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Growth inhibitory and proapoptotic effects of L-asparaginase from *Fusarium culmorum* ASP-87 on human leukemia cells (Jurkat)

Running title: proapoptotic effects of L-asparaginase

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ABSTRACT

The objective of this study was to evaluate the anticancer properties of L-asparaginase purified from fungal isolate *Fusarium culmorum* ASP-87 against human T-cell leukemia cell line (Jurkat). The growth inhibitory and proapoptotic effects of purified L-asparaginase on Jurkat cell lines were investigated by determining its influence on cell viability, colony formation, DNA fragmentation and cell cycle progression. The results revealed that purified L- asparaginase showed significant decrease in cell survival with IC_{50} value of 90μg/ml(9 IU/ml). The enzyme inhibited colony formation and showed characteristic laddering pattern on agarose gel thereby confirming the induction of apoptosis. Further, cell cycle analysis revealed that the enzyme induced apoptotic cell death by arresting the growth of cells at G_2-M phase. However, the enzyme did not elicit any toxic effects on human erythrocytes. L-asparaginase purified from Fusarium culmorum ASP-87 showed significant and selective cytotoxic and apoptotic effects on human T-cell leukemic cells in dose dependent manner. Results of the study give leads for the anticancer effects of fungal L-asparaginase and its potential usefulness in the chemotherapy of leukemia.

Keywords: Apoptosis; cell cycle; DNA fragmentation;*Fusarium culmorum*; leukemia

INTRODUCTION

Therapeutic enzymes are enzymes which can be used medically with other therapies for the treatment of various diseases. A variety of enzymes have therapeutic applications. A potential application of therapeutic enzymes is in the chemotherapy of cancer. L-asparaginase is an important therapeutic enzyme which causes the hydrolysis of L-asparagine to L-aspartic acid and ammonia. The most common therapeutic indications of L-asparaginase are in the treatment of Hodgkin's disease, acute lymphocytic leukemia, acute myelocytic leukemia, acute myelomonocytic leukemia and chronic lymphocytic leukemia, lymphosarcoma and

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melanosarcoma [1-7]. The clinical effect of asparaginase is related to depletion of serum asparagine, an amino acid required for the growth of leukemic cells. The administration of L-asparaginase results in rapid and complete deamination of the amino acid (asparagine) in the plasma. Normal cells however are able to synthesize L-asparagine and are less affected by its rapid depletion. Asparagine depletion inhibits protein and RNA synthesis and also induces cell cycle arrest and apoptosis in leukemic cell lines [8,9].The bacterial strains such as *Escherichia coli* and *Erwinia chrysanthemi* are reported to produce L-asparaginase and have been extensively used for the treatment of ALL [10,11]. The use of L-asparaginase from bacterial origin has been reported to cause pancreatitis, liver dysfunction, neurological seizures that may guide to intracranial thrombosis or haemorrhage and hypersensitivity leading to allergic reactions and anaphylaxis [12-15]. In view of the fact that L-asparaginase from eukaryotic microorganisms is known to have less adverse effects [16] our laboratory was interested in evaluating the cytotoxicity of L-asparaginase purified from a fungus, *Fusarium culmorum* ASP-87 [17] by performing an array of *invitro* assays on human T-cell leukemic cell line (Jurkat).

MATERIALS AND METHODS

Chemicals

RPMI 1640 culture medium, Dulbecco's Modified Eagle Medium (DMEM), RNase A, proteinase K were purchased from Sigma-Aldrich (St Louis, USA). Fetal bovine serum (FBS) phosphate-buffered saline (PBS) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and Propidium Iodide (PI) were purchased from Himedia (Mumbai, India). L-asparaginase used in the study was isolated from a tropical soil fungus *Fusarium culmorum* ASP-87.

Production of L-asparaginase by *Fusarium culmorum* ASP-87

The fungal culture was maintained on potato dextrose agar (PDA) slant at 4°C and sub cultured on PDA plates, incubated at 30°C for 6 days and used as inoculum. The culture medium used for the study was modified Czapek-dox medium containing g/l of, Glucose,2.0; L-asparagine,10.0; KH_2PO_4,1.52; KCL,0.52; $MgSO_4.7H_2O$,0.52; $CuNO_3.3H_2O$,trace; $ZnSO_4.7H_20$,trace; $FeSO_4.7H_20$,trace; pH 7.5 [18]. Modified Czapek-dox broth (200 ml) was prepared, sterilized

