Chapter V
5. In - Vitro cytotoxicity activity of pterin deaminase against HeLa cell line

5.1. Introduction

Cancer is the major cause of human’s death because of high incidence and mortality. Cancer may be caused by carcinogens, tobacco smoke, radiation, chemicals or infectious agents, especially some viruses. Cancers cause annually more than 13 % of all human deaths. Deaths from cancer world wide continue to rise, with an estimated 12 million deaths in 2030 (WHO estimate). The conventional modality for cancer therapy includes surgery, chemotherapy and radiotherapy, separately or in combination but all of these have wide range of deficiency and side effects. These factors highlight the essential prospects for novel therapies or therapeutic combinations to improve the survival of cancer individuals. An effective anticancer agent should kill cancer cells without affecting abnormal-to-normal cells. Hence, the identification of new cytotoxic drug with low side effects on immune system is essential in the area of immunopharmacology.

The field of cancer research has few enzymes that are used as therapeutics against cancer. They are PEGylated arginine deaminase used against human melanoma and hepatocellular carcinoma (Ensor et al., 2002), PEGylated aspargase (oncaspar) and asparaginase used against acute lymphoblastic leukemia in children (Avrami et al., 2002). Except for aspargase, the other two enzymes belong to the family of enzyme class hydrolase. In this streak, another atypical enzyme called pterin deaminase, a folate deaminating enzyme, has also been reported to have antitumour activity (by depleting folate level among cancerous cells) against leukemic cell line L5178888Y and melanoma B16 induced in C57BL mice (Kusakabe et al., 1979).

The folate metabolic pathway is regarded as an important target for chemotherapy as tetrahydro folic acid and its derivatives are very essential for cell metabolism (Omura et al., 1985). Folate coenzymes are critical for de novo synthesis of purines and thymidine and for interconversion of amino acids. Folate deficiency inhibits cellular proliferation, disturbs cell cycling (Borman and Branda, 1989) causes genetic damage (Libbus et al., 1990; Branda and Blickensderfer, 1993) and eventually results in cell death. The underlying cell death mechanisms associated with these observed phenomena remain elusive.
Antifolates, a class of antimetabolite drugs, is a part of anti-cancer chemotherapy regimen that inhibit folate dependent enzymes and cellular processes by inducing biochemical cascades associated with apoptosis resulting in cell death (Priest and Bunni, 1995; Schweitzer et al., 1990). Though antifolates cause an irreversible damage to cancer cells leading to death, cells develop resistance to drugs by up regulating the activity of dihydrofolate reductase (DHFR) causing a major demerit of using it as drugs (Thompson, 1995). Among the antimetabolic drugs, one of the most important and widely used drugs is methotrexate (MTX). MTX is a chemical agent that acts by inhibiting the enzyme dihydrofolic acid reductase, which catalyses the conversion of folic acid to its active form folinic acid, by binding to it (Huennekens, 1994; Kamen, 1997).

Though antifolates are found to be efficient anticancer drugs, cancer cells develop resistance against these chemotherapeutics. In order to circumvent the problem of resistance, several new antifolates viz., ZD1694, Multitargeted antifolate (MTA) or LY231514, GW1843U89, AD9331, AG337, PT523, PEM disodium have been developed (Morotti et al., 2012). These above line of limitations constantly stimulate the researchers to search new antifolate chemotherapeutics against cancer. Consistent to these, pterin deaminase, identified to be an exceptional candidate owing to the vital property exhibited as antifolate. The enzyme induces a condition of folate deficiency by directly utilizing folic acid, converting it into their corresponding lumazine forms that could not be further used for DNA synthesis. Since, the mechanism of action is directly on folic acid and independent of up regulation of folate dependent enzymes there are only few chances of development of resistance among the cancer cells.

