# Evaluation of cytotoxic potential of L-asparaginase from *Scopulariopsis brevicaulis* on cell lines *in vitro*

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## ABSTRACT

**Introduction and Aim:** This study reports the cytotoxic potential of L-Asparaginase isolated from the fungus *Scopulariopsis brevicaulis*.

**Materials and Methods:** Extracellular L- Asparaginase was isolated from *Scopulariopsis brevicaulis* and purified by ammonium sulfate precipitation, followed by dialysis, ion exchange and gel filtration chromatography. Varying concentrations (31.25, 62.5, 125, 250, 500 µg/ml) of purified L-Asparaginase was tested on MCF7, HeLa, HepG2 and 3T3L1cell lines by MTT assay. Curcumin was maintained as a positive control.

**Results:** Minimum inhibition of 23.57% was observed at an enzyme concentration of 31.25 µg/ml and maximum inhibition (66.41%) was observed at 500 µg/ml against MCF7 cell line. Minimum inhibition of 2.87% was observed at an enzyme concentration 31.25 µg/ml and maximum inhibition (58.49%) was observed at 500 µg/ml against HeLa cell line. Minimum inhibition of 4.58% was shown at an enzyme concentration of 31.25 µg/ml and maximum inhibition (46.14 %) was observed at 500 µg/ml against HepG2 cell line. Minimum inhibition of 1.4% was shown by enzyme concentration 31.25 µg/ml and maximum inhibition (50.9%) was observed at 500 µg/ml against 3T3L1 cell line.

**Conclusion:** We report for the first time the cytotoxic potential of L-Asparaginase from *Scopulariopsis brevicaulis*.

**Keywords:** L-Asparaginase; cytotoxicity; MCF-7; HeLa; HepG2; 3T3L1 cell lines; MTT; *Scopulariopsis brevicaulis.*

## INTRODUCTION

L- Asparaginase (L-asparagine amidohydrolase EC 3.5.1.1) is a hydrolase that plays a major role in the metabolism of all living organisms specifically catalyzing the hydrolysis of L-asparagine to L-aspartic acid and ammonia (1). The reaction is irreversible when maintained at physiological conditions. The enzyme is found widespread in the nature and has been isolated from various sources such as plant tissues, bacteria, fungi, yeasts, actinomycetes, algae and the serum of rodents (2, 3).

L-Asparaginases has been isolated and purified from several different bacteria such as *E. coli* (3), *Serratia marcescens* (4), Vibrio *succinogens* (5), *Pseudomonas acidovorans* (6), *Pseudomonas geniculata* (7), *Corynebacterium glutamicum* (8) and Staphylococcus *sps.* (9). Fungal sources of the enzyme include *Alternaria sps.* (10), *Aspergillus nidulans* (11), *A. niger* (12), *A. tamarii* (13),

*Fusarium roseum* (14). Among plant species, L - asparaginase enzyme has been reported in *Pisum sativum* (15) and *Withania somnifera* (16). Among mammals L-Asparaginase was detected in guinea pigs serum (17) and agouti (2).

The enzyme is proven to have cytotoxic activity and is being used in the treatment of acute lymphoblastic leukemia (18). This is due to the fact that the leukemic cells do not have the property to produce L-asparagine, which is a non-essential amino acid, whereas the normal cells can produce their own (19). Therefore, the leukemic cells are deprived from L-asparagine which is their source of nutrition and they are prevented from malignant growth.

L-Asparaginase from bacterial origin can cause hypersensitivity in the long term leading to allergic reactions in the tissues of patients, resulting in anaphylactic shock (20). Therefore, the search for a new serologically different L-asparaginase with similar therapeutic role and less adverse effects is highly recommended. The study on purification of L-Asparaginase from *Scopulariopsis brevicaulis* has not been reported so far. This prompted to study further on cytotoxic property of the enzyme with reference to therapeutic purposes. Hence, attempt has been made to purify the L-Asparaginase from strain *Scopulariopsis brevicaulis* and study its anti-neoplastic effect.