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and inoculated with *F. culmorum* ASP-87 spore suspension (10^6/ml). The cultures were incubated at 30°C under shaken condition (120 rpm) for 4 days. The crude L-asparaginase extracted from the fungus was partially purified by ammonium sulphate precipitation (salting out). A four day old culture filtrate (200ml) of *F. culmorum* ASP-87 grown in modified Czapek-dox broth was collected after centrifugation at 8000 rpm for 10 min at 4°C. All subsequent purification steps were carried out at 4°C. The crude enzyme was subjected to ammonium sulfate precipitation and the protein precipitates were collected at 0 to 40 %, 40 to 70 % and 70 to 90% salt saturation. The precipitates at each saturation was collected by centrifugation at 10,000 rpm for 15min and resuspended in 0.01M Tris-HCl buffer (pH-7.2). The protein precipitates obtained after each saturation were tested for L-asparaginase activity and the precipitates showing maximum activity was dialyzed overnight against 0.01M Tris-HCl buffer [19].The precipitate was purified by size exclusion chromatography and ion exchange chromatography. It was further purified by SDS-PAGE by passive diffusion method [20] (*Figure* 1). The purified sample was run along with standard L-asparaginase (procured from sigma Cat no. 3809) in precoated TLC plates. The test compound appeared as a single spot in TLC plate and the test sample was found to possess the same Rf value (0.266/90 kDa). The purity of the extracted L-asparaginase was found to be approximately 98% [17].

Cell lines and cell culture

The human T-cell leukemia cell line Jurkat was procured from ATTC (USA). The cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated FBS and cultured at 37°C under 5% CO_2 and 95% humidity. Cells were used in exponential growth throughout all the experiments.

Cell viability assay

One hundred microliter (100 µl) of Jurkat cells (1×10^4 cells/well) in RPMI-1640 medium were seeded onto 96-well culture plate and incubated overnight at standard conditions. The cells were treated with different concentrations of L-asparaginase (1.56 to 100 µg/ml) and incubated for 24 h. Cells treated with culture medium served as a negative control. The supernatant from each well was removed and the cells were treated with 20 µl of MTT solution (5 mg/mlin PBS) and incubated for 4h at 37°C in a CO_2 incubator. The formazan crystals formed were solubilized by

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adding 100µl of Di-methyl Sulphoxide (DMSO) and the plates were read at 570nm using a microplate reader [21]. All experiments were performed in quadruplicate, and the cell viability is expressed as the percent relative to control cells.

Clonogenic assay

Clonogenic assay is a test routinely performed in the laboratory to determine the effectiveness of cytotoxic agents [22]. Jurkat cells (5×10^3 cells/dish) was plated on dishes containing RPMI complete media and incubated at standard conditions for 24 h. After 24 h, cells were treated with 50 and 100 µg/ml of L-asparaginase for 24 h. Cells were grown for 21 days and fresh media is added on the seventh day. On the 21st day the media was removed from the dishes and washed once with PBS (pH 7.2). Cells were fixed with 6 % glutaraldehyde for 5 min, followed by staining the colonies with 1 ml of 0.5% crystal violet in PBS for 30 minutes on a rocking platform. The dishes were rinsed three times with PBS and gently air-dried, and the colonies were counted manually.

DNA fragmentation analysis

DNA fragmentation assay is a qualitative method for assessing cell death by detecting DNA fragments using agarose gel electrophoresis. Jurkat cells (5×10^5/ml) were treated with L-asparaginase at 50 µg/ml and 100 µg/ml concentrations and incubated at 37° C for 24 h. The cells were transferred into 1.5-ml sterile microcentrifuge tubes and centrifuged at 2000 rpm for 5 minutes at 4°C and supernatant was discarded. The cell pellet was dissolved in 20 µl of TES lysis buffer. 10 µl of DNase-free RNase A was added to above mixture and incubated for 30 minutes at 37°C. 10 µl of proteinase K was added and incubated at 50°C for 90 minutes. 5 µl of DNA loading buffer was added and the DNA samples were loaded into wells of 1% agarose gel containing 0.5 µg/ml of ethidium bromide. DNA ladders were visualized under UV transilluminator and photographed [23].

Cell cycle analysis

Cell cycle analysis was performed by flow cytometric analysis after staining Jurkat cells with propidium iodide. Jurkat cells (1×10^6/ml) were cultured in a p35 culture dishes containing 1 ml of medium under standard conditions. After 24h, cells were treated with 50 µg and 100 µg/ml L-

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asparaginase and incubated for 18h. Wells containing Dimethyl sulfoxide (DMSO) suspended in Dulbecco's Modified Eagle Medium (DMEM) (1 ml/well) served as control.After incubation, the cells were harvested and washed twice with PBS, fixed in 70% ethanol overnight and stored at -20° C. The cells were resuspended in 20 μg/ml propidium iodide (PI) containing 20 μg/ml RNase A in PBS and incubated at 37° C for 30 min. The percentage of cells in various stages of cell cycle in L-asparaginase treated and un-treated populations were determined using Fluorescence Activated Cell Sorting (FACS) and analyzed by flow cytometry [24]. The results were expressed as percentage of the cells in each phase.

