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Evaluation of Antineoplastic Activity of Extracellular Asparaginase Produced by Isolated *Bacillus circulans*

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Abstract L-Asparaginase is an important component in the treatment of acute lymphoblastic leukemia in children. Its antineoplastic activity toward malignant cells is due to their characteristic nature in slow synthesis of L-asparagine (Asn), which causes starvation for this amino acid, while normal cells are protected from Asn starvation due to their ability to produce this amino acid. The relative selectivity with regard to the metabolism of malignant cells forces to look for novel asparaginase with little glutaminase-producing systems compared to existing enzyme. In this investigation, the role of the extracellular asparaginase enzyme produced by an isolated bacterial strain was studied. Biochemical characterization denoted that this isolated bacterial strain belongs to the *Bacillus circulans* species. The strain was tested for L-asparaginase production, and it was observed that, under an optimized environment, this isolate produces a maximum of 85 IU ml^{-1} within 24-h incubation. This enzyme showed less (60%) glutaminase activity compared to commercial *Erwinia* sp. L-asparaginase. The partially purified enzyme showed an approximate molecular weight of 140 kDa. This enzyme potency in terms of antineoplastic activity was analyzed against the cancer cells, CCRF-CEM. Flow cytometry experiments indicated an increase of sub-G1 cell population when the cells were treated with L-asparaginase.

Keywords L-Asparaginase · Antineoplastic activity · CCRF-CEM cells · Enzyme production

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Introduction

L-Asparaginase is an important component in the treatment of children with acute lymphoblastic leukemia [1]. Its antineoplastic activity is associated with the property of depleting the circulating pool of L-asparagine by the asparaginase catalytic activity. Malignant cells with low L-asparagine levels are killed due to lack of an exogenous supply of this amino acid combined with an impaired protein synthesis mechanism [2]. However, normal cells are protected from L-asparagine-starvation due to their ability to produce this amino acid [3]. Based on this, L-asparaginase has also been included in most contemporary, multi-agent regimens for adult acute lymphoblastic leukemia (ALL). Broome, while working in Kidd's laboratory, succeeded in 1961 in demonstrating that the antilymphoma activity in guinea pig sera was due to L-asparaginase [4]. Subsequently, Yellin and Wriston [5] partially purified two isoforms of L-asparaginase from the serum of guinea pig. Since production of sufficient quantities of the enzyme from the guinea pig serum is difficult, the scientific community was in search for alternative methodologies. It was a breakthrough when Mashburn and Wriston in 1964 [6] and Campbell and Mashburn [7] in 1969 reported the purification of *Escherichia coli* L-asparaginase and demonstrated that its tumoricidal activity was similar to that of guinea pig sera proving a practical base for large-scale production of this enzyme for pre-clinical and clinical studies [8]. Though several L-asparaginases of bacterial origin have been developed and their potential usage in clinical trials have been studied to prevent the progress of L-asparagine-dependent tumors, mainly lymphosarcomas, the success hitherto has been rather limited, and most of the treatments must be interrupted due to severe side effects and immunological reactions in the patients. Since 1970 onward, several microbial strains like *Aspergillus tamari*, *Aspergillus terreus* [9], *E. coli* [10, 11], *Erwinia aroideae* [12], *Pseudomonas stutzeri* [13], *Pseudomonas aeruginosa* [14], *Serratia marcescens* (*Vibrio succinogenes*) [15], and *Staphylococcus* sp. [16] having potential for L-asparaginase production have been isolated and studied in detail. Literature reports indicated that the enzyme biochemical and kinetic properties vary with the genetic nature of the microbial strain analyzed [17, 18]. For example, *Erwinia* L-asparaginase exhibited less allergic reactions compared to the *E. coli* enzyme. However, *Erwinia* asparaginase had a shorter half-life than *E. coli* [19], suggesting the need to discover new L-asparaginases that are serologically different but have similar therapeutic effects. This may require the screening of soil samples from various sources for isolation of potential microbes, which have the ability to produce the desired enzyme. Hence, studies are continued and focused on abatement of immune reactivity either by modifying the L-asparaginase or by exploring the exotic environment L-asparaginases with novel properties. In this context, a bacterial strain belonging to the *Bacillus circulans* species has been isolated in our laboratory. Our preliminary investigation indicated that this strain has the potential to produce extracellular L-asparaginase. Keeping this in view, in this investigation, we have studied the antineoplastic activity of this extracellular L-asparaginase and report that this enzyme has potential for being used as antileukemia drug.