## MATERIALS AND METHODS

The screening, isolation, identification and preparation of crude enzyme extract of *Scopulariopsis brevicaulis* was carried out as mentioned in the earlier publication (21). Modified Czapek –Dox media was employed to culture the organisms by submerged fermentation. The organism produced maximum enzyme on the $10^{th}$ day. Optimum temperature for the growth of the organism was found to be 37°C and pH was found to be 7.2.

### Purification of L-Asparaginase

The purification of the crude extract was carried out at 4°C according to the method (6). Finely powdered ammonium sulfate was added to 80% saturation. The mixture was left overnight for 12hrs at 4°C, followed by centrifugation at 8000 rpm for 20min at 4°C. The precipitate was dissolved in 0.05M Tris HCl buffer pH 7.2 and dialyzed overnight against the same buffer at 4°C. The dialyzed sample was further purified by passing through a column of DEAE cellulose previously equilibrated with 0.05M Tris HCl buffer, pH 7.2. A total of 55 fractions were collected at the flow rate of 3 mL per 15min. Fractions showing high activity were pooled together and dialysed against the same buffer. The dialysed sample was lyophilized and concentrated. 1ml of the lyophilized sample was loaded on to the Sephacryl S-200 gel filtration column and was eluted by using the Tris HCl buffer pH 7.2. A total of 45 fractions were collected at the rate of 3mL/15min. Fractions showing high activity were pooled together dialyzed, lyophilized and used for further studies. Total and specific enzyme acitivity was determined using the method briefly described (22).

### *In vitro* cytotoxicity study

Human Cervical Cancer cell line (HeLa), Human Breast Cancer Cell line (MCF7), Human Liver Cancer Cell line (HePG2) and Mouse Embryo Fibroblast Cell lines (3T3L1) were obtained from NCCS, Pune. The cells were maintained in DMEM medium supplemented with 10% FBS and Penicillin (100U/ml) in a humidified atmosphere of 50µg/ml $CO_2$ at 37°C. The cytotoxicity of the sample on the cell lines was determined by MTT assay (23). 200µl of cell suspension was seeded in a 96 well plates at required cell density (20,000 cells per well), without the test agent. The cells were allowed to grow for about 24 hours. The various concentrations of the sample (31.25, 62.5, 125, 250,500 µg/mL) were added. The plates were incubated at 37°C for 48hrs in a 5% $CO_2$ atmosphere.

After the incubation period, the spent media was removed and MTT reagent was added to a final concentration of 0.5 mg/mL of total volume. The plates were returned to the incubator and incubated for 3 hours. The MTT was removed and 100 µl of solubilisation solution (DMSO) was added. The absorbance was read on an ELISA reader (ELX800, Bioteck) at 570nm and 630 nm used as reference wavelength. The $IC_{50}$ value was determined graphically. All experiments were performed in triplicates.

**Assessment of Cell Morphology**

The cytotoxicity induced by purified asparaginase was confirmed microscopy. The cell lines were treated with different concentrations of the purified enzyme (31.25, 62.5, 125, 250, 500 µg/mL), after 24 h of treatment cells were subjected to investigation for morphological changes (24, 25).

## RESULTS

**Purification of *Scopulariopsis brevicaulis* L-Asparaginase**

The purification profile of L-Asparaginase from *Scopulariopsis brevicaulis* is represented in (Table 1). It was purified to 107.42-fold, with a specific activity of 116.02 (IU/mL/mg) and a yield of 11.03.

**Table 1: Purity profile of *L- Asparaginase***

***In vitro* cytotoxicity studies**

| Steps | Total activity (IU/mL) | Total protein Conc. (mg) | Specific activity (IU/mL/mg) | Fold purification | Yield |
|---|---|---|---|---|---|
| Crude enzyme | 74,900 | 68,720 | 1.08 | 1 | 100 |
| Ammonium sulphate (80%) Precipitation | 62,414 | 18,945 | 3.2944 | 3.05 | 83.32 |
| Dialysis | 38762 | 4623 | 8.38 | 8.18 | 51.75 |
| DEAE cellulose chromatography | 9482.94 | 196.91 | 48.1580 | 44.5 | 12.66 |
| Sephacryl S-200 gel filtration chromatography | 8268.76 | 71.26 | 116.02 | 107.42 | 11.03 |