Isolation of erythrocytes

One to Five ml of blood was collected from healthy volunteers in the age group of 20 to 25 following the Helsinki protocol. The blood samples were collected in tubes containing EDTA and centrifuged at 3000 rpm for 10 min at 4°C. The erythrocytes were washed three times with PBS, pH 7.2 for 5 min. Washed erythrocytes were stored at 4°C and used within 6 h for the haemolysis assay.

Haemolysis assay

Haemolytic assay was performed to investigate the effect of purified L-asparaginase on normal cells. The haemolysis experiments were carried out in erythrocyte suspension. The effect of enzyme was assessed by preincubating the erythrocyte suspensions with different concentrations of L-asparaginase (10 μg – 320 μg/ml) at 37° C for 60 min. 100 μl of PBS was used as negative control with 0 % haemolysis and 1% SDS was used as positive control with 98 % haemolysis. After incubation, the erythrocyte suspensions were centrifuged at 3000 rpm for 10 min. The percentage of haemolysis was measured in a UV-visible spectrophotometer by analyzing the concentration of hemoglobin released into supernatant at 540 nm. The haemolysis results were presented as the percentage of complete haemolysis [25].

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Statistical analysis

All the experiments were carried out in triplicates and statistical analysis was performed using SPSS software (version 20). Values were expressed as mean ±SD. The statistical difference between mean values were assessed by student *t* test at a significance level of ($p<0.05$) and comparisons were made between the control and the other groups.

RESULTS

Cell viability assay

The MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide) assay is a reliable method to analyze the growth inhibitory properties of test compounds. It is a non radioactive, fast and economical assay widely used to quantify cell viability and proliferation. Hence in the current study effect of different concentrations of L-asparaginase (1.56 µg – 100 µg/ml) on the growth and viability of Jurkat cells was assessed by MTT assay. The results revealed that L-asparaginase decreased the cell viability of Jurkat cells in a dose dependent manner with an IC_{50}value of 90µg/ml is equal to 9 IU/ml. The results are shown in (*Figure* 2). This gives an indication about the inhibitory effect of L-asparaginase on the growth and proliferation of Jurkat cells, thereby indicating its antileukemic activity.

Clonogenic assay

The clonogenic cell survival assay determines the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony. We investigated the effect of L-asparaginase on clonogenic survival of Jurkat cells. The cells treated with L-asparaginase, showed a lower survival rate of Jurkat cells which was found to be dose dependent. The cells treated with lower dose of L-asparaginase (50µg/ml) showed moderate inhibition on colony formation. However, higher concentration (100µg/ml) showed appreciable inhibition on colony (*Figure* 3a and b).

DNA fragmentation analysis

Apoptosis is an organized process that signals the cell to self destruct for renewal or to control aberrant cell growth. In order to delineate the mechanism of cell death mediated by L-asparaginase, DNA fragmentation assay was performed. One of the hallmark features of apoptosis is the cleavage of genomic DNA into oligonucleosomal fragments in the form of

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ladder. Jurkat cells treated with different concentrations of L-asparaginase (50 and 100 µg/ml) showed dose dependent characteristic laddering pattern on agarose gel thereby conforming the induction of apoptosis, but there was no ladder formation observed in control cells (*Figure* 4).

Cell cycle analysis

The cell cycle is a recurring sequence of events that includes the duplication of cell contents and its subsequent division. The effect of L-asparaginase on cell cycle progression was evaluated by flow cytometry. The results revealed that Jurkat cells treated with L-asparaginase exhibited cell cycle arrest at G_2-M phase. The cell cycle changes were confirmed by significant increase in the accumulation of cells in G_2-M phase and a marked decrease in the number of cells in G_0-G_1 phase (*Figure*5). It was observed that when treated with L-asparaginase at a concentration of 50 µg/ml and 100 µg/ml the cells in G_2-M phase exhibited a population of 12.57 % and 19.78 % respectively as against 9.07 % in the untreated control. Interestingly, this was concomitant with a significant decrease in the cell population in G_0-G_1 phase (75.39 % at 50 µg/ml and 64.19 % at 100 µg/ml concentration of L-asparaginase as against 81.76 % in the control). No significant changes were observed in the population of cells in the S phase in the L-asparaginase treated groups as compared to control (*Figure*6). This suggests that treatment with L-asparaginase arrested the cells in G_2-M phase thereby preventing them from entering into subsequent phases of the cell cycle. The results of flow cytometry analysis confirmed the induction of apoptosis and cell cycle arrest at G_2-M phase.