Materials and Methods

Microorganism and Culture Conditions

A bacterial strain producing L-asparaginase was isolated from soil samples of sea shore by growing on agar-based M9 medium (composition for 1 l: 6.0 g $Na_2HPO_4 \cdot 2H_2O$; 3.0 g

KH_2PO_4; 0.5 g NaCl; 5.0 g L-asparagine; 0.5 g $MgSO_4.7H_2O$; 0.014 g $CaCl_2.2H_2O$; 2.0 glucose (w v^{-1}) and 20.0 g agar) supplemented with phenolphthalein (few drops) as an indicator. The inoculated agar plates were incubated at 37 °C in an incubator. Several microbial strains were isolated based on the size of the phenol-red reactivity zone they showed. One of the strains with the largest zone was used for this study. After purification, the culture was characterized using standard biochemical tests and identified as *B. circulans*. The culture was maintained in slants of the above medium at 4 °C after growth. The agar slants were sub-cultured at fortnightly intervals.

Estimation of L-Asparaginase Activity

L-Asparaginase enzyme assay was performed by a colorimetric method according to Wriston and Yellin [17] at 37 °C using a CECIL UV–Visible spectrophotometer by estimating the ammonia produced during L-asparaginase catalysis using Nessler's reagent. A reaction mixture consisting of 0.5 ml of 0.08 ML-asparagine, 1.0 ml of 0.05 M borate buffer (pH 7.5), and 0.5 ml of enzyme solution was incubated for 10 min at 37 °C. The reaction was stopped by the addition of 0.5 ml of 15% trichloroacetic acid solution. The liberated ammonia was coupled with Nessler's reagent and was quantitatively determined using a standard curve. One unit of the L-asparaginase (IU) is defined as the amount of enzyme capable of producing 1 μmol of ammonia per minute at 37 °C.

Purification of L-Asparaginase from Fermentation Broth

One liter of fermentation culture was subjected to centrifugation at 10,000 rpm for 10 min at 4 °C using Sigma-3 K30, USA. The supernatant (cell free broth) was concentrated 10 times by ultrafiltration using 100 kDa PVDF (poly vinyl divinyl fluoride) membrane. The enzyme present in 100 ml of retentate was precipitated using 80% ammonium sulfate. The precipitate was dissolved in 20 ml of 50 mM Tris buffer (pH 8.6) and dialyzed for 24 h at 4 °C. The dialyzed material further subjected to lyophilization using Lyophilizer (VIRTIS, USA). The obtained enzyme was stored for further studies.

Determination of L-Asparaginase Molecular Weight and Homogeneity by Electrophoresis

The lyophilized asparaginase was subjected to native polyacrylamide gel electrophoresis (PAGE) to estimate the molecular weight of the protein. PAGE was performed on a slab gel containing 5% polyacrylamide [20]. To 20 μl of protein sample, 10 μl of sample buffer was added and loaded onto the gel. Proteins were detected using Coomassie brilliant blue G. The molecular weight of the purified L-asparaginase was determined in comparison with standard molecular weight markers.

Assay of L-Asparaginase Cytotoxic Activity In Vitro

CCRF-CEM cells (human T lymphocytes) were obtained from ATCC, USA. These cells were cultured in RPMI-1640 media containing 10% fetal bovine serum at 37 °C, in a CO_2 incubator in the presence or absence of test compounds. Cytotoxicty was measured using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, according to the method of Mosmann [21]. Briefly, the cells (2×10^4) were seeded in each well containing 0.1 ml of RPMI medium in 96-well plates. After 24 h, different test concentrations (3.125 to 50 μg ml^{-1}) of L-asparaginase RPMI medium were added, and

cell viability was assessed after 2 days, adding 10 μl per well of MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; 5 mg ml^{-1}; stock solution, Sigma) was added to the wells. The plates were incubated at 37 °C for additional 4 h. The medium was discarded, and the formazan blue crystals, which formed in the cells, were dissolved with 100 μl DMSO. The rate of color production was measured at 570 nm in a spectrophotometer (Spectra MAX Plus; Molecular Devices; supported by SOFT max PRO-3.0). All experiments were conducted under the standard laboratory illumination. The percent inhibition of cell viability was determined with reference to the control values (without test compound). The data were subjected to linear regression analysis, and the regression lines were plotted for the best fit. The IC_{50} (inhibition of cell viability) concentrations were calculated using the respective regression equations.