The purified enzyme was tested for cytotoxicity on MCF-7, HeLa, HepG2 and 3T3L1 cell lines (Table2)

**Table 2: $IC_{50}$ value against various cell lines**

| Sl. No. | Cell lines | $IC_{50}$ value (µg/ml) L-Asparaginase | Curcumin concentration (5µM) |
|---|---|---|---|
| 1 | MCF-7 | 310.12 | 51.87 |
| 2 | HeLa | 377.66 | 45.58 |
| 3 | HePG2 | >500 | 35.06 |
| 4 | 3T3L1 | 454.47 | 60.7 |

**Assessment of cell morphology**

Results showed that morphological changes were increased with increasing dose of L-Asparaginase. Symptoms of apoptosis such as cellular rounding

up, cell shrinkage, membrane blebbing and loss of cell adhesion were observed **(**Fig 1: b, d f, h). The amount of blebbing and shrinkage of the cells were found to increase dramatically at higher concentrations of enzyme treatment. However, morphological changes were not observed with untreated cells (Fig 1: a, c, e, g). These results further suggest that purified asparaginase induced potential apoptotic effect in dose dependent manner.

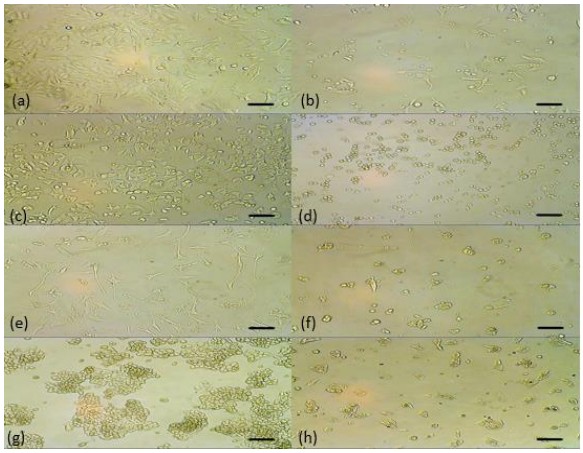


**Fig. 1** Effect of L- Asparaginase on cancer and normal cell lines (a) untreated HeLa cell lines (b) treated HeLa cell lines (c) untreated MCF cell lines (d) treated MCF cell lines (e) untreated 3T3L1 cell lines (f) treated 3T3L1 cell lines (g) untreated HepG2 cell lines (f) treated HepG2 cell lines. Scale bar = 1mm

## DISCUSSION

L-Asparaginases are effective against acute lymphocytic leukemia, acute myeloid leukemia and chronic myeloid leukemia (1). Tumor cells synthesize L-asparagine slowly and are dependent on an exogenous supply. L-Asparaginase destroys extracellular source of L-asparagine and inhibits protein synthesis in lymphoblasts resulting in apoptosis. Normal cells on the other hand can synthesize L-asparagine by Asparagine synthetase and therefore tend to resist L-Asparaginase.

The anti-proliferative effects of L-asparaginase from *A. terreus* (PC-1.7 A) was evaluated after 24, 48, 72 and 96 h of incubation on two leukemia cell lines (RS4;11 and HL-60) and PBMC. There was no effect on proliferation of PBMC, whereas 50% reduction in cell viability was observed after 72 hours on the cell line HL-60 and after 96 hours on the cell line RS4; 11 (26). The cytotoxic effect of L-Asparaginase from *Aspergillus flavus* (KFF20) was studied on MCF – 7 cells and the $IC_{50}$ value were found to be 120.875 µg/ml (27). The incubation of Hep-G2 with gradual doses of *Penicillium brevicompactum* NRC 829 L-asparaginase lead to a gradual inhibition in the cell growth with a low $IC_{50}$ values of 76.4 µg/ml (28).

The purified L-Asparaginase induces apoptosis in human cancer cell lines (HL-60, MOLT-4, MDA-MB-231 and T47D.) Morphological changes during apoptosis include membrane blebbing, cell shrinkage, chromatin condensation, formation of apoptotic and scattered apoptotic bodies (29). Morphological analysis of our cell lines after L-Asparaginase treatment revealed that the cell population had reduced significantly in number. Cells undergoing apoptosis were characterized by cellular rounding up, shrinkage, membrane blebbing and loss of cell adhesion.