Haemolysis assay

Hemolysis refers to the damage of red blood cells leading to the release of intracellular erythrocyte content into blood plasma. *In vitro* hemolytic activity of L-asparaginase purified from *Fusarium culmorum* was studied on human erythrocytes at different concentrations ranging from (10µg – 320µg/ml). It was observed that purified L-asparaginase was nontoxic to erythrocytes even at higher concentrations (320µg/ml). The results are shown in (*Figure* 7). This indicates that L-asparaginase do not posses any toxic effects on normal cells.

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DISCUSSION

Leukemia, the cancer of white blood cells is the most common malignancy in children and adolescents. L-asparaginase an enzyme which catalyzes the hydrolysis of amino acid L-asparagine to L-aspartic acid and ammonia, has been widely used in the treatment of leukemia. The current study reveals the growth inhibitory and antiproliferative effects of L-asparaginase from *F. culmorum* ASP-87 on human T-cell leukemia cell line (Jurkat). The cytotoxicity of purified L-asparaginase was tested against human leukemic cell lines and found decrease in the cell viability of Jurkat cells in a dose dependent manner. Several reports earlier suggest the *in vitro* cytotoxicity of purified L-asparaginase against different cell lines such as human myeloid leukemia HL-60 [26], human breast cancer MCF-7 and human myelogenous leukemia K-562 [27], human colon cancer CACO-2 and human cancer prostrate PC-3 [28], gastric cancer (AGS) [29], Hepato cellular carcinoma (Hep-G2) [30] and human gastric cancer cell line (MKN-28) and human T lymphoblastic leukemia cell line (MOLT-4) [31]. L-asparaginase purified from *Penicillium brevicompactum* NRC 829 and *Aspergillus flavus* (KUFS20) was cytotoxic to hepatocellular carcinoma (Hep-G2) and human breast cancer cells (MCF-7) with IC_{50} value of 43.3μg/ml and 120.87μg/ml respectively [30, 32]. However, commercially available L-asparaginase from *E. coli* and *Erwiniacarotovora* have been reported to have IC_{50} value of 1.0 IU/ml and 7.5 IU/ml respectively on the similar cell lines [33]. The results of clonogenic assay were in line with the results obtained by MTT assay. The DNA fragmentation results were in concordance with earlier reports by Story et al. which showed characteristic fragmentation of DNA of mouse lymphoma cell line (LY-TH) after L-asparaginase treatment indicating apoptosis as the mode of cell death [34]. L-asparaginase purified from *Enterobacter cloacae* also showed characteristic laddering pattern of DNA of human myeloid leukemia HL-60 cell line [26]. The fragmentation of DNA may be due to the activation of caspase 3 which inactivates DFF-45 (DNA fragmentation factor) and ICDA (Inhibitor of caspase-activated DNase) which in turn release active DFF-40 or CAD (Caspase activated DNase) [35, 36].

Induction of apoptotic death may be due to elevation of some cell cycle inhibitors that have been reported to suppress cell cycle progression, resulting in G_2-M phase arrest in P388 leukemia cells [37]. However, earlier reports by Shimizu et al. and Ueno et al. suggested that L-asparaginase purified from bacterial source *Escherichia coli* induced apoptosis of leukemia cells in association

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with cell cycle arrest at G_1 phase [38, 39]. But, on the contrary Pagliardi et al. reported that L-asparaginase induced apoptosis of lymphoblastic leukemia cells with cell cycle arrest at S phase [40]. The cell cycle is regulated by activation of a family of cyclin dependent kinases (CDKs) and cyclins [41, 42]. King et al. reported that the G2-M phase transition was regulated by cyclin A and cyclin B [43].It is possible that the purified L-asparaginase would have exerted modulatory activity on cyclin A and cyclin B which plays a key role in regulating G_2-M phase transition. The major setback with cancer chemotherapy is the absence of selective cytotoxic effects leading to cytotoxic effects on all (normal and cancerous) actively proliferating cells. This frequently results in dreadful side effects in patients including a drastic drop in cell counts (WBC and RBC) and severe anemic conditions. Although erythrocytes are not good models for normal cells, to understand whether asparaginase is haemocompatible, haemolysis assay was performed on erythrocytes [44].The *in vitro* haemolysis assay results were in concordance with earlier reports of Husain et al. and Mahajan et al. which showed L-asparaginase purified from *Enterobacter cloacae* and *Bacillus licheniformis* RAM-8 was not toxic to human erythrocytes at higher concentrations [26, 27]. This result may suggest that L-asparaginase may exert selective cytotoxicity only on leukemic cells, making it an ideal candidate for cancer chemotherapy especially for leukemia.