Analysis of the Cell Cycle

CCRF-CEM cells (1×10^6 in 100-mm dishes) were incubated with or without L-asparaginase enzyme at various concentrations (100 to 300 IU ml^{-1}). After treatment (24 h), the cells were collected and fixed in 70% cold ethanol (−20 °C) overnight. The cells were washed twice and resuspended in PBS (10 mM phosphate and 150 mM NaCl, pH 7.0). The endogenous cellular RNA in the cells was digested with RNase A (0.5 mg ml^{-1}) at 37 °C for 1 h. Finally, the cells were stained with propidium iodide (PI) at 2.5 μg ml^{-1}. The cellular DNA content was then analyzed by a FACScalibur flow cytometer (Becton Dickinson, USA). All experiments were performed in triplicates and the average values were reported.

Microscopic Analysis

For morphological evaluation using light microscopy, control and treated cells were examined and photographed on an inverted Nikon microscope using phase contrast and bright-field optics. Images of cells in culture at indicated time points were obtained using an inverted phase contrast microscope, attached to a video camera, and captured using a Nikon NIS-Elements image Software (Japan). Flourescence analysis was performed using the cell nuclear DNA. For nuclear DNA staining, cells were separated and centrifuged and washed twice with PBS at 1,000×*g*. The cells were fixed with 2.5% paraformaldehyde for 20 min at room temperature and then stained with 50 μg/ml Hoechst 33242 (in PBS). The dye was washed out with PBS, and the cells were photographed under a fluorescence microscope (Nikon) with DAPI filter.

Results and Discussion

Several bacterial strains were isolated using soil samples collected from Trivendrum, Lakshwadeep, Visakapatanam, and Machilipatanam sea coast using the serial dilution method. The soil samples were serially diluted with sterile deionized water and inoculated to develop the natural bacterial colonies on M9 agar medium. L-Asparaginase-producing microbial strains were identified by a pink color zone around the colony (Fig. 1). Forty different microbial strains were isolated. A secondary screening for isolated colonies was further performed by streaking on M9 agar medium with phenol red as an indicator for the detection of L-asparaginase-producing colonies.

One of the colonies, which showed fast growth with a clear pink color zone, was selected for further studies to understand its growth behavior and its L-asparaginase

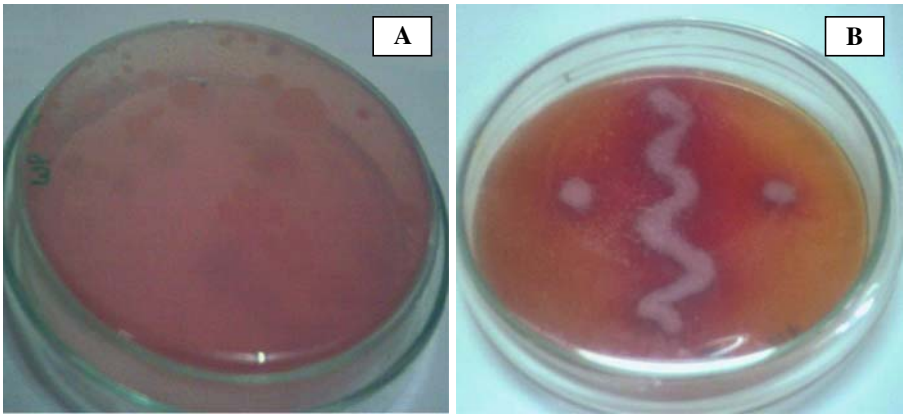

Fig. 1 Agar plates showing primary (**a**) and secondary (**b**) screening of microbial colonies producing L-asparaginase enzyme and its detection zone

