

IN VITRO STUDY OF APOPTOSIS INDUCTION BY L-ASPARAGINASE OF *ASPERGILLUS FUMIGATUS* (ASPF-6) ON HUMAN LEUKEMIA CELL LINES

VIPINA R*, VIJAYA CHITRA A

Department of Microbiology, See Narayana Guru College, Coimbatore, Tamil Nadu, India. Email: vipinaprasad@gmail.com

Received: 24 January 2019, Revised and Accepted: 23 May 2019

ABSTRACT

Objective: L-Asparaginase is a relatively widespread enzyme used in the lymphoblastic leukemia chemotherapy. In the present investigation, we report new potential fungal L-asparaginase producer *Aspergillus fumigatus* from Kadalundi mangrove forest, Kerala. The aim of the present investigation was to evaluate the concentration-dependent cytotoxicity and apoptosis-inducing the effect of extracted enzyme and its ability to induce apoptosis against human leukemia cell lines-HL-60.

Methods: The fungal strain was isolated by serial dilution method from rhizosphere soil of mangrove forest. The isolated enzyme purified by 80% ammonium sulfate precipitation, dialysis, and ion exchange chromatography. To determine cytotoxicity and level of apoptosis, three different tests were performed: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, JC-1 flow cytometry, and Annexin V/propidium iodide (PI) flow cytometric DNA fragmentation. The test compound efficiency compared with positive drug doxorubicin against HL-60 cell lines.

Results: The test compound exhibited a concentration-dependent cytotoxic effect on proliferation of HL-60 cell lines (IC-50 12.39 U) with a different level of apoptosis induction (LR-11.3%).

Conclusion: *A. fumigatus* derived L-asparaginase may be clinically useful and results in better utilization for limited fungal-derived drug resources and improve financial feasibility as a therapeutic option for human leukemia.

Keywords: L-asparaginase, *A. fumigatus*, 3-(4,5-dimethyl thiazol-2-)-2,5-diphenyl tetrazolium bromide assay, Annexin V/PI and JC-1 Fluorocytometry.

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INTRODUCTION

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) is an enzyme present in many animal tissue, bacteria, and plants and serum of certain rodents, but not in mankind. L-Asparaginase hydrolyzes the amidase bond in L-asparagine to L-aspartic acid and ammonia. L-asparagine is required for both normal and cancer cell. Normal cell acquires L-asparagine through diet or synthesized using asparagine synthetase by protein synthesis. Tumor cell depends on the absorption of L-asparagine from the blood. When L-asparaginase was administered, as a therapeutic agent, its catalytic activity results in depletion of L-asparagine into ammonia and aspartic acid. The enzyme widely used in cancer therapeutics and has shown effective results for acute lymphoblastic, leukemia treatment of children [1].

L-asparaginase derived from fungi has been less studied than bacteria. Fungi have able to produce and secrete this enzyme [2]. Filamentous fungi belonging to the genera *Aspergillus*, *Penicillium*, *Fusarium*, and some yeast species can produce this enzyme [3]. Based on the reduction of 3-(4,5-dimethyl thiazol-2-)-2,5-diphenyl tetrazolium bromide (MTT) to a formazan product by living but not by dead cell has been used for large scale cytotoxic studies [4]. Anti-proliferative effect of recombinant L-asparaginase from thermotolerant *E-coli* strain was reported on leukemic cell line like and HL-60 [5,6].

In the present study, anticancer activity of L-asparaginase from *Aspergillus fumigatus* was observed on leukemia cell lines, HL-60. The mitochondrial potential of the treated cell was indicated using JC-1 flow cytometry and apoptosis by Annexin V/propidium iodide (PI) fluorocytometry modern techniques.

MATERIALS AND METHODS

Sample collection and isolation

The soil sample was collected from mangrove rhizosphere soil. The dilution plate method was employed for the isolation of fungal strains. The isolated microorganisms were maintained on Czapek-Dox medium (Glucose-2 gm, L-asparagine-10 gm, KH_2PO_4 -1.52 gm, KCl-0.52 gm, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -0.52 gm, $\text{CuNO}_3 \cdot 3\text{H}_2\text{O}$ -trace, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ -trace, and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ -trace/l) at pH 6.2 and 0.09% phenol red as an indicator to the media [7].