CONCLUSION

The current study reveals the growth inhibitory and antiproliferative effects of L-asparaginase from *F. culmorum* ASP-87 on human T-cell leukemia cell line (Jurkat). The observed cytotoxic effect of L-asparaginase on Jurkat cells is due to the induction of cell cycle arrest and apoptosis. The enzyme was found to be selectively cytotoxic to cancer cells with no observed adverse effects on erythrocytes suggesting that the enzyme was much haemocompatible for drug delivery applications and hence could be considered as a potential candidate for further pharmaceutical applications as an anticancer drug.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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FIGURES

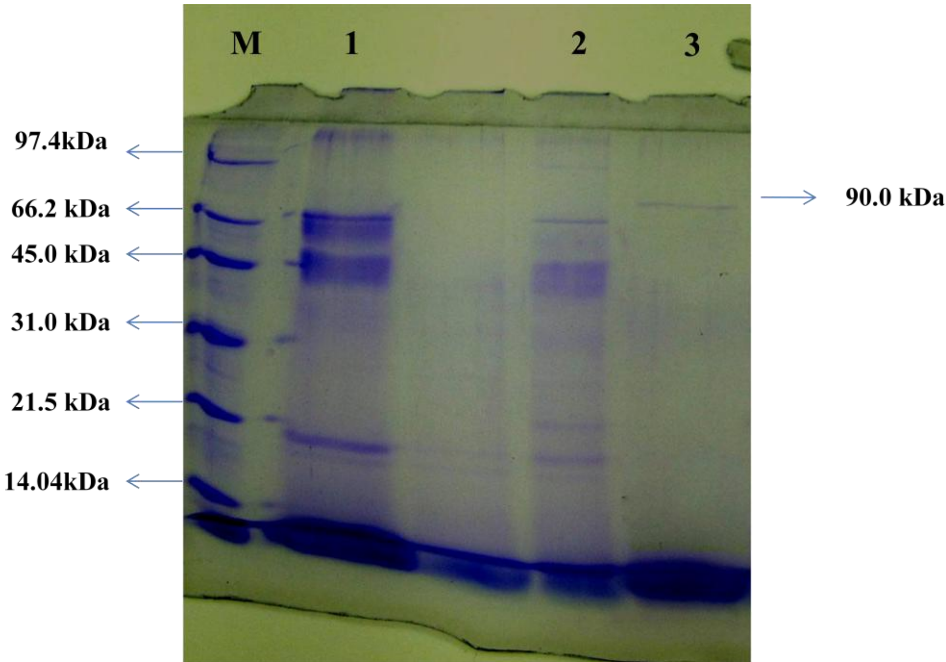

Figure 1

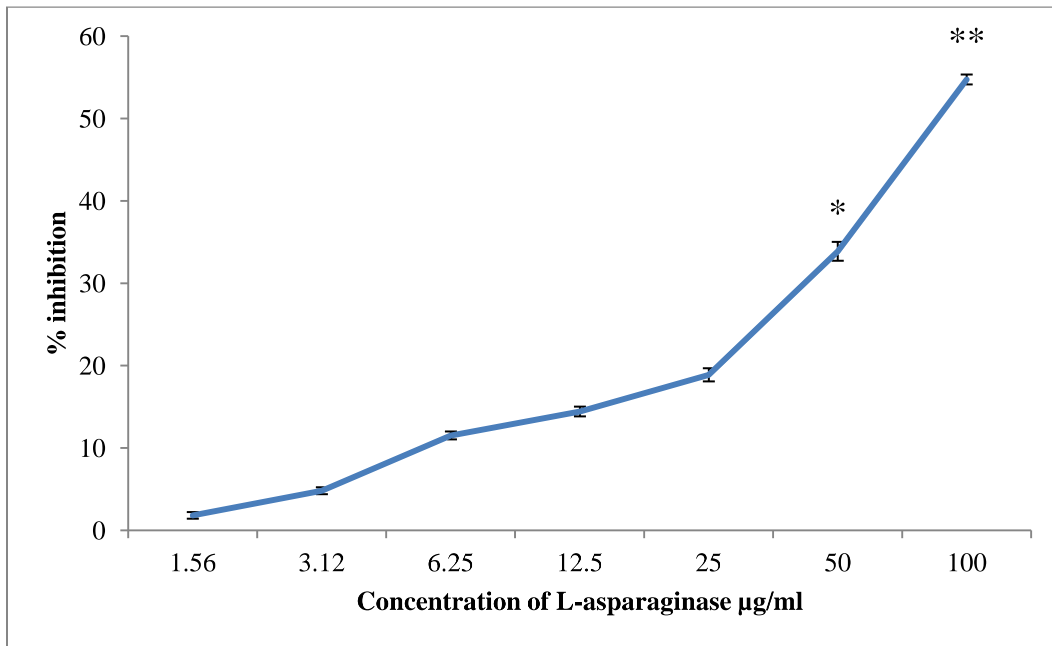

Figure 2

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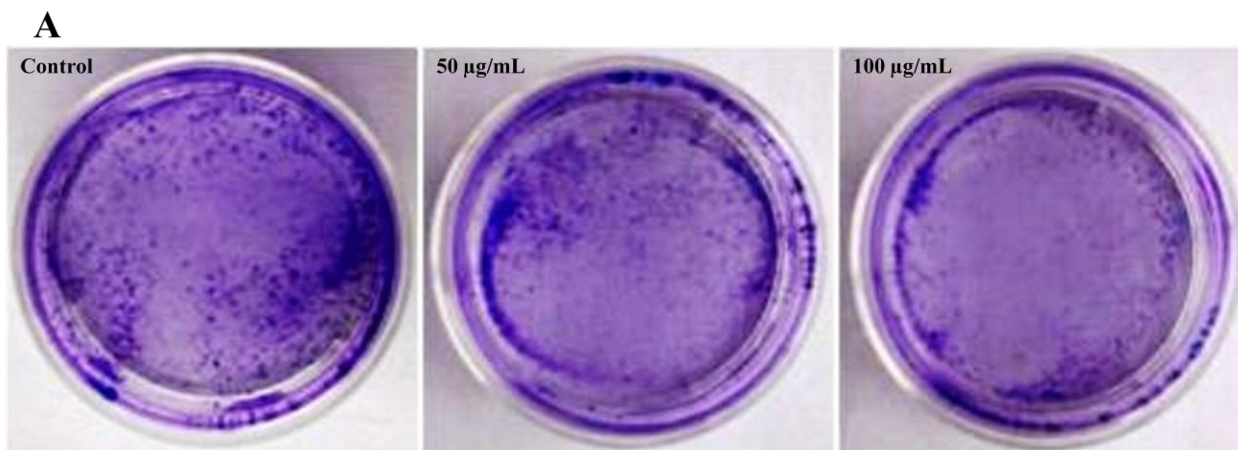

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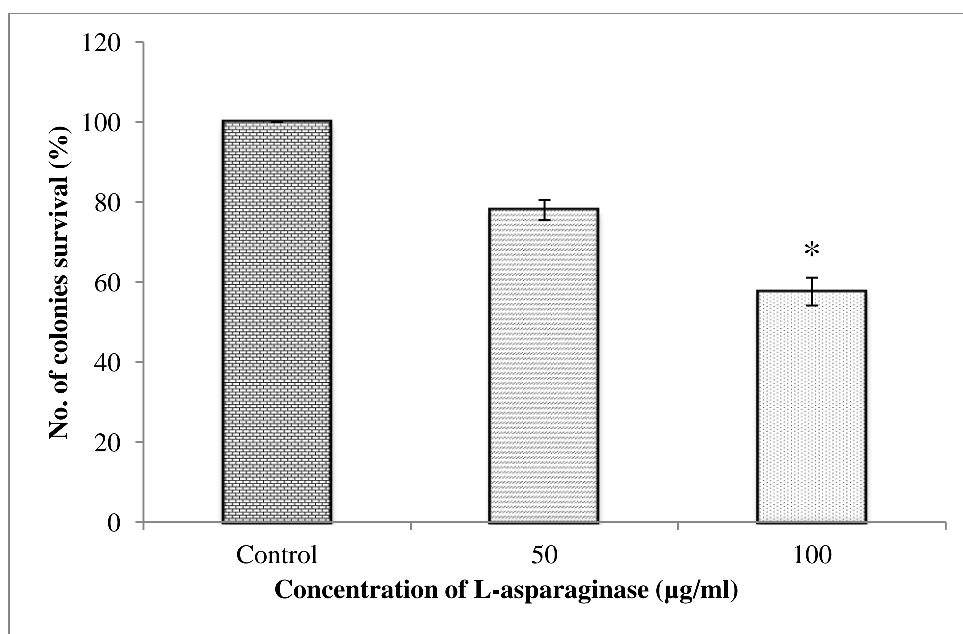