production levels using an L-asparaginase-producing medium under a submerged fermentation environment (Fig. 1). Preliminary characterization of this bacterial strain revealed that this strain showed effective growth with the medium consisting of 5.0 g tryptone, 5.0 g yeast extract, 1.0 g glucose, 1.0 g K_2HPO_4, 5.0 g NaCl, and 20 g agar in L, pH 7.0 and incubated at 37 °C. The selected bacterial strain grew well and showed maximum growth after 18 h of fermentation. Analysis of the enzyme production pattern during fermentation indicated that enzyme synthesis also occurs along with microbial strain growth and produces a maximum at 24-h fermentation, indicating that enzyme production is growth-associated in this bacterial strain (results not shown). The enzyme production was further optimized using conventional methodology and under an optimized environment. This strain produced a maximum of 85 IU/ml in a production medium consisting of 6.0 g Na_2HPO_4, 3.0 g KH_2PO_4, 0.5 g NaCl, 5.0 g asparagine, 0.2 g glucose, and 10 g NH_4Cl at pH 7.0. The strain was further identified as *B. circulans* based on biochemical characterization by IMTECH, Chandigarh.

Native PAGE separation indicated a band representing a molecular weight of 140 kDa (Fig. 2). Such data are in accordance with the literature data where L-asparaginase molecular weight was reported to range from 133 to 141 kDa [22, 23]. It is also evident from Fig. 2 that no other band appeared. This may be due to the fact that all low molecular weight proteins produced during fermentation and proteins contributing from the medium are dialyzed out. This enzyme being of high molecular weight, retained during dialysis and had 100 kDa cut-off PVDA membrane.

The antineoplastic activity of the L-asparaginase produced by the isolated bacterial strain was studied using tumor cell lines. This was performed based on the fact that lymphatic cells demand huge quantities of L-asparagine in order to have rapid malignant growth as these tumor cells lack or have very low expression levels of L-asparagine synthetase and depend on the extracellular pool of this amino acid unlike normal cells. The presence of an external L-asparaginase enzyme in the growth medium causes depletion of asparagine due to the catalysis of the supplemented enzyme and kills tumor cells by depriving them of an essential factor required for protein synthesis [23–25]. CCRF-CEM cells were selected for this study and evaluated for L-asparaginase antineoplastic activity. In order to find out the IC_{50} value of L-asparaginase, the MTT assay was performed with freshly grown CCRF-CEM cells, and it was observed that the IC_{50} value was 100 IU million^{-1} cells. These cells

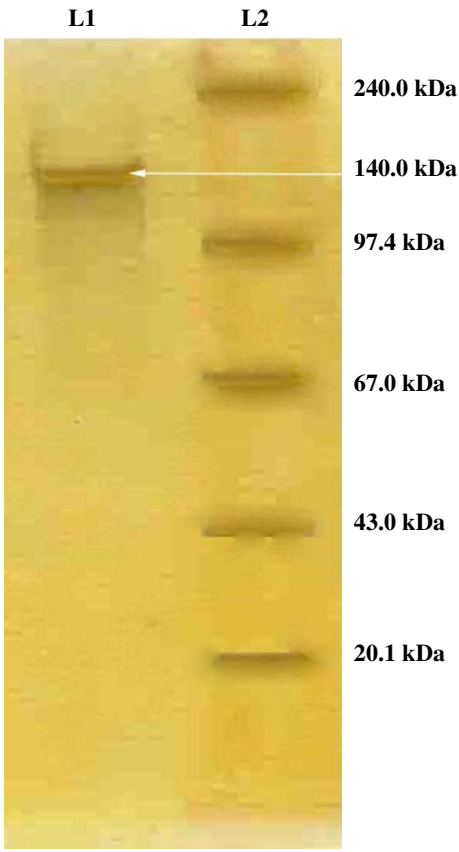

Fig. 2 Native polyacrylamide gel electrophoresis of purified (*L1*) asparaginase and marker (*L2*)

