of cells can result in excessive uric acid production and excretion. Several investigators (Naziroglu et al., 2004; Atessahin et al., 2005) reported that the alterations induced by cisplatin in the kidney functions were characterized by signs of injury, such as increase of creatinine and urea levels in plasma. In study conducted by them, it was shown that administration of cisplatin to rats caused a reduction in glomerular filtration rate, which correlated with increased creatinine and urea in plasma.
4.3.5. Antioxidant enzymes

In our study, the activity of SOD, catalase and GPx was found to be decreased in the tumor induced mice when compared with normal. The activities of all the three antioxidant enzymes have been restored to normal on treatment with 6-propionylpterin. Oxygen free radicals are known to stimulate cancer development at all three stages: initiation, promotion and progression. A depleted endogenous antioxidant enzyme with enhanced free radicals generation and MDA is well documented in carcinogenesis. The reactive oxygen species (ROS) have potential to cause damage to lipids, proteins and DNA. Thus, to protect against the potentially damaging effects of ROS, cells possess several antioxidant enzymes such as SOD, Catalase (CAT) and GPx. SOD is an important defense enzyme which catalyzes the dismutation of superoxide radicals to hydrogen peroxide thereby reducing the likelihood of superoxide anion interaction with NO to form reactive peroxynitrite (Maritim et al., 2003). Cancer cells can generate large amounts of hydrogen peroxide that in vivo may also contribute to their ability to damage normal tissues and facilitate tumor growth and invasion (Szatrowski and Nathan, 1991). Hydrogen peroxide is successively metabolized into water and non reactive oxygen species by the activities of catalase and GPx (Mates and Sanchez-Jimenez, 1999). Marklund et al. (1984) reported that DLA bearing mice treated with methanolic extract of *Triumfetta rhomboidea* appreciably elevated the catalase activity. Mice administered with *L. inermis* root extract significantly enhanced the activities of catalase, glutathione peroxidase and glutathione S transferase and also increased the level of vitamin C and E and reduced glutathione, which may be responsible for antioxidant properties. In EAC bearing mice, the decrease in SOD activity may be due to loss of both Mn$^{2+}$ containing SOD activity in EAC cells and mitochondria, leading to a decrease in total SOD activity in the liver (Gupta et al., 2004; Natesan et al., 2007). Ehrlich tumor growth induces inhibition of SOD and CAT. Senthilkumar et al. (2008) reported that treatment with MECA significantly increased the levels of SOD and CAT in liver and kidney tissue homogenates compared to tumor control, further indicating its antioxidant nature. Balamurugan et al. (2010) found that the tumor implants have decreased the activities of enzymes like SOD, CAT, GPx, GST and GSH levels in the liver. Again treatment with various doses of SNV peptide was found to restore the above levels to near normal.
4.3.6. RT-PCR

Apoptosis is widely known as programmed cell death that is part of normal development, senescence, and other diverse biologic processes. Apoptosis is an evolutionary conserved process that contributes to development and maintenance of virtually all cell types. Accumulation of normal and abnormal tissue depends on the delicate balance between cell proliferation and apoptosis; as such, it is difficult to assess the importance of apoptosis without careful measurements of both proliferative and apoptotic components. Apoptosis is a pathway that may potentially be disrupted in tumor cells conferring a survival advantage (Fisher, 1994; Thompson, 1995). The survival of a tumor cell is dependent on the acquisition of resistance to cell death and escape from immune-surveillance (Vaux and Strasser, 1996). In most cases, the machinery of death is intact within the cells and ready to be activated by signals that trigger the death process.

Further studies involving a semi-quantitative RT-PCR analysis had been performed to determine the involvement of apoptotic genes in anti-tumor activity. Interestingly, the expression of genes involved in the apoptotic pathway namely iNOS, p53 and caspase-3 had not altered but the expression of MDM 2 gene had been up-regulated (Fig. 4.11). Additionally, the housekeeping gene β-actin which acts as an internal control remained unaltered.