Figure 3b

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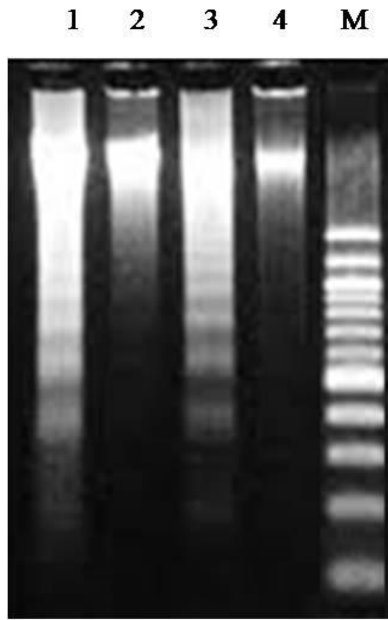

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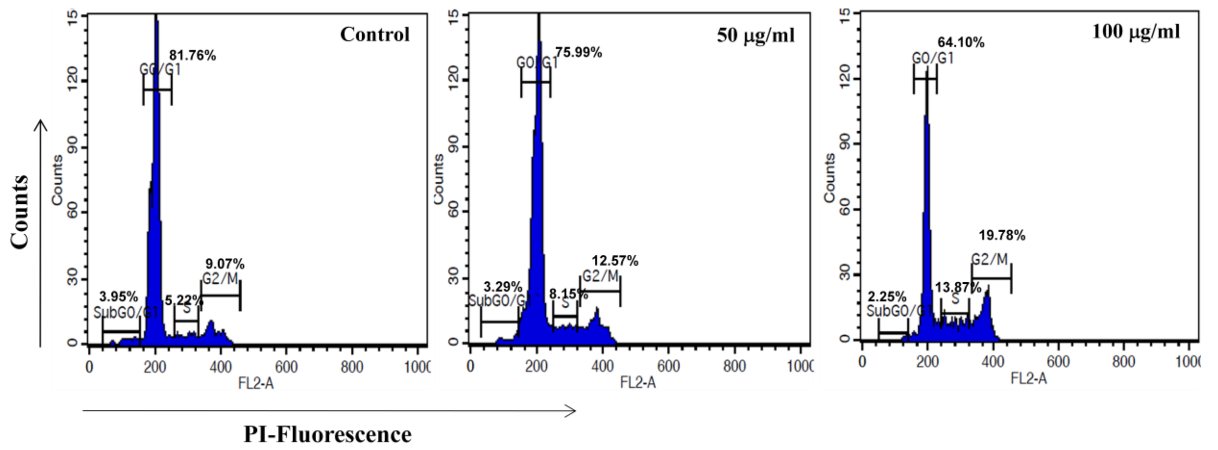

Figure 5

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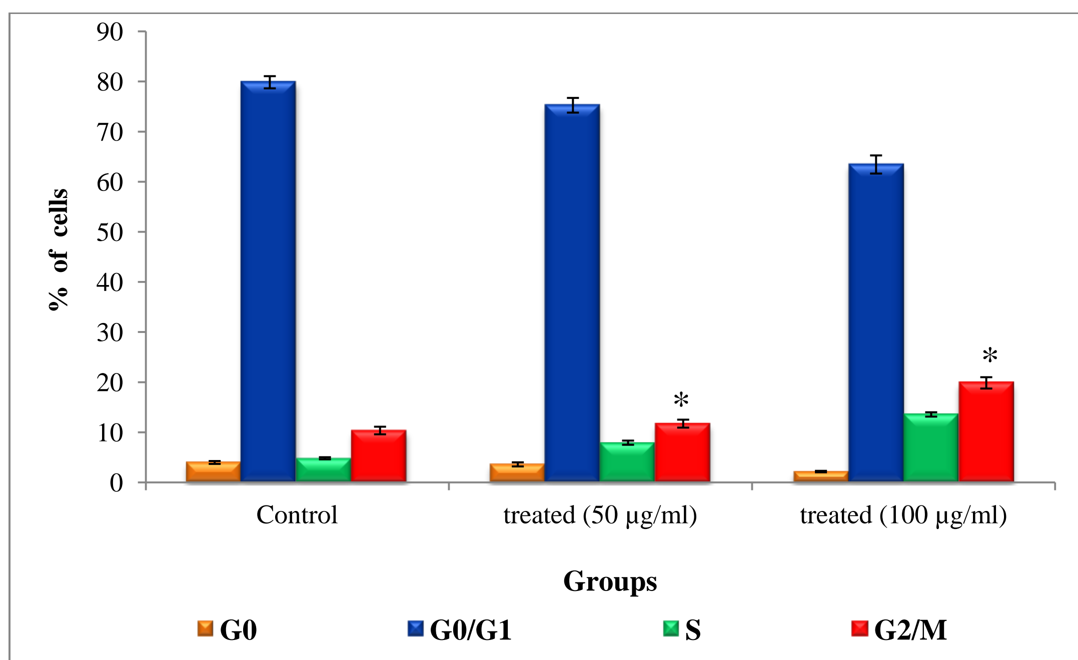