Screening of L-asparaginase producing fungi

The isolated fungi were subjected to rapid screening for L-asparaginase production by plate assay [8] in Czapek-Dox agar. Control plates were of modified Czapek-Dox medium containing NaNO_3 instead of L-asparagine as nitrogen source and phenol dye as an indicator. The isolates that showed pink-zone around the colonies after incubation at 30°C for 48 h were selected for determination of enzyme activity.

L-asparaginase production by submerged fermentation

The cultures were inoculated in Erlenmeyer flask containing 50 ml of modified Czapek-Dox medium. The flask incubated at 30°C at 150 rpm for 8 days. The enzymatic activity was measured at a subsequent interval. Uninoculated media serve as a control. The cultures were harvested by centrifugation at 1800 rpm for 15 min at a refrigerator condition (4°C), and the clear supernatant was used as the crude enzyme extract to determine the enzyme activity and protein assay.

L-asparaginase assay

The rate of hydrolysis of L-asparaginase was determined by measuring the released ammonia using Nessler's reagents [9]. The reaction was

started by adding 100 µl enzyme extracts into 200 µl of 0.05 M Tris HCl Buffer (pH 8.6) and 1.7 ml of 0.01 M asparagine, incubated for 10 min at 37°C. The reaction was stopped by addition of 500 µl of 1.5 M TCA. After centrifugation at 10,000 rpm at 4°C, 0.5 ml of the culture supernatant was diluted to 7 ml with distilled water and followed by addition of 1 ml of Nessler's reagent. Allowed 10 min for color reaction and OD was checked at 425 nm with a spectrophotometer. The ammonia liberated was entrapped in a curve derived with ammonium sulfate as a standard curve. 1U of L-asparaginase was defined as the amount of enzyme which liberates 1 µM of ammonia per minute under the assay condition at pH 8.6 at 37°C.

Purification of enzyme extract

The enzyme was purified using ammonium sulfate precipitation, dialysis and applied to ion-exchange chromatography through sulfopropyl-sepharose and carboxy methyl-sepharose. The purified extract was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein content was determined by Lowry's method using bovine serum albumin as the standard [10].

Antitumor activity of L-asparaginase on HL-60 cell line

Tumor cell lines

HL-60 (Human leukocytes) cell lines were collected from the National Center for Cell Science, Pune, India. L-asparaginase is an anticancer enzyme widely used against leukemia cancer cell. HL-60 cell line is floating and grows in suspension. The cells were maintained in RPMI-1640 [11] supplemented with 10% fetal bovine serum, streptomycin (1%), and penicillin (1%) which were added to inhibit bacterial contamination [12]. The cells were kept in a CO₂ incubator at 37°C.

Experimental design

The cells were dissociated by washing with PBS (×1). Extracellular matrix was removed using Trypsin and it is continued for 2-4 min in CO₂ incubator. After trypsinization cells were suspended in fresh serum to neutralize the trypsin [13]. The solution subjected to centrifugation at 25,000 rpm/4 min and the pellet was collected.

Cell counting

The cells are counted using hemocytometer. The cell suspension was mixed with dye trypan blue solution in the ratio of 1:1 [14,15].

Calculation

Total number of Viable cell = A × B × C × 10⁴

Total number of dead cells = A × B × D × 10⁴

Total cell count = Viable cell count + dead cell count

$$\% \text{ of viability} = \frac{\text{Viable cell count} \times 100}{\text{Total cell count}}$$

Where,

A = Volume of cells

B = Dilution factor in trypan blue

C = Mean number of unstained cells

D = Mean number of dead or stained cells

MTT assay

MTT assay is a colorimetric method for sensitive quantification of viable cells in proliferation and cytotoxicity assay [16,17]. It is based on reduction of the yellow colored water-soluble tetrazolium dye MTT to formazan crystals that are insoluble in cell culture media. The conversion brought about by mitochondrial lactate dehydrogenase produced by live cells reduces MTT to insoluble formazan crystals, which on dissolution into an appropriate solvent exhibits purple color, the intensity directly proportional to number of viable cells and measured spectrophotometrically at 570 nm [18,19].