To study the ability of chemotherapeutic agents to induce apoptosis, it is essential to accurately define this mode of cell death (Oh et al., 2001). Generally, the following traditional criteria are required: 1) typical morphological changes, including chromatin condensation and nuclear fragmentation, must be observed under light microscopy and 2) a DNA ladder must be demonstrated by agarose gel electrophoresis. Several new potentially useful assays to detect apoptosis have recently been developed on the basis of biochemical events associated with apoptosis, such as DNA fragmentation by flow...
cytometry analysis (Otsuki et al., 2003; Kurokawa and Kornbluth, 2009). Unlike other enzymes reported as anticancer chemotherapeutics, pterin deaminase null probability of developing resistance by cancer cells. In spite of these novelties, their pharmacological properties and bioactive constituents were left uncharacterised. In the present study, we investigated the cytotoxic and apoptotic effects of fungal enzyme pterin deaminase in *Hela* cells.

### 5.2. Materials and Methods

#### 5.2.1. Cell line and culture

*HeLa* cell lines were obtained from National Centre for Cell Sciences (NCCS) Pune, India. The cells were maintained in Minimal Essential Media supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO₂ at 37 °C.

#### 5.2.2. In vitro assay for cytotoxicity activity (MTT assay)

The cytotoxicity of samples on *HeLa* cells was determined by MTT assay (Mosmann et al., 1983). Cells (1 × 10⁵/well) were plated in 5ml of medium/well in 6-well plates. After 48 h incubation the cell reaches the confluence. Then, cells were incubated in the presence of pterin deaminase at 7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 µg / ml respectively for 24 to 48 h at 37°C. After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 1ml/well of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide cells (MTT) phosphate- buffered saline solution was added. After 4 h incubation, 0.04M HCl / isopropanol were added. Viable cells were determined by the absorbance at 570 nm. Measurements were performed and the concentration required for 50% inhibition of viability (IC₅₀) was determined graphically. The absorbance at 570 nm was measured with UV- Spectrophotometer using cells without sample treatment as blanks. The effect of samples on the proliferation of *HeLa* cells was expressed as % cell viability, using the following formula:

\[
\text{% cell viability} = \frac{A_{570}}{A_{570}} \times 100\%.
\]
5.2.3. DNA fragmentation analysis

Dispensed 0.5 ml of cell suspension and centrifuged cells at 200 xg at 4°C for 10 min. TTE solution (TE buffer pH 7.4 (A1) with 0.2% Triton X-100 stored at 4°C) of 0.5 ml was added to the pellet and vortexed vigorously. This procedure allowed the release of fragmented chromatin from nuclei, after cell lysis (due to the presence of Triton X-100 in the TTE solution) and disruption of nuclear structure (following Mg²⁺ chelation by EDTA in the TTE Solution). To separate fragmented DNA from intact chromatin, tubes were centrifuged at 20,000 xg for 10 min at 4°C. Carefully transferred the supernatants to new tubes labelled T (Top). Added 0.5ml of TTE solution to the small cell pellet in tubes. Added 0.5 ml of ice-cold 5 M NaCl and vortexed vigorously. The addition of the salt should be able to remove histones from DNA. Added 0.7 ml of ice-cold isopropanol and vortex vigorously. Allowed precipitation to proceed overnight at -20 °C. The step can be shortened by putting samples in a dry ice for 1h. After precipitation, DNA was recovered by pelleting for 10 min at 20,000 xg at 4°C. Discarded the supernatants by aspiration and carefully removed any drops or fluid remaining adherent to the wall of the tubes with a paper towel corner. This can be critical step because the pellet could be loosened and transparent, hard to be seen.

Rinsed the pellets by adding 0.5 - 0.7 ml ice-cold 70% ethanol. Centrifuged the tubes at 20,000 xg for 10 min at 4°C. Discarded supernatants by aspiration. Carefully removed any drops or fluid remaining adherent to the wall of the tubes by inverting tubes over an absorbent paper towel for 30 min. Air dried the tubes in upright position for at least 3 h before proceeding. Dissolved the DNA by adding to each tube 20-50 µl of TE solution (10 mM Tris, pH 7.4, 1 mM EDTA) and place the tubes at 37°C. Mixed the samples of DNA with loading buffer (20 % Ficoll 400, 0.1M EDTA (pH 8.0), 1% SDS, 0.25% bromophenol blue, and 0.25% xylene cyanol) by adding 10x loading buffer to a final concentration of 1x. The addition of loading buffer to samples allows to load gel wells more easily and to monitor the run of samples. The marker used in the range of 100 - 1500 bp. The electrophoresis was run in standard TBE buffer (dissolve 800 ml of H₂O, 108 g Tris base (89mM) , 55g boric acid (89 mM), 40 ml 0.5M EDTA, pH8.0 (2mM), bring to 1litre with H₂O. Use diluted 1:10) after setting the voltage to the desired level. Agarose (1%) gel was prepared with ethidium bromide for electrophoresis. During
electrophoresis it is possible to monitor the migration of samples by following the migration of bromophenol blue dye contained in the loading dye. Terminated the electrophoresis when the dye reached about 3 cm from the end of the gel. To visualize DNA, the gel was placed on a UV Transilluminator and documented the picture (Khodarev et al., 1998).

