ORIGINAL ARTICLE

# Cytotoxic activity of L-asparaginase isolated from endophytic aspergillus nomius of *Justicia adhatoda* on A549 cell lines

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## Abstract

**Introduction:** L-Asparaginase belonging to a group of amidase possesses a broad spectrum of antitumor activity. It catalyzes the hydrolysis of L-asparagine to ammonia and L-aspartic acid. **Objective:** In the present study, L-asparaginase producing endophytic fungi were isolated from *Justicia adhatoda* and cytotoxicity studied on A549 cell line. **Methodology:** The screening modified Czapek-Dox media contained asparagine as the source and phenol red as the indicator. The positive zones were identified and sub cultured to obtain pure culture. **Results:** A quantitative assay of asparaginase was carried out and the level of production was found to be 1.8916 (μmol/ml). The molecular weight of the enzyme was 66 kDa. MTT assay and dual staining with acridine orange/ethidium bromide were done to assess the type of cell death induced by the enzyme in A549 cells. The percentage of apoptotic cells after treatment showed a drastic increase in apoptotic cells ($P < 0.001$) to 62% and 84%, respectively. Flow cytometry analysis was performed to evaluate the cell cycle arrest phase induced by different concentration of partially purified sample (80 μg/ml and 160 μg/ml). Lower concentration (80 μg/ml) showed arrest at S phase with 19% cells accumulated. At higher concentration (160 μg/ml), it showed an increased cell population at S phase with 30% with alterations in the other phase of cell cycle. **Conclusion:** The above observations show that the isolated asparaginase exhibited a marked cytotoxic activity against A549 cell lines. Further animal model studies, toxicity assays and pharmacological studies of this asparaginase from the endophyte would help to authenticate the use of this enzyme as an anticancer drug.

**Key words:** A549 cell lines, asparaginase, *Aspergillus nomius*, endophytic fungi, *Justicia adhatoda*

## INTRODUCTION

L-asparaginase (E.C.3.5.1.1 L-asparagine amido hydrolase) from fungal source is a potential therapeutic agent for treating acute lymphoblastic leukemia, an hematologic disorder occurring nearly around 25% among teenagers and children's.[1,2] The enzyme converts L-asparagine to L-aspartate and ammonia. Its anti-leukemic effect results in decreasing L-asparagine that circulates in the blood, which are required for the malignant lymphoblastic cells.[3] They are clinically approved anticancer agent since the L- asparagine in the blood serum is removed by L-asparaginase, depriving the tumor growth.[4] At present, L- asparaginase isolated from bacterial sources are used for therapeutic purpose. Conversely, these preparations are associated with side effects leading to the search of alternative eukaryotic source of the enzyme.[5]

The "endophytes" are group of organism grown in the plants tissue without causing damage to the host they live in.[6] Globally an estimated number of about 300,000 plants species are being host to endophytes.[7] The endophyte is used as a potential source in medicine, industry, and agriculture has they provide survival and defense environment to plants by producing plethora substance.[8] Fungal endophytes remain alive without being harmful to the host plant, their mutualistic interaction benefits both the organism and the host. Medicinal plants like *Justicia adhatoda* are used in various diseases which were their barks and leaves are used as

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medical ingredients.[9] The previous studies have reported the broad spectrum of bioactivity of the endophytic fungi isolated from *J. adhatoda*. As a part of the ongoing studies, the present study dealt with isolating and characterizing L–asparaginase from endophytic fungi derived from *J. adhatoda*.

## MATERIALS AND METHODS

### Isolation of Endophytic Fungi

The leaves from *J. adhatoda* were collected. The leaf samples were washed with 70% ethanol and 5% of sodium hypochlorite solution for few minutes and were rinsed with distilled water to remove surface contamination. The leaves were cut into pieces and were transferred to potato dextrose agar (PDA), supplemented with chloramphenicol (50 µg/ml). The plates were incubated at 27°C for 7–14 days. The fungi were further sub cultured till the fungal growth was seen and was preserved at 4°C.[10]

### Molecular Characterization

Fungal genomic DNA was isolated using the InstaGene™ Matrix Genomic DNA isolation kit. The isolated fungal was suspend in 1 ml of sterile water and centrifuged at 10,000 rpm for 2 min. 200 µl of InstaGene matrix was added to the pellet and incubate at 56°C for 15 min and was Vortex at a 100°C in heat block for 10 min. The mixture was spun at 10,000 rpm for 2 min. 20 µl of the supernatant was used for PCR reaction.

Single-pass sequencing was performed on each template using below 18s rRNA ITS universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).The phylogeny analysis of the sequence with closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences.[11] The program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as substitution model. The program Tree Dyn 198.3 was used for tree rendering.[12]

### Screening for L-asparaginase

The endophytic fungi isolated were screened on modified Czapek-Dox (MCD) agar pH 7.0. The MCD media contained (g/l) L-asparagine (10), glucose (1%), sodium nitrate (2), ferrous sulfate (0.01), magnesium sulfate (0.5), potassium chloride (0.5), di-potassium phosphate (1.0), agar (15), and phenol red. The fungal culture was inoculated on MCD agar plates at 28°C for 5–7 days and monitored for formation of pink zone after every 24 h for 7 days.[13]

The fungal culture with positive zone for production of asparaginase was grown on MCD broth under aseptic conditions and was incubated in shaker incubator at 120 rpm, 28°C for 7–10 days.[14] The cell free culture filtrate was obtained by separating fungal mycelium by filtration followed by centrifugation at 12,000 rpm for 15 min.

### Determination of L-ASN Activity

Cold acetone precipitation was carried out to precipitate the extracellular protein.[15] Pellet obtained was dissolved in 1 ml of 0.5 M phosphate buffer (pH 7.0) and stored at –80°C till further use.

The activity of L-asparaginase was quantified by direct nesslerization.[16] To, 0.1 ml of L-asparagine (40 mM), 1 ml of crude sample was added. 0.5 ml of 0.5 M phosphate buffer (pH 7.0) and 0.4 ml of $dH_2O$ were added and mixed gently. The mixture was incubated at 37°C for 30 min. After incubation 0.5 ml of 1.5 M trichloroacetic acid was added to stop the reaction and was centrifuged at 10,000 rpm for 5 min. To 0.1 ml of supernatant solution, 3.7 ml of distilled water was added. 1 ml of Nessler's reagent was added, vortexed and then incubated for 10 min. Blank was prepared containing all the other reagents except enzyme. Absorbance was measured at 450 nm.[16] The amount of ammonia liberated from protein sample of interest is calculated from standard curve of ammonia.

The total protein was estimated using Lowry's method using bovine serum albumin as standard.[17] The partial purification of the asparaginase was carried out by dialysis. The molecular weight determination was done by standard SDS PAGE, with molecular weight markers of 5–100 kDa.

### Cytotoxicity Studies

The A549 lung adenocarcinoma cell line was procured from National Centre for Cell Science, Pune, India, with the passage number of 24. Cells were maintained in Dulbecco's Minimum Essential Media supplemented with 10% fetal bovine serum, with 100 units/mL penicillin and 100 µg/mL streptomycin. Proliferation of A549 cells was assessed by MTT assay. Cells were plated in 96-well plate at a concentration of 5