As mentioned earlier, the propionyl pterin elicited tumour suppression and increased lifespan via NOS induced Fas/Fas ligand pathway. MDM 2 oncoprotein has been reported to promote apoptosis in p53 deficient human medullary thyroid carcinoma cells (Dilla et al., 2000). Also caspase-3 activation has been reported to have no role in MDM2 induced apoptosis. In that view, the unaltered expression of iNOS, p53 and caspase 3 is justifiable.

Recent studies have demonstrated that BH\textsubscript{4} can inhibit the proliferation and migration in rat aortic smooth muscle cells, independently of its ability to regulate NO synthase activity (Jiang et al., 2010). Sepiapterin was found to inhibit cell proliferation and migration of ovarian cancer cells via down-regulation of p70\textsuperscript{S6K} –dependent VEGFR-2 expression (Cho et al., 2011).
But, the roles and molecular mechanisms by which sepiapterin regulates angiogenesis are not fully understood. Similarly the molecular mechanism behind the inhibition of angiogenesis and metastasis, cytotoxicity of 6-propionylpterin has to be elucidated further.

4.4. Conclusion

Cancer is expected to claim 9 million deaths worldwide by the year 2015. A major problem in the use of chemotherapeutic or chemopreventive agents in cancer treatment is the potential toxicity of these drugs to normal cells. There is a rapid screening for potential therapeutic agents from plant derived substances as well as from the microbial origin.

The results of present investigation is encouraging which explores the potent anticancer activity of 6-propionylpterin, by exhibiting cytotoxic effect on the lymphoma cells, inhibition of angiogenesis by preventing the formation of new blood vessels and formation of cell aggregation. Decrease in body weight by inhibiting the ascitic fluid accumulation along with increase in life span, enhanced antioxidant status provided confirmatory evidence for protection against Dalton’s Lymphoma. Further significant restoration of haematological parameters, biochemical parameters involving the liver and kidney functions towards normal value upon the treatment with 6-propionylpterin indicated its efficacy in the protection of vital organs from damage induced by DLA. More detailed investigations has to be carried out in finding the exact molecular pathway by which 6-propionylpterin exert its anti-tumor activity.
4.5 References


Table 4.1: Primer sequence used for apoptotic signaling genes

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Genes</th>
<th>Primer Sequences</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Beta-actin</td>
<td>Forward: 5’-CTGTCTGGCCGCACCACCACCAT-3’</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-GCAACTAAGTCATAGTCCGC-3’</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>p53</td>
<td>Forward: 5’-TGCCCCCTCCTCAGCATTTAT-3’</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-GTTGGGCAGTGCTCCTAGTG-3’</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Caspase-3</td>
<td>Forward: 5’-TTTGTTTTGTTGCTCTGAGCC-3’</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-ATTCTGTGACCGCCACCTTCGG-3’</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>iNOS</td>
<td>Forward: 5’-CTTCAACACCAAGGTTGCTGAT-3’</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-CAAGACGCGAAAAACGTACTGTA-3’</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>MDM 2</td>
<td>Forward: 5’-GCTTTGTTAACGCGGCTT-3’</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5’-CGAAGGTCCAGCATCTT-3’</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.2: Effect of 6-propionylpterin on the body weight of mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Increase in body weight</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 3</td>
<td>Day 6</td>
<td>Day 9</td>
</tr>
<tr>
<td>Group 1</td>
<td>1.0±0.05</td>
<td>1.32±0.06</td>
<td>2.15±0.05</td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>0.92±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.12±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.75±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Group 3</td>
<td>1.15±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.38±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.96±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Group 4</td>
<td>1.79±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.20±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.45±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Group 5</td>
<td>1.54±0.01&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>2.75±0.03&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>4.18±0.02&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Group 6</td>
<td>1.34±0.02&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>2.52±0.04&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>3.84±0.01&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Change of body weight of animals in different intervals was calculated by comparing the body weight at Day 0 of the experiment.