5.2.4. Cell cycle analysis by flow cytometry

Cell cycle analysis was performed by flow cytometric measurements of the DNA content of the cells based on propidium iodide staining. For cell cycle analysis, HeLa cells treated with indicated concentrations of pterin deaminase of Aspergillus terreus JQ436691 (62.5, 125 and 250 μg mL-1) for 48 h. The control, comprising cells without any treatment, served as blank. Cells were harvested, fixed in ice-cold 70% ethanol, stored at 4°C, washed with phosphate-buffered saline (PBS) (pH 7.2), treated with 25 μg/ml RNase A at 37 °C for 15 min. For flow cytometric analysis, a FACS Calibur flow cytometer (Becton Dickinson, NJ) equipped with a single argon ion laser was used. The excitation wavelength was 488 nm and the emission filters were 515-545BP, 572-588BP and 600LP. DNA content of 10,000 cells per analysis was monitored using the FACS Calibur system (Kim et al., 2009). DNA fluorescence of PI-stained cells was evaluated by excitation at 488 nm and monitoring through a 630/22 nm band pass filter. A minimum of 10,000 cells per sample was used for analysis performed using Cell Quest software. Apoptotic nuclei were identified as a subploid DNA peak, and were distinguished from cell debris on the basis of forward light scatter and PI fluorescence. Representative flow cytometry pattern were shown. Cells in different phases of the cell cycle were analysed for both the control cells and the cells treated with extracts (Dassonneville et al., 2000).

5.3. Results and discussion

The aim of present study was to determine the cytotoxic effects of pterin deaminase on cell cycle arrest and apoptosis of human cervix cancer cell line (HeLa). The reason for selecting human cervix cancer cell line is that this is the second most common cancer in woman worldwide (Krauss et al., 2001). According to the Indian National Cancer Registry Programme of ICMR (1997), cervical cancer is the most
common cancer in Indian women, followed by breast, oesophagus, ovary and stomach and the incidence is increasing with an estimated rate of 100,000 new cases per year (Sinha et al., 2003).

Earlier reports an atypical enzyme called pterin deaminase, a folate deaminating enzyme, to have antitumour activity (by depleting folate level among cancerous cells) against leukemic cell line L5178Y and melanoma B16 induced in C57BL mice (Kusakabe et al., 1979). However, very little is known regarding the molecular mechanisms by which they may exert their anti cancer effect. In the present study apoptosis induction by pterin deaminase effect was analysed using various assay viz., cell viability using MTT, DNA fragmentation and flow cytometry analysis (Pillai et al., 2004).

5.3.1. In vitro anticancer studies (MTT assay)

The antiproliferative activity of the purified enzyme pterin deaminase was analysed by MTT assay. The MTT assay has greater applicability in the detection of cells which are not dividing but are still metabolically active. It can, therefore be used to distinguish between proliferation and cell activation (Gerlier, et al., 1986). An additional advantage to the assay is that it can be used in suspended or monolayer cell preparations (Henriksson et al., 2006).