Assay controls

- i. Medium control (medium without cells)
- ii. Negative control (medium with cells but without the experimental drug/compound)
- iii. Positive control (medium with cells and with 10 µM of CPT)

Same media were used in control as well as test wells [20,22].

Procedure for determining cell cytotoxicity

Two hundred microliters cell suspension at a required cell density of 20,000 cells per well was added in 96 well plates and incubated for 12 h. Test agent L-asparaginase was added at a concentration of 1, 10, 15, and 20 µg/ml, respectively. The plates were incubated at 37°C in CO₂ atmosphere for about 48 h. Spent media were trashed out, and MTT reagent was added to a final concentration of 0.05 mg/ml of total volume. The plate was wrapped with aluminum foil to avoid exposure to light. The plate was incubated for 3 h and MTT reagent was removed and to it 100 µl of solubilization solution was added. The plate was subjected to stirring in a gyratory shaker, and absorbance was read on a spectrophotometer at 570 nm [24,26].

Determination of IC₅₀ value

IC₅₀ was a quantitative measure which indicates how much of a particular drug or other substance needed to inhibit a given biological process by half. It is the half maximal (50%) inhibitory concentration of a substance [27,29]. The percentage of inhibition calculated using the formula.

$$\frac{\text{Mean OD of untreated cell (control)} - \text{Mean OD of treated cell}}{\text{Mean OD of treated cell}} \times 100$$

The graph can be plotted with the percentage of inhibition on Y-axis and the concentration of drug on X-axis and IC₅₀ concentration was determined.

Mitochondrial membrane potential assay by JC-1 flow cytometry method

The effect of L-asparaginase on mitochondrial membrane-potential in HL-60 was evaluated using JC-1 dye [30,31]. In normal mitochondria, JC-1 forms aggregate and emit red fluorescence. The dead population with low mitochondrial membrane potential dye remains in a monomeric form which emits green fluorescence. 96-well plate was seeded with the cell at a density of 3×10⁵ cells/ml and incubated in CO₂ incubator at 37°C for 24 h, keeping first well as blank, and second well as a positive control (doxorubicin). The cell treated with 20 µg/ml of L-asparaginase and incubated, harvesting and centrifuged at 300 ×g at 25°C washed with PBS [32,33]. JC-1 dye at a concentration of 0.5 ml was added and vortexed to disrupt cell to cell clumping. After incubation at 37°C in CO₂ incubator for 10–15 min, cells were washed 2 times with 1× assay buffer and centrifuged at 400 ×g for 5 min and discarded the supernatant. The cell pellet suspended in 1× assay buffer and analyzed by flow cytometry [34,35].

Detection of apoptosis

Early apoptotic events were detected using Annexin V/PI fluoro cytometric study [36,37]. The cells were cultured in a 6 well plate at a density of 3×10⁵ cells/1mL and incubated in a CO₂ incubator overnight at 30°C for 24 h. The cells were then treated with experimental compound L-asparaginase and positive control doxorubicin, in 1 mL RPMI medium and again incubated for 24 h. The cells were transferred to 12 × 75 mm polystyrene tubes and centrifuged at 300 ×g at 25°C for 5 min, and the pellet was collected. The cells were then washed twice with PBS and treated with 5 µl of FITC Annexin V [38]. The suspension was vortexed and incubated for 15 min at room temperature in the dark. Five microliters of PI and 400 µl of 1 × binding buffer was added to each tube and gently vortexed buffer to each tube and analyzed immediately by flow cytometry after addition of PI.