Values are expressed as mean ± SEM (n=6). Values were found out by using one way ANOVA followed by Duncan’s multiple range test at p<0.01 significance level. Group 1: Normal; Group 2: Normal mice treated with low dose of 6-propionylpterin; Group 3: Normal mice treated with high dose of 6-propionylpterin; Group 4: DLA Induced; Group 5: DLA induced mice treated with low dose of 6-propionylpterin; Group 6: DLA induced mice treated with high dose of 6-propionylpterin. <sup>a</sup>P<0.01 with respect to Group 1; <sup>b</sup>P<0.01 with respect to Group 4; <sup>c</sup>P<0.01 with respect to Group 1.
Table 4.3: Effect of 6-propionylpterin on life span, tumour volume of mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
<th>Group 5</th>
<th>Group 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Survival Time (MST)</td>
<td>39.75±0.15</td>
<td>38.10±0.18</td>
<td>17.60±0.32</td>
<td>30.32±0.15</td>
<td>32.56±0.3</td>
<td></td>
</tr>
<tr>
<td>Increase in life span (% ILS)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>71.49±0.31</td>
<td>84.16±0.14</td>
</tr>
<tr>
<td>Reduction in tumor volume (%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>55.4±0.10</td>
<td>60.11±0.43</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM (n=6). Values were found out by using one way ANOVA followed by Duncan’s multiple range test at p<0.01 significance level. Group 1: Normal; Group 2: Normal mice treated with low dose of 6-propionylpterin; Group 3: Normal mice treated with high dose of 6-propionylpterin; Group 4: DLA Induced; Group 5: DLA induced mice treated with low dose of 6-propionylpterin; Group 6: DLA induced mice treated with high dose of 6-propionylpterin. a P<0.01 with respect to Group 1; b P<0.01 with respect to Group 4; c P<0.01 with respect to Group 1.
Fig. 4.1: Physical appearance of DLA tumor bearing mice and normal mice

a. DLA tumor bearing mice  
b. Normal mice
Fig. 4.2: AO/EB staining of DLA induced mice and 6-propionylpterin treated groups

DLA induced untreated group

DLA induced mice treated with low dose of 6-propionyl pterin

DLA induced mice treated with high dose of 6-propionyl pterin
Fig. 4.3: DNA fragmentation assay

a. DLA induced untreated group
b. DLA induced mice treated with low dose of 6-propionylpterin
c. DLA induced mice treated with high dose of 6-propionylpterin
Fig. 4.4: Solid clump formation in 6-propionylpterin treated DLA induced groups

DLA induced mice treated with low dose of 6-propionyl pterin

DLA induced mice treated with high dose of 6-propionyl pterin

 Indicates the clumping of tumor cells in the ascites fluid
Fig. 4.5.a: Angiogenesis in normal and 6-propionylpterin treated groups

Normal (uninduced)  Normal + low dose treated  Normal + high dose treated

Fig. 4.5.b: Angiogenesis in DLA induced and 6-propionylpterin treated groups

DLA induced  DLA induced low dose treated  DLA induced high dose treated
Fig. 4.6a: Effect of 6-propionylpterin on RBC count

Each bar represents mean ± SEM. Values were found out by using one way ANOVA followed by Duncan’s multiple range test at p<0.01 significance level.  

- a P<0.01 with respect to Normal;
- b P<0.01 with respect to Induced;
- c P<0.01 with respect to Normal.

Fig. 4.6b: Effect of 6-propionylpterin on WBC count
Fig. 4.7a: Effect of 6-propionylpterin on SGOT level

![Graph showing effect of 6-propionylpterin on SGOT level.]

Fig. 4.7b: Effect of 6-propionylpterin on SGPT level

![Graph showing effect of 6-propionylpterin on SGPT level.]

Each bar represents mean ± SEM. Values were found out by using one way ANOVA followed by Duncan’s multiple range test at p<0.01 significance level. 

\[ \text{a} \] P<0.01 with respect to Normal ; 
\[ \text{b} \] P<0.01 with respect to Induced ; 
\[ \text{c} \] P<0.01 with respect to Normal.
Fig. 4.8: Effect of 6-propionylpterin on LDH level

Each bar represents mean ± SEM. Values were found out by using one way ANOVA followed by Duncan’s multiple range test at p<0.01 significance level. \(^a\) P<0.01 with respect to Normal ; \(^b\) P<0.01 with respect to Induced ; \(^c\) P<0.01 with respect to Normal.
**Fig. 4.9a: Effect of 6-propionylpterin on the level of urea**

![Graph showing the effect of 6-propionylpterin on urea levels in different groups.](image)

Each bar represents mean ± SEM. Values were found out by using one way ANOVA followed by Duncan’s multiple range test at p<0.01 significance level. 