were further analyzed using microscopic visualization. A change in morphology of the cells was observed as seen in both light and fluorescence microscopy (Fig. 3). In fact, morphological analysis of Hoechst-stained CCRF-CEM cells indicated that they had undergone remarkable morphological changes. After a 24-h exposure, the cells showed typical apoptotic properties, including cell shrinkage, chromatin condensation, and loss of normal nuclear architecture. The Hoechst-stained nuclei contain nicked DNA, a characteristic exhibited by cells in apoptotic cell death (Fig. 4). This apoptotic cell death was noticed to be related to the concentration of L-asparaginase used during the experimentation. This could be evidenced based on the observation that only the cells treated with 100 and 200 IU million^{-1} cells showed toxicity (Fig. 5). In addition, cell death was also found to be incubation time dependent (Fig. 5). Such data are in accordance with the results obtained by Asselin et al. [26] who quantified cell killing both in vitro and in vivo in patients with ALL undergoing treatment with L-asparaginase as a single agent. Further, cell growth was monitored in the presence and absence of L-asparaginase. Analysis of these cells indicated a consistent increase in the sub-G1 cell population in flow cytometry as shown in Fig. 4, suggesting that the growth phase of the cell cycle was affected by this novel L-asparaginase. These observations were similar to other studies where a cell cycle arrest in the G1 phase has been reported in the murine L5178Y cell line [27] and the MOLT-4 human T-lymphoblastoid line [28], resulting in apoptosis.

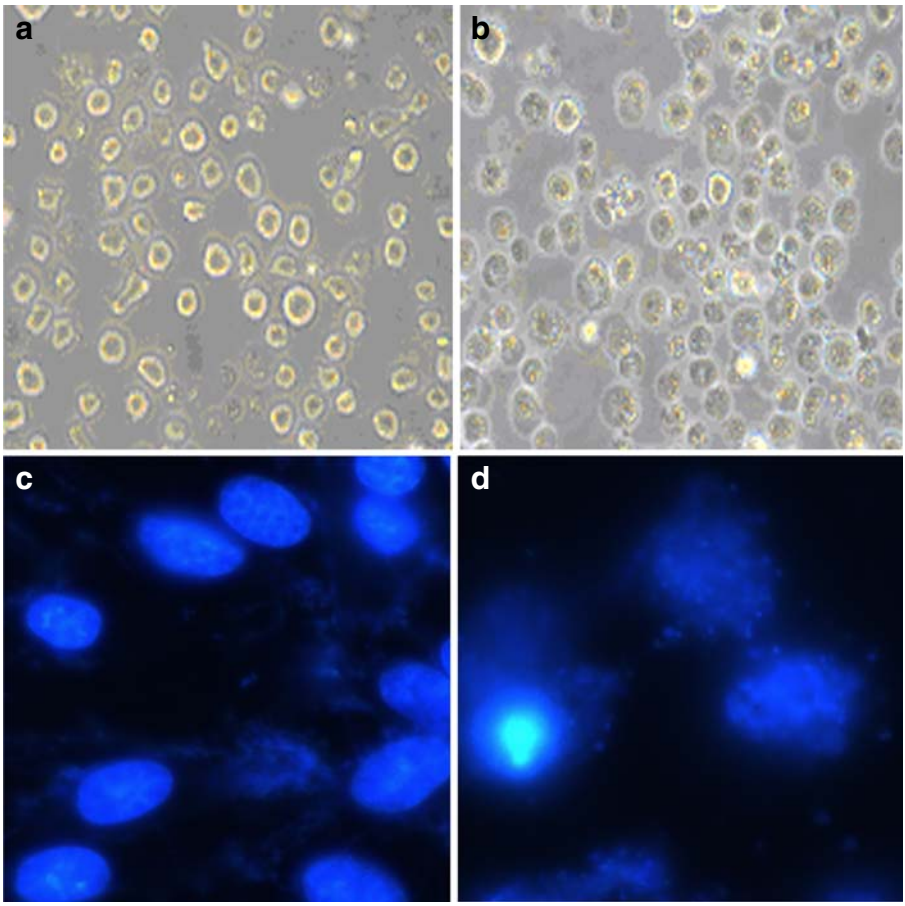

Fig. 3 Micrographs of CCRF-CEM cells before and after treatment with L-asparaginase. **a** Normal (untreated) cells under phase contrast microscopy, **b** L-asparaginase-treated cells under phase contrast microscopy, **c** normal (untreated) cells under fluorescent microscopy, and **d** L-asparaginase treated cells under fluorescent microscopy