RESULTS

Isolation and screening of microorganisms

A total of 18 fungal cultures isolated from rhizosphere mangrove rhizosphere soil. The potential strain was selected on the basis of the pink zone around the colony by plate assay method. The strain that showed complete color change was selected for secondary screening. The maximum enzyme activity of 32.8 µ/ml was observed in secondary screening.

Purification of enzyme extract

The enzyme was purified 2.6-fold with a recovery of 5.6% and a specific activity of 23.96 U/mg of protein. The concentrated-active fraction was further purified by CM-Sepharose column chromatography. After the final purification, the enzyme was purified three-fold with a recovery of 2.7% and specific activity of 27.25 U/mg of protein. The purified L-asparaginase appeared as a single protein band in SDS-PAGE and molecular weight of 56 KDa.

In vitro cytotoxic activity

The results of the *in vitro* cytotoxicity using MTT assay are depicted in Fig. 1. The decrease in cell count was observed with increase in the concentration of the enzyme extracts. *In vitro* exposures of HL-60 cells with various concentration of L-asparaginase significantly suppressed HL-60 cancer cell growth in a dose-dependent manner. The maximum inhibition of HL-60 due to exposure to L-asparaginase was found at 20 µg/ml of the enzyme extracts and 73.66% inhibition in viability [Fig. 2]. IC₅₀ graph plotted in Fig. 3.

Mitochondrial membrane potential study of the compound L-asparaginase against HL-60 cell line

JC-1 expression study of the test compound (L-asparaginase) against HL-60 cell line is represented in Table 1 and Fig. 4. Given test compound (L-asparaginase) showed significant reduction of mitochondrial membrane integrity against the HL-60 by showing the shift of cells from upper right (UR) quadrant-red (FL 2) to lower right (LR) quadrant-Green (FL 1) represented in Fig. 5.

Apoptosis study of L-asparaginase treated HL-60 cell lines

In the apoptotic cell, loss of plasma membrane asymmetry is one of the earliest features of apoptosis. In the apoptotic cell, the membrane phospholipid phosphatidylserine (PS) is translocate from the inner membrane to outer and exposed to the outer cellular environment. 36-36 kDa Annexin V is a Ca²⁺ dependent phospholipid-binding protein with

high affinity for phospholipid PS and binds to exposed PS on the apoptotic cell surface. Annexin V can be conjugated to fluorochrome which retains its high affinity for PS and serves as a sensitive probe for flow-cytometric analysis of cells undergoing apoptosis. In untreated samples, the majority of cells were viable (96.2%) and nonapoptotic (Annexin V-PI). However, when cells were treated with positive drug doxorubicin with 15 µM [Table 2] for 4 h, 27.98 % of Annexin V-PI cells were observed. Test compound L-asparaginase exhibited 50.96 of Annexin V-PI. There was an increase in early apoptotic cell populations (AnnexinV⁺PI⁻) from untreated to treated cells such as 73% to 9.83% in doxorubicin positive drug and 11.3% in test compound L-asparaginase [Table 3].

HL-60 cell lines treated with standard drug Doxorubicin and test compound Lasparaginase is represented in Fig. 6. Fig. 6 shows proof-of-principle data from the HL-60 untreated, treated with the standard drug, and test