In our studies we observed that the four cell lines showed varied levels of inhibition as well as resistance to L-Asparaginase enzyme. MCF-7 cell lines showed an $IC_{50}$ value of 310.12(µg/ml), HeLa cell lines 377.66(µg/ml), whereas the HepG2 cell lines were completely resistant. 3T3L1 cell lines which are normal cells also showed

resistance to L-Asparaginase. Probably the differences might be due to the differences in the level of purity of the enzymes used in the study. Further purification of *Scopulariopsis brevicaulis* L-Asparaginase might prove to be useful. It is not clear why in our study the cell lines appeared to be comparatively resistant to *Scopulariopsis brevicaulis* L-Asparaginase compared to other fungal Asparaginases. This is the first report on the selective cytotoxicity of L-Asparaginase from *Scopulariopsis brevicaulis.*

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## REFERENCES

1. Lincoln, L., and More, S. S. Isolation and production of clinical and food grade L-Asparaginase enzyme from fungi. Journal of Pharmacognosy and Phytochemistry. 2014; 3(3): 177-183.
2. Broome, J. D. Evidence that the L-asparaginase of guinea pig serum is responsible for its antilymphoma effects. J Exp Med, 1963; 118 (1): 99-120.
3. Batool, T. E., Makky, A., Jalal, M., and Yusoff, M.M. A Comprehensive Review on l-Asparaginase and Its Applications. Applied Biochemistry and Biotechnology. 2016; 178 (5): 900-923.
4. Whelan, H. A., and Wriston, J. C. Purification and properties of l-asparaginase from Serratia marcescens. Biochimica et Biophysica Acta (BBA) - Protein Structure. 1974; 365 (1): 212-222.
5. Davidson, L., Brear, D. R., Wingard, P., Hawkins, J., and Kitto, G. B. Purification and Properties of an L-Glutaminase-L- Asparaginase from Pseudomonas acidovorans. J. Bacteriol.1977; 129:1379-1386.
6. Distasio, J. A., Niederman, R. A., Kafkewitz, D., and Goodman, D. Purification and characterization of L-asparaginase with anti-lymphoma activity from *Vibrio succinogenes*. J. Biol. Chem. 1976; 251(22): 6929-6933.
7. Kitto, G. B., Smith, G., Thiet, T. Q., Mason, M., and Davidson, L. Tumor Inhibitory and Non-Tumor Inhibitory L-Asparaginases from Pseudomonas geniculata. Journal of Bacteriology. 1979; 137(1): 204 -212.
8. Mesas, J. M., Gil, J. A., and Martin, J. F. Characterization and Partial Purification of L-Asparaginase from Corynebacterium Glutamicum. Journal of General Microbiology. 1990; 136(3): 515-519.
9. Mikucki, J., Szarapińska-Kwaszewska, J., and Krzemiński, Z. Factors Influencing L-Asparaginase Production by Staphylococci. Central Journal of Bacteriology, Parasite Science, Infectious Diseases and Hygiene. Second Natural Science Department: General, Agricultural and Technical Microbiology. 1977; 132 (2): 135-142.
10. Nagarajan, A., Thirunavukkarasu, N., Suryanarayan, T. S., and Gummadi, S. N. Screening and Isolation of Novel Glutaminase Free L-asparaginase from Fungal Endophytes. Research Journal of Microbiology. 2014; 9(4):163-176.
11. Saxena, R. K., and Sinha, U. L-asparaginase and glutaminase activities in the culture filtrates of *Aspergillus nidulans.* Current Science. 1981; 50(5): 218-219.
12. Mishra, A. Production of L-Asparaginase, an Anticancer Agent, From *Aspergillus niger* Using Agricultural Waste in Solid State Fermentation. Applied Biochemistry and Biotechnology. 2006; 135(1): 33-42.
13. Sarquis, de M. M. I., Morais Oliveira, E. M., Silva Santos, A., and Lara da Costa, G. Production of L-asparaginase by filamentous fungi. Memórias do Instituto Oswaldo Cruz. 2004; 99(5): 489-492.
14. Nakahama, K., Imada, A., Igarasi, S., and Tubaki, K. Formation of L-Asparaginase by Fusarium Species. Journal of General Microbiology. 1973; 75(2): 269-273.
15. Sleciechowicz, K. A., and Joy, K. W. The effect of plant age on asparaginase activity and amino acid levels in developing leaves of *Pisum sativum.* Canadian Journal of Botany. 1989; 67(3): 732-736.
16. Oza, V. P., Trivedi, S. D., Parmar, P. P., and Subramanian, R. B. *Withania somnifera* (Ashwagandha): a Novel Source of L-asparaginase. Journal of Integrative Plant Biology. 2009; 51(2): 201-206.
17. Cooney, D. A., and Hands chumacher, R. E. L-Asparaginase and L-Asparagine Metabolism. Annual Review of Pharmacology. 1970; 10(1): 421-440.
18. Jain, R., Zaidi, K. U., Verma, Y., and Saxena, P. L-Asparaginase: A Promising Enzyme for Treatment of Acute Lymphoblastic Leukiemia. People's Journal of Scientific Research. 2012; 5(1):29-35.
19. Verma, N., Kumar, K., Kaur, G., and Anand, S. L-Asparaginase: A Promising Chemotherapeutic