- \(^a\) P<0.01 with respect to Normal; 
- \(^b\) P<0.01 with respect to Induced; 
- \(^c\) P<0.01 with respect to Normal.

**Fig. 4.9b: Effect of 6-propionylpterin on the level of uric acid**

![Graph showing the effect of 6-propionylpterin on uric acid levels in different groups.](image)
Fig. 4.9c: Effect of 6-propionylpterin on the level of creatinine

Each bar represents mean ± SEM. Values were found out by using one way ANOVA followed by Duncan’s multiple range test at p<0.01 significance level.  

\(^a\) P<0.01 with respect to Normal ;  

\(^b\) P<0.01 with respect to Induced ;  

\(^c\) P<0.01 with respect to Normal.
Fig. 4.10a: Effect of 6-propionyl pterin on SOD activity

Fig. 4.10b: Effect of 6-propionylpterin on catalase activity

Each bar represents mean ± SEM. Values were found out by using one way ANOVA followed by Duncan’s multiple range test at p<0.01 significance level.  

- P<0.01 with respect to Normal ;  
- P<0.01 with respect to Induced ;  
- P<0.01 with respect to Normal.
Fig. 4.10c: Effect of 6-propionylpterin on GPx activity

![Graph showing the effect of 6-propionylpterin on GPx activity.](image)

Each bar represents mean ± SEM. Values were found out by using one way ANOVA followed by Duncan’s multiple range test at p<0.01 significance level. 

- \(^a\) P<0.01 with respect to Normal
- \(^b\) P<0.01 with respect to Induced
- \(^c\) P<0.01 with respect to Normal
Fig. 4.11: Expression of genes in semi-quantitative RT-PCR for tumor induced groups

1. DLA induced untreated group
2. DLA induced treated with low dose of 6-propionyl pterin
3. DLA induced treated with high dose of 6-propionyl pterin
Summary

In the dynamic new era, approximately one in three people contract cancer and around one in four of those die from the disease. The worldwide incidence of cancer is set to double from ten to twenty million over the next two decades and the death rate would increase from six to ten million [Paul and Kaye, 2002]. Normally cells will grow and divide limited number of times in the body, where the regulation of cell division is an intricate process requiring many cellular check points, different enzymes and a variety of cellular process. Generally the proliferation mechanisms are tightly regulated. Cancer cells possess six essential characters which are observed in virtually all types of cancer. They are generation of self-stimulatory growth signals, insensitivity to inhibitory growth signals, resistance to apoptosis, unlimited potential for proliferation, capacity for angiogenesis and tissue invasion and metastasis. Intensive research in cancer therapy has become the need of the hour. Though effective drugs are available, the search of novel drugs is still a priority goal for cancer therapy, due to the rapid development of resistance to multiple chemoetherapeutic drugs. In addition, the high toxicity usually associated with cancer chemotherapeutic drugs and their undesirable side effects increase the demand for novel antitumor drugs active against untreatable tumors, with fewer side effects and /or with great therapeutic efficiency. Based on these facts, the present study focused on isolation and screening of pterin producing microorganism from industrially contaminated soil sample and determination of its anti-cancer efficacy against Daltons Lymphoma Ascites lymphoma.

Objective 1: Isolation of cyanate and cyanide degrading microorganisms from industrially contaminated soil and water samples

Based on the colony morphology and their stability during subculturing around 72 organisms have been isolated from soil samples collected from various regions of SIPCOT industrial area and nearby Vanappadi Lake. Out of which 58 isolates were bacteria and 14 isolates were actinomycetes. The organisms were designated based on the area of sample collection.
The isolates were determined for its ability to degrade sodium cyanate. Out of 72 isolates 46% of the organisms were able to utilize 10 mM sodium cyanate as nitrogen source. 33% and 22% of the organisms tolerated 20 mM and 30 mM of sodium cyanate respectively. Bacterial isolates VPW3 and MW1 alone were able to tolerate upto 35 mM of sodium cyanate. The 16 isolates which were able to tolerate upto 30mM of sodium cyanate were subjected to secondary selection by cyanide degradation. Out of these isolates, 12 isolates were able to utilize sodium cyanide as sole nitrogen source.