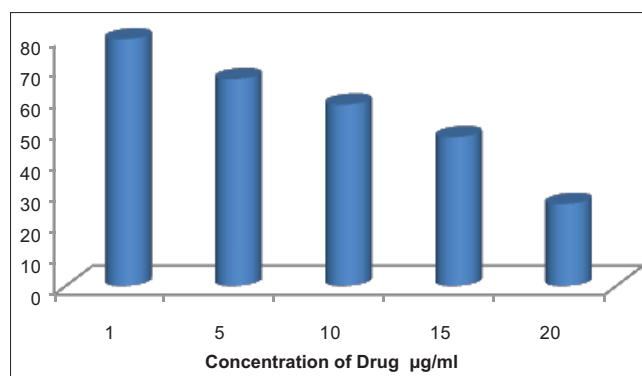


Fig. 1: Percentage of viability

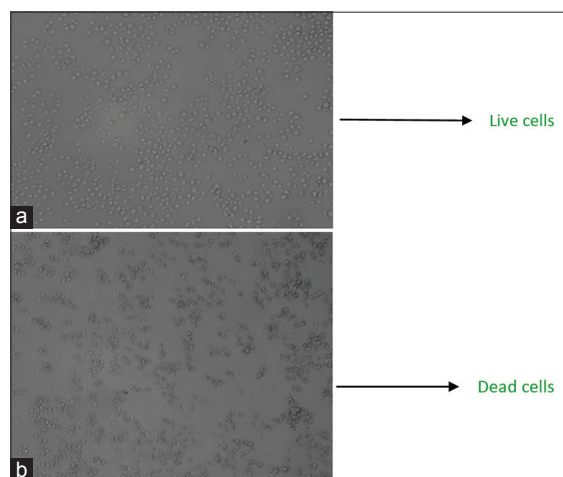


Fig. 2: HL-60 cell images after treatment. (a) Control. (b) L-asparaginase at 20 µg/ml

Table 1: The percentage of cells of arrested in different stages of apoptosis in the untreated, standard, and test compound, namely L-asparaginase treated HL 60 cells and overlay of the results plotted in bar graph

Quadrant	UR	LR
Label	FL1-JC1	FL2-JC1
Untreated HL 60 cells	99.96	0
STD	33.13	66.85
Test compound-1 (L-asparaginase)	52.04	47.82

UR: Upper right, LR: Lower right, STD: Standard and test compound, FL1: Fluorescence channel1, HL: Human acute promyelocytic leukemia cell line, JC-1: 5,5',6,6' tetrachloro-1,1',3,3'-tetra ethylbenzimidazol carbocyanineiodide

Table 2: Details of drug treatment

S.No	Test compounds	Cell line	Concentration treated to cells
1	Untreated	HL 60	No treatment
2	STD (doxorubicin)	HL 60	15 µg/ml
3	Test-1 (L-asparaginase)	HL 60	12.39 unit

STD: Standard and test compound, HL: Human acute promyelocytic leukemia cell line