Asparaginase-induced immunosuppression is generally imparted to glutaminase activity of L-asparaginase enzyme [29]. Hence, glutaminase activity of *Bacillus circulans* L-asparaginase was measured to evaluate its potential for pharmaceutical application. The data revealed a ratio of 0.06 for L-glutaminase/L-asparaginase activity (results not shown) for L-asparaginase obtained from *B. circulans*. This ratio was reported to be 0.03 for *E. coli* and 0.10 for *Erwinia* sp. L-asparaginases [29], suggesting that the enzyme produced by *B. circulans* has pharmaceutical importance and might be better than *E. coli* L-asparaginase, which is commercially available in the brand name of Elspar or Kidrolase [30]. Comparative evaluation of L-asparaginase activity further indicated that the enzyme activity was almost identical 240–260 IU mg^{-1} protein for *B. circulans* (present study) and *E. coli* asparaginases [29].

Conclusions

An L-asparaginase-producing microbial strain belonging to the *B. circulans* species has been isolated from soil samples collected from exotic environments and studied for

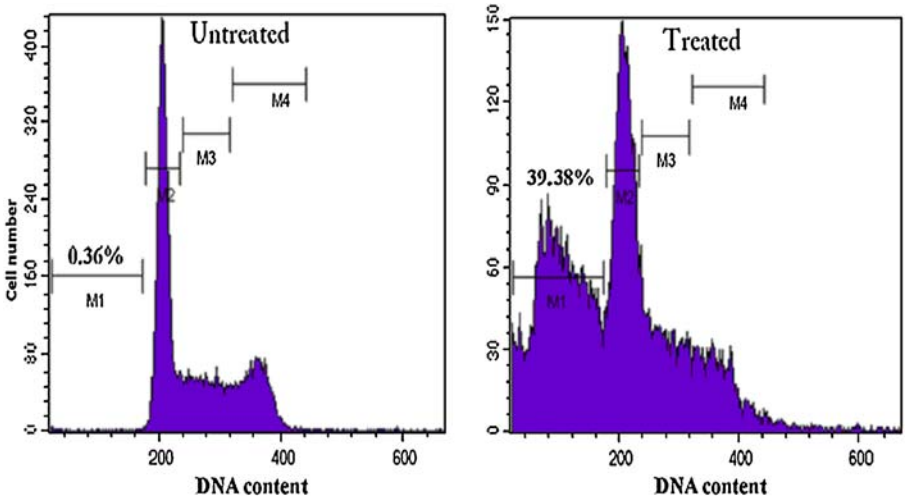

Fig. 4 FACS analysis of L-asparaginase untreated and treated CCRF-CEM cells. FACS analysis images were reproduced as generated by instrument software and hence have different *Y*-axis scale

extracellular enzyme production and its antineoplastic activity using CCRF-CEM cells. This microbial strain showed effective enzyme production up to 85 IU ml^{-1} under submerged fermentation environments, indicating its potential for industrial application. The purified enzyme showed a molecular weight of 140 kDa. L-Asparaginase activity (240–260 IU mg^{-1} protein) was almost comparable for *B. circulans* (present study) and *E. coli*. L-Glutaminase activity of this enzyme was one-fold higher to that of *E. coli* and 40% less compared to *Erwinia* L-asparaginases. Antineoplastic studies revealed that this enzyme has application potential in the pharmaceutical sector.

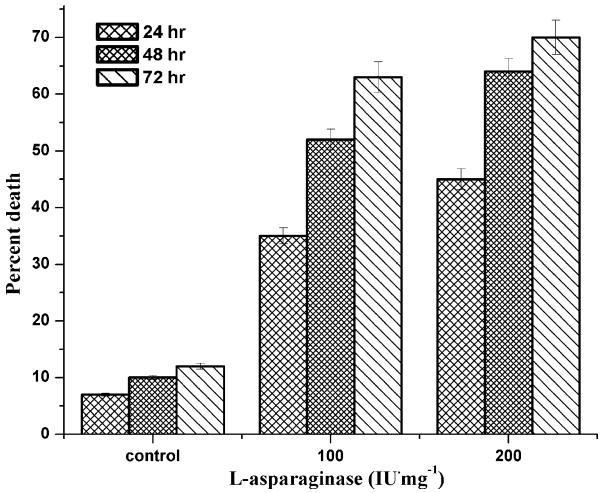

Fig. 5 Antineoplastic activity of extracellular L-asparaginase produced by *B. circulans*

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