**Objective 2: Screening and identification of pterin producing microorganism**

There are different modes of cyanide degradation. One of the mechanisms is the oxygenolytic cleavage of cyanide which is dependent on cyanide monoxygenase enzyme. This enzyme obligately requires pterin cofactor for its activity. Based on this, the organisms had been subjected to screening for the presence of pterin. Out of 12 organisms capable of utilizing cyanide 10 organisms had the blue fluorescent compound in their cell extract. The cell extracts of 10 organisms has been subjected to HPTLC for the further confirmation along with the standard pterin. All the ten organisms possessed peak similar to that of standard pterin with the Rf value of 0.76.

Based on the incubation period, cell biomass yield, peak height and area VPW3 has been selected for further study. The organism has been identified as *Bacillus subtilis* based on morphological, physiological, biochemical characteristics and 16 rRNA gene sequencing. The sequence has been submitted in the GenBank under the accession number JN989651.

**Objective 3: Extraction, purification and characterization of pteridine derivative from the selected bacterium Bacillus subtilis**

For further confirmation, the cellular extract was prepared, deproteinized, oxidized with iodine and injected into HPTLC. The compound had a blue fluorescence. The Rf value of the sample was 0.73 which was concurrent to the Rf value of the standard pterin 0.74 when scanned at 254 nm.
The concentrated sample has been subjected to further purification in conventional column chromatography and preparative HPLC. The sample corresponding to the pterin standard has been collected and dried in vacuo.

The UV-Vis spectrum of the sample absorbed maximally at 254 nm and 360 nm. The compound showed emission at 450 nm when excited at 360 nm. The IR spectra implied that the compound to be an aromatic compound with the presence of amino and ketone group. The ESI-MS spectrum of the compound implied the mass of the compound to be 219. The $^1$H NMR of the compound coincided with the earlier report and was found to be 6-propionylpterin. Based on all these observations, the compound was found to be 6-propionylpterin (2-Amino-6-propionyl-3H-pteridin-4-one).

Objective 4: Evaluation of in vivo anticancer activity of purified pteridine using Daltons’ Lymphoma Ascites tumour model in mice

The efficiency of the compound against lymphoma has been elucidated by in vivo Daltons Lymphoma Ascites tumor model in mice. There was a well documented visual development of tumor in the peritoneal region DLA induced mice. Whereas in the DLA induced treated mice, the development of tumor has been inhibited and the tumor volume was less. Thus the compound had inhibited the accumulation of ascites fluid and thereby controlled the development of tumor.

There was a significant increase in life span of DLA induced treated mice when compared to the normal. The percentage of cytotoxicity in the low and high dose of 6-propionylpterin treated was 10.91 and 11.20% respectively. There was visual clumping of tumor cells and also the angiogenesis has been stopped when compared to the induced group.

The hematological and the antioxidant enzyme functions had been disturbed in the induced mice when compared to the normal mice. Treatment with 6-propionylpterin has found to restore the activity near to its normal value. In case of hepatoprotectivity, the low dose of the compound was found to be more potent than the high dose. The SGOT, SGPT and LDH analysis of the serum implied that high dose exerted mild toxicity to liver.
The RT-PCR for the genes in the apoptotic pathway namely iNOS, p53 and caspase 3, revealed no change in expression whereas there was increased expression of MDM2 gene.

Based on all these observations, it was found that the compound exerted anti-tumor activity by exhibiting cytotoxic effect on the lymphoma cells, inhibition of angiogenesis by preventing the formation of new blood vessels and also metastasis by the formation of cell aggregation. Thus the compound proved its efficacy as potent anti-tumor agent.

**Future perspectives**

The anti-cancer efficacy of the compound 6-propionylpterin on lymphoma has been exploited. The compound exerted cell aggregation on tumor cells and also inhibited angiogenesis. Therefore the genes involved in the apoptotic and anti-angiogenic pathway has to be elucidated further.