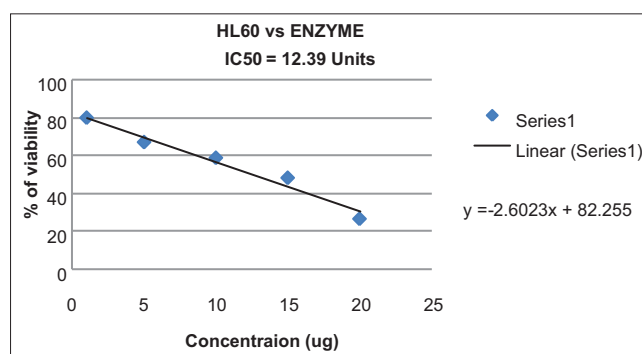


Fig.3: IC₅₀ graph

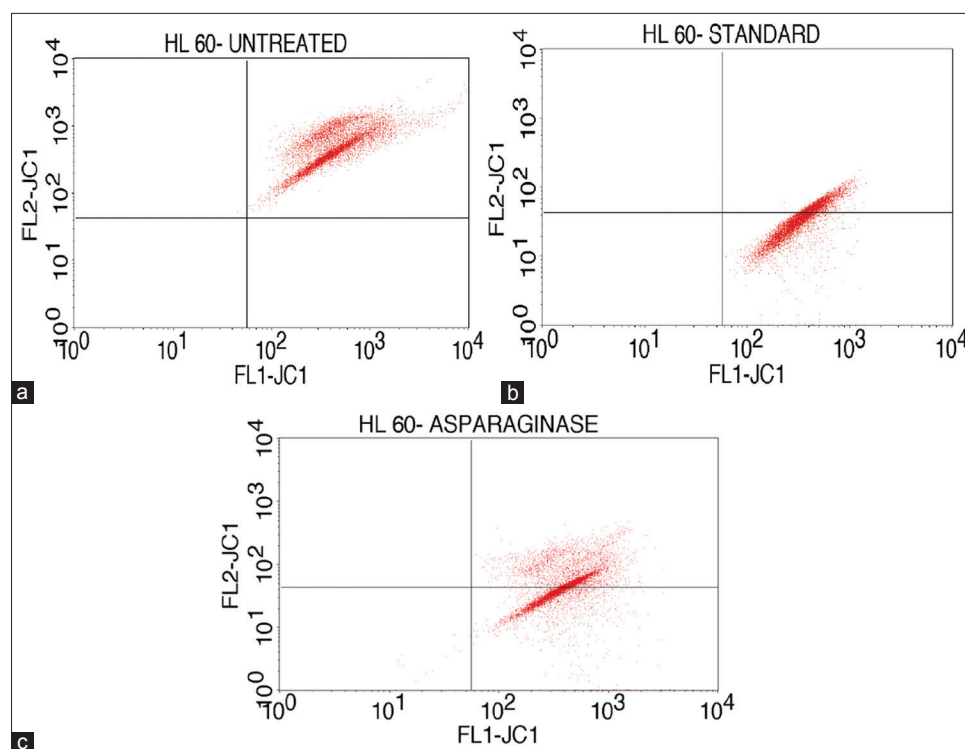


Fig. 4: Mitochondrial membrane potential study of the test compound-1 (L-asparaginase) against HL 60 cell line: JC1 expression study of test compound (L-asparaginase) on HL 60 cells using BD FACS caliber, Cell Quest Pro Software (Version: 6.0). Quadrants showing the expression of JC1 stain in the given untreated HL 60 Cells (a), standard drug (Doxorubicin) (b) and L-asparaginase treated HL 60 cells (c)

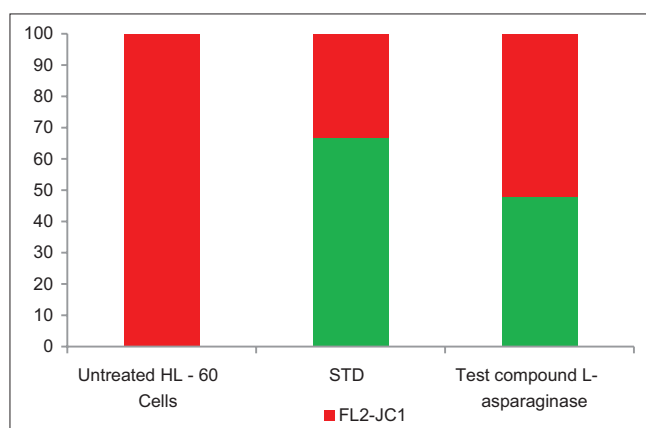


Fig. 5: The percentage of healthy and unhealthy cell s after treatment

compound Lasparaginase as Annexin V FITC versus PI counter plots with quadrant gates showing four populations. In the untreated control sample, the majority of (96.2%) cells were viable and non-apoptotic, and standard drug-treated cell line (Fig. 7a,b) and test compound L-asparaginase treated cells Fig. 7c shows decrease in the Annexin V-PI-populations and increase in cells undergoing early apoptotic (Annexin V⁺ PI⁺)

Lower left quadrant population (LL) represents the viable cells. About 96.20%, 27.98%, and 50.96% of cells were found in untreated, standard, and test compound namely L-asparaginase, respectively, in the LL quadrant. Upper left (UL) quadrant represents cells debris or necrotic cells. About 2.24%, 0.43%, and 5.75% of cells were found in untreated, standard, and test compound L-asparaginase, respectively, in the UL quadrant. UR quadrant represents late apoptotic cells. About 0.82%, 61.75%, and 31.99% of cells were found in untreated, standard, and test

UL	UR
Percentage of necrotic cells	Percentage late apoptotic cells
LL	LR
Percentage viable cells	Percentage of early apoptotic cells
UR: Upper right, LR: Lower right, UL: Upper left, LL: Lower left	

Fig. 6: Meaning of the quadrant layout in apoptosis study

compound, namely L-asparaginase, respectively, in the UR quadrant. LR quadrant represents early apoptotic cells. About 0.74%, 9.84%, and 11.30% of cells were found in untreated, standard, and test compound, namely L-asparaginase, respectively, in the LR quadrant (Fig.8).