In the present investigation, HeLa cells were treated with different concentrations of pterin deaminase (7.8, 15.6, 31.2, 62.5, 125, 250, 500 and 1000 µg /ml) and the cell viability was measured by MTT reduction assay. The IC$_{50}$ dose was determined by exposing cells to various concentrations of pterin deaminase for 48 h which showed 50% less growth compared to control. Among the eight concentrations tested, the IC$_{50}$ dose was determined to be 52 µg /ml (Table 5-1, Fig 5-1B). The purified pterin deaminase reduced the cell viability upto 8.33 % at a concentration of 1000 µg/ml for HeLa cell line (Table 5-1 ; Fig.5- 1A-e) which was very high and the inhibition was time and dose dependent manner. It indicated that purified pterin deaminase from Aspergillus terreus JQ436691 has better antiproliferative activity towards HeLa cell line at higher concentration. Rimpler et al. (1996) described the ability of fungi in the families Tricholomataceae and Polyporacea to produce antitumor activity.
The untreated *HeLa* cells appeared elongated and attached smoothly on the culture surface (Fig. 5-1A-a). Following treatments for 48 h, the cells became rounded and lost cell contacts at higher concentration of 1000 μg/ml (Fig. 5-1A-e). In particular, the surface morphological changes leading to cell detachment were observed. At lower concentration of pterin deaminase (31.2 μg/ml) treated *HeLa* cells showed similar appearance (Fig.5-1A-b) to that of control and no changes were observed. Similar results were reported by Leah *et al.* (2006) in the study of *in vitro* cytotoxic activities of fungal products against different cell lines including hepatoma cell line HepG2 (Saint *et al.*, 2006). An increase in the concentration of pterin deaminase resulted in increasing morphological changes (cell death) and a greater decrease of viable cells were confirmed by MTT methods (Fig. 5-1A-e).

For DNA fragmentation *HeLa* cells were treated with three different concentrations of pterin deaminase (62.5, 125 and 250 μg/ml) based on the IC₅₀ that was predetermined by MTT assay. Agarose gel electrophoresis of DNA from *HeLa* cell treated with three different concentrations of pterin deaminase for 48 h showed no DNA ladder pattern (Fig. 5-2 Lane 2, 3, 4), similar to untreated cells (Fig 5-2 Lane 1). The cytotoxicity might be due to TNF receptor activated pathway without any DNA fragmentation. Similar type of results have been reported with MCF-7 cells treated with various apoptotic stimuli (e.g. transforming growth factor-β1, etoposide and TNF) that undergo cell death in the absence of DNA fragmentation (Oberhammer *et al.*, 1993; Janicke *et al.*, 1998). Vantieghem *et al.* (1998) reported that no fragmentation was observed when 1 μM photoactivated hypericin was used and no clear DNA laddering could be visualized in apoptotic *HeLa* cells, an observation which has also been reported by others using different apoptotic stimuli (Lock and Stribinskiene, 1996).

5.3.2. Flow cytometry analysis

Cancer is a disease where regulation of the cell cycle goes awry and normal cell behaviour is lost. Once these crucial cell cycle genes start behaving abnormally, cancer cells start to proliferate widely by repeated, uncontrolled mitosis (Beijersbergen and Bernards, 1996; Bernards, 1997). If cell division is understood completely, it might be
possible to control many forms of cancer. Basically, the cell cycle is the program for cell growth and cell division (proliferation). The distribution of cells among different phases of their growth cycles was achieved by using a standard method with slight modifications (Nicoletti et al., 1991).

The quantitative analysis of cell cycle is very important in the study of molecular mechanism of cell death and cell cycle progression (Tao et al., 2004). Untreated and treated HeLa cells were evaluated for apoptosis by measuring the amount of apoptotic cells using of DNA flow cytometry (FCM). Flow cytometric analysis of cell cycle measures the apoptotic changes in cells by staining them with DNA dyes (Telford et al., 1994). Apoptotic cells, due to a change in membrane permeability, showed an increased up-take of the vital dye, propidium iodide compared to live cells (Nicoletti et al., 1991; Telford et al., 1994). This method is useful for quantitative estimates of the fractions of cells in the different phases of the cell cycle (Ali et al., 2011).

In this study, cell cycle analysis provides rapid and convenient assay for cell cycle and cell proliferation. For normal cells, the content of DNA is changed with the process of cell cycle, observed DNA stained by dyes using flow cytometry to calculate percentage of G0/G1, S, and G2/M. In the present study, the number of cells in the G0/G1 phase was significantly augmented by treatment of pterin deaminase (250 µg/ml); therefore, the cell cycle arrest in the G0/G1 phase might be one mechanism of the anticancer effects of pterin deaminase. The dying cells were observed to be accumulated in the sub G1 phase in a dose dependent manner. The G1 (Gap1) phase is characterised by gene expression and protein synthesis. This is really the only part of the cell cycle regulated primarily by extra cellular stimuli (like mitogens and adhesion) any way; this phase enables the cell to grow and to produce all the necessary proteins for DNA synthesis. It primes the cell to enter the next phase.