Figure 6

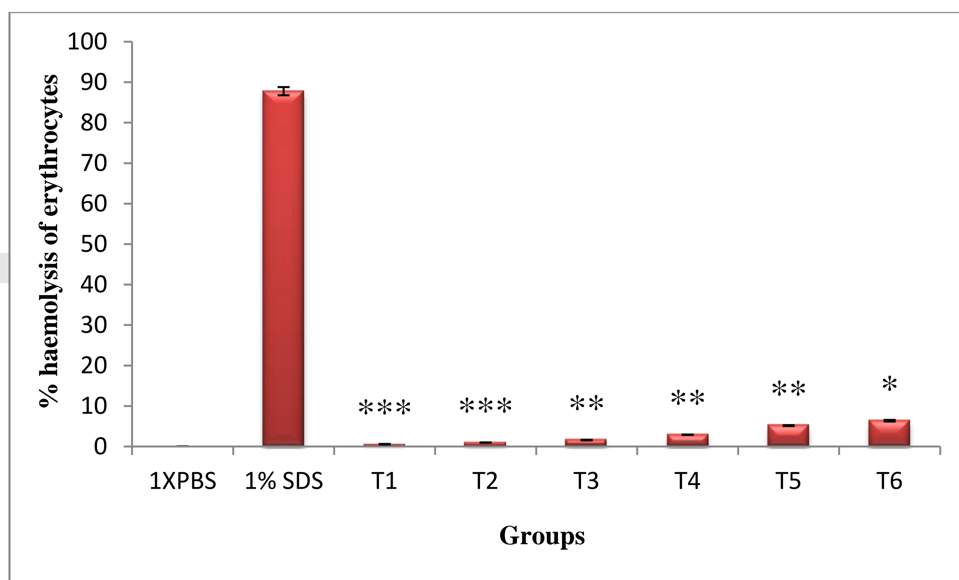

Figure 7

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FIGURE LEGENDS

Figure 1 SDS-PAGE analysis of purified L-asparaginase from *F. culmorum* ASP- 87. **M** = Protein molecular weight marker. **Lane 1** = Crude preparation. **Lane 2** = Ammonium sulfate precipitated fraction. **Lane 3** = Ion exchange fraction (Purified protein).

Figure 2 Effect of L-asparaginase on the viability of Jurkat cells; the cells were treated with or without L-asparaginase (1.56 -100 µg/ml) and cell viability was determined by MTT assay. Values were expressed as mean ± SD. (*P< 0.05, **P< 0.01) represents the statistical significance between control and L-asparaginase treated cells by student's *t*-test.

Figure 3 Effect of L-asparaginase on colony formation in Jurkat cells. (a)L-asparaginase treatment shows suppression of colony formation. (b) Graphical representation of number of colonies.Values were expressed as mean ± SD. (*P<0.05) represents the statistical significance between control and L-asparaginase treated cells by student's *t*-test.

Figure 4 Detection of apoptosis by DNA fragmentation assay in Jurkat cells treated with L-asparaginase from *F. culmorum* ASP-87. Agarose gel electrophoresis of DNA extracted from Jurkat cells. **Lane 1 and 3** represents L-asparaginase treated cells (50 and 100 µg/ml) showing oligonucleosomal DNA fragments. **Lane 2 and 4** represents untreated control cells. **Lane M** is DNA molecular weight marker.

Figure 5 Effect of L-asparaginase on the cell cycle progression in Jurkat cells. The percentage of cells in each phase was determined by flow cytometry after propidium iodide staining. Decrease in the proportion of cells in the G_1 phase of the cell cycle and increase in the S and G_2-M phase was observed.

Figure 6 Histogram of cell cycle distribution in control cells and cells treated with L-asparaginase.Values were expressed as mean ± SD. (*P<0.05) represents the statistical significance between control and L-asparaginase treated cells by student's *t*-test.

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Figure 7 Effect of purified L-asparaginase on haemolysis of human erythrocytes. **T1 to T6**: Cells treated with different concentrations of L-asparaginase (10, 20, 40, 80, 160 and 320 µg/ml).Values were expressed as mean ± SD. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) represents the statistical significance between control and L-asparaginase treated cells by student's *t*-test.

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