DISCUSSION

The first reviews on L-asparaginase demonstrate anti-leukemic activity, but progress has been made in the therapeutic practical that combined L-asparaginase with chemotherapeutic drug [12]. The current investigation is the first report in the isolation of L-asparaginase from *A. fumigatus* species. Antiproliferative effect of L-asparaginase from *A. fumigatus* was evaluated after 48 h of incubation on HL-60 cell lines. The L-asparaginase caused 74% reduction in cell viability after 48 h of treatment. Efficacy of the antiproliferative effect of L-asparaginase from *Streptomyces enissocaesilis* on different cell lines at a concentration of 5.0 IU/mL showed cell inhibition such as 82.3% on MCF-7, 71.7% on MDA-MB4355 (Breast metastatic cancer), 64.2% and 60% in HeLa cell lines and Human small lung cancer cell lines H69PR, and in 52.1% in COLO205 cell lines [21]. *L-asparaginase* from *Enterobacter clocae* against human myeloid Leukemia cell line, MDA-MB-2311 at the concentration of 2,5,10 and 15 IU/ml [23], showed IC50 value 3.1, 7.1 and 11.8 IU ml⁻¹. L-asparaginase from *Salinococcus* spp. showed cytotoxicity against HL-60 cell lines [25,28]. In this study, we attempted to understand the effect of

Table 3: The percentage of cells of arrested in different stages of apoptosis in the untreated, standard, and test compound L-asparaginase

Quadrant	Percentage of necrotic cells	Percentage of late apoptotic cells	Percentage of viable cells	Percentage of early apoptotic cells
Label	UL	UR	LL	LR
Untreated cells	2.24	0.82	96.2	0.74
STD (doxorubicin)	0.43	61.75	27.98	9.84
Test compound	5.75	31.99	50.96	11.3
L-asparaginase				

STD: Standard and test compound, UR: Upper right, LR: Lower right, UL: Upper left, LL: Lower left