The cell population in G1 phase increased steadily from 77.4 to 80.2 µg/ml which was accompanied with a slight decrease in S (7.8 to 9.9 µg/ml) and G2 (14.2 to 16.7 µg/ml) phases (Table 5-2; Fig. 5-3 and Fig. 5-4). Pellicciari et al. (1996) reported that the phase in which vepesid will inhibit cell division depended on the cell type and concentration of the drug employed. Chatterjee et al. (1996) in their study with prostate cancer cells,
found the cells to be inhibited at the G₁ phase, similar to our results. *Alfalfa saponin* arrested cell cycle at G₁ phase and stimulated apoptosis at G0/G1 phase and inhibited the synthesis of DNA and RNA and protein synthesis in a dose and time dependant manner (Hirano, *et al.*, 1996). In the flow cytometric analysis involving vepesid, Nizami Duran *et al.* (2001), observed a larger proportion of the cell division to have been inhibited at the G₁ (86%) and S (12%) phases similar to our results. Hence, the present investigation clearly demonstrated that pterin deaminase targets the actively proliferating cancer cells by inducing the apoptotic pathway and mechanism by which the induction is made yet to be resolved. In the present study, there may be decreased purine and pyrimidine bases available for DNA synthesis, due to folic acid depletion. Folic acid is needed for purine and pyrimidine synthesis. The decrease in dNTP pools might retard DNA synthesis and hence S phase cells were not seen. The cells were stopped in the earlier G₁ phase. Our findings are consistent with other studies reported by Ho and Huang (1997) who observed repletion with 2 µg mol/L folate caused folate-deficient HepG2 cells to exit from S-phase arrest and resulted in the diminution of DNA fragmentation as well as DNA laddering. dNTP pool imbalance may be considered to cause the failure of DNA replication and/or repair and leads to mutagenesis (Kunz and Kohalmi, 1991). Folate deficiency induced dNTP imbalance in lymphocytes (James *et al.* 1994) and Chinese hamster ovary cell lines (James *et al.* 1994).

5.4. Conclusion

The regulation of apoptosis in normal and malignant cells has become an area of intensive study in cancer research. The therapeutic application of apoptosis is currently being considered as a model for the development of antitumour drugs. It is therefore essential to identify novel apoptosis inducing compounds that are candidate anti-tumour agents. In conclusion, the present study demonstrated the potent antiproliferative and antitumor properties of purified enzyme pterin deaminase from *Aspergillus terreus* JQ436691.

On treatment of *HeLa* cells with a variety of concentrations (7.8 -1000 µg/ml) of pterin deaminase, the higher concentration 1000 µg/ml showed potential antiproliferative effect on *HeLa* cells. The IC₅₀ for *HeLa* cell line was found to be 52 µg/ml. *HeLa* cells
treated with pterin deaminase (62.5, 125 and 250 μg/ml) for 48 h showed no DNA ladder formation for three different concentrations studied. Cell cycle analysis revealed that the pterin deaminase treatment at a concentration of 250 μg/ml, accumulated the cells in the sub-G1 phase in a dose-dependent manner. The cells accumulated in the G1 phase indicated that the clear mechanism of action pterin deaminase as antifolate inhibiting the DNA synthesis. Thus, pterin deaminase exerts antiproliferative action and growth inhibition in Hela cancer cells (Hela cell line).

In summary, data from this study suggest that pterin deaminase from Aspergillus terreus may be efficient in treating cervical cancer. Our study for the first time proved the anticancer and cytotoxic potentials of pterin deaminase from Aspergillus terreus JQ436691 on HeLa cells by inducing apoptosis in these cancer cells. However, before coming to conclusive statement more research will be needed to fully delineate the part they play in cancer and molecular mechanism of pterin deaminase action, which is not yet clearly understood.
Table 5 – 1. Determination of cytotoxicity of pterin deaminase on HeLa cell line by MTT assay

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Concentration (µg/ml)</th>
<th>Dilutions</th>
<th>Absorbance (O.D)</th>
<th>Cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cell control</td>
<td>-</td>
<td>0.48</td>
<td>100.0</td>
</tr>
<tr>
<td>2</td>
<td>7.8</td>
<td>1:64</td>
<td>0.45</td>
<td>93.7</td>
</tr>
<tr>
<td>3</td>
<td>15.6</td>
<td>1:32</td>
<td>0.42</td>
<td>87.5</td>
</tr>
<tr>
<td>4</td>
<td>31.2</td>
<td>1:16</td>
<td>0.40</td>
<td>83.3</td>
</tr>
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<td>5</td>
<td>62.5</td>
<td>1:80</td>
<td>0.36</td>
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<tr>
<td>6</td>
<td>125.0</td>
<td>1:40</td>
<td>0.25</td>
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</tr>
<tr>
<td>7</td>
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<td>1:20</td>
<td>0.20</td>
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<tr>
<td>8</td>
<td>500.0</td>
<td>1:10</td>
<td>0.11</td>
<td>22.9</td>
</tr>
<tr>
<td>9</td>
<td>1000.0</td>
<td>Neat</td>
<td>0.04</td>
<td>8.33</td>
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Table 5-2. Effect of pterin deaminase (control, 62.5, 125, 250 µg/ml) on cell cycle progression in HeLa cancer cell lines after 48 h.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Cell cycle phase</th>
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<th>125 µg/ml</th>
<th>250 µg/ml</th>
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<td>HeLa cell lines</td>
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<td>69.7</td>
<td>75.6</td>
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<td></td>
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<td>20.8</td>
<td>18.67</td>
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</table>

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Fig. 5-1A. and 5-1B. Anticancer effect of pterin deaminase on *HeLa* cell line

1A- a. Normal *HeLa* cell line

1A- b. Toxicity- 31.2 µg/ml

1A-c. Toxicity- 62.5 µg/ml

1A-d. Toxicity- 125 µg/ml

1A- e. Toxicity- 1000 µg/ml

Fig. 5-1B

Antiproliferative effect of pterin deaminase on *HeLa* cancer cell line. **Fig. 5-1A:** *HeLa* cell line exposed to pterin deaminase, lost their morphology, demonstrating round shape and some of which were lysed or replaced by debris. **Fig. 1B:** Cell viability in percentage and inhibition of growth in percentage. **IC**<sub>50</sub> estimation in the *Hela* cell line was 52 µg/ml for pterin deaminase.
HeLa cells were incubated with pterin deaminase for 48 h. DNA fragmentation was analysed by agarose gel electrophoresis as described in materials and methods. DNA size calibration markers were from fermentas. Lane 1 HeLa cells un treated pterin deaminase: Lane2 HeLa cells treated with 62.5 µg/ml of pterin deaminase: Lane 3 HeLa cells treated with 125 µg/ml of pterin deaminase: Lane 4 HeLa cells with 250µg/ml of pterin deaminase and Lane 5 DNA size calibration were from fermentas.
Fig 5-3. Effect of pterin deaminase of *Aspergillus terreus* JQ436691 on the cellular content of *HeLa* cells

Flow cytometric analysis of DNA fragmentation. Cells were grown in the absence (control), presence of pterin deaminase of *Aspergillus terreus* JQ436691 (62.5, 125 and 250 μg/ml) for 48 h, stained with propidium iodide and analyzed by flow cytometry for DNA content. Each plot is representative of three similar experiments.
Fig 5-4. Effects of pterin deaminase on the cell growth of *HeLa* cells

Effects of pterin deaminase of *Aspergillus terreus* JQ436691 on *HeLa* cell growth. Cells treated with 0, 62.5, 125 and 250 μg/ml of pterin deaminase from *Aspergillus terreus* JQ436691 for 48 h. Control cells maintained in the vehicle for the indicated time periods.
5.5. References


In-Vitro Cytotoxicity Activity of Pterin Deaminase Against HeLa Cell Line