Agent. Critical Reviews in Biotechnology. 2007; 27(1): 45-62.
20. Elshafei, A. M., and El-Ghonemy, D. H. Screening and Media Optimization for Enhancing L-asparaginase Production, an Anticancer Agent, from Different Filamentous Fungi in Solid State Fermentation. British Biotechnology Journal. 2015; 9(3):1–15.
21. D'Souza, R. M., Pinto, S. K., and Abraham, A. Identification of a Novel Strain of Extracellular L-Asparaginase Producing *Scopulariopsis brevicaulis* (Sacc.) Bainier using Molecular Techniques," International Journal of Scientific Research in Computer Science Applications and Management Studies. 2018; 7(6): 1-5.
22. Ghose, T. K. Measurement of Cellulase Activities. International union of Pure and Applied Chemistry. 1987; 59 (2): 257-268.
23. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods. 1983; 65(2): 55-63.
24. Bhat, M., and Marar, T. Cytotoxic Effect of Purified L-asparaginase from Salinicoccus sp. M KJ997975, International Journal of Current Microbiology and Applied Sciences. 2015; 4(4): 701–712.
25. Aljewari, H. S., Nader, M. I., Alfaisal, A. H. M., Natthida, W., and Sahapat. High Efficiency, Selectivity against Cancer Cell Line of Purified L-Asparaginase from Pathogenic *Escherichia coli.* International Journal of Medical, Health, Biomedical, Bioengineering and Pharmaceutical Engineering. 2010; 4(5): 416-421.
26. Battiston Loureiro, C., Silva Borges, K., Faria Andrade, A., Gonzaga Tone, L., and Said, S. Purification and Biochemical Characterization of Native and Pegylated Form of L-Asparaginase from Aspergillus terreus and Evaluation of Its Antiproliferative Activity. Advances in Microbiology. 2012; 2(2): 138-145.
27. Rani, S. A., Sundaram, L., and Vasantha, P. B. *In vitro* Antioxidant and Anticancer Activity of L-Asparginase from *Aspergillus flavus* (KUFS20). Asian Journal of Pharmaceutical and Clinical Research. 2011; 4(2): 174-177.
28. Elshafei, A., Hassan, M. M., Abd-Elmontasr Abouzeid., M. A., Mahmoud., D. A., and Elghonemy, D. H., Purification, Characterization and Antitumor Activity of L-asparaginase from *Penicillium brevicompactum* NRC 829. British Microbiology Research Journal. 2012; 2(3): 158-174.
29. Husain, I., Sharma, A., Kumar, S., and Malik, F. Purification and Characterization of Glutaminase Free Asparaginase from Enterobacter cloacae: *In-Vitro* Evaluation of Cytotoxic Potential against Human Myeloid Leukemia HL-60 Cells. PLoS One. 2016; 11(2): 1-27.