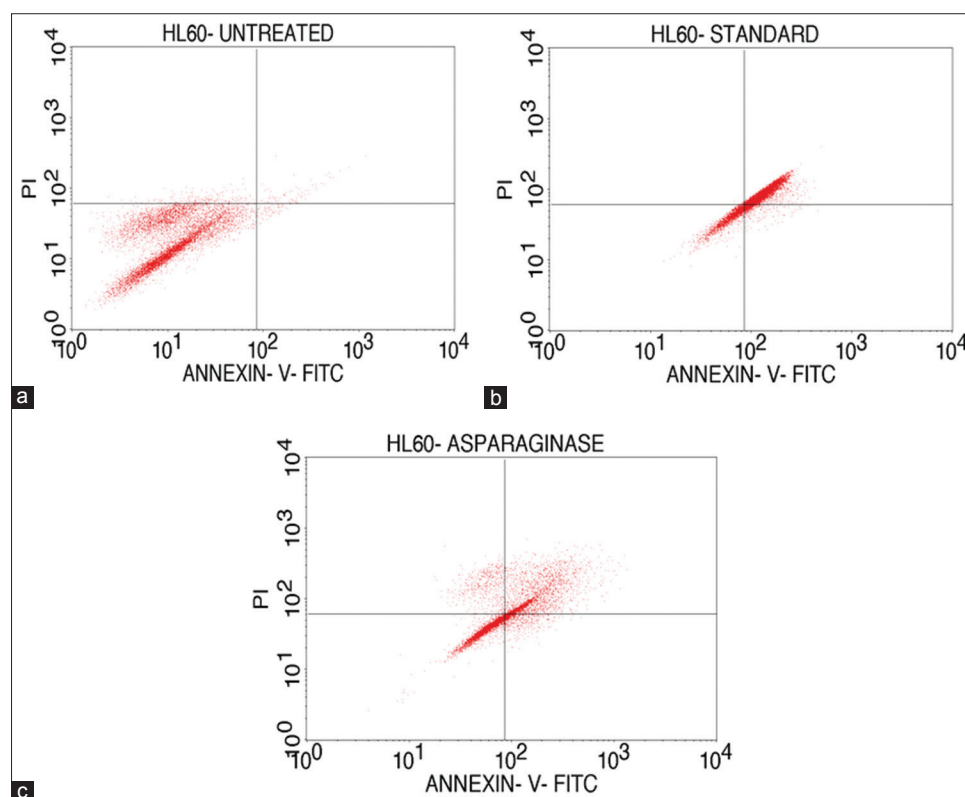


Fig. 7: Annexin V-PI expression study of test compound (Asparaginase) on HL 60 cells using BD FACSCalibur, Cell Quest Pro Software (Version: 6.0). Quadrants showing the expression of given untreated HL 60 Cells (a) and standard drug (Doxorubicin-15uM) treated HL 60 cells (b) and test compound-1 (L-asparaginase) treated HL 60 cells against the Annexin V-FITC and propidium iodide stain

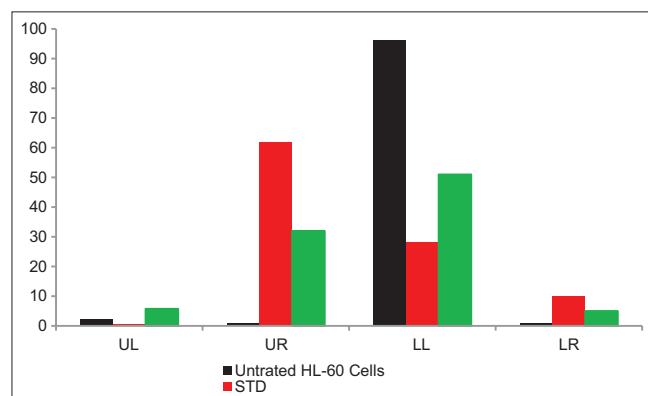


Fig. 8: The percentage of apoptotic cells gated in the different samples

L-asparaginase alone on mitochondrial membrane permeability in HL-60 cells, and there is an increased loss of mitochondrial membrane potential and likely of inducing apoptosis in HL-60 cell lines, after 48 h of treatment.

CONCLUSION

The treatment of cancer is largely based on the use of the chemotherapeutic drug that affects the viability and growth rate of tumor cells. Extraction and purification of natural product L-asparaginase from fungal species have attracted much attention, due to the cost-effective and eco-friendly in nature. Although there are many therapeutic bacterial derived L-asparaginase present in the market, recent studied pointed out that asparaginase from fungal species might be more efficient and also exhibited fewer side effects. Our study demonstrates that L-asparaginase isolated from *A. fumigatus* ASPF-6 has enhanced anti-proliferative and exhibited a higher degree of early apoptosis, compare to necrosis or late apoptosis on the human leukemia cell line, HL-60. The needs of new sources for therapeutic enzyme have much attention in both medicine and biotechnology.

ACKNOWLEDGMENTS

We would like to thank Unibiosis Research Lab Pvt., Ltd., Kochi, and NCCAE Centre in Bharathidasan University for providing the lab facility and support to conduct the present study.

AUTHORS' CONTRIBUTIONS

Vipina and Vijaya Chitra designed and performed the experiment, derived the models, and analyzed the data. Vipina performed the experiment under the guidance and supervision of Vijaya Chitra. Vipina drafted the manuscript and designed the figures. Vijaya Chitra aided in interpreting the results and worked on the manuscript. Two authors discussed the results and commented on the manuscript.

CONFLICTS OF INTEREST STATEMENT

Authors declared that there is no competing interest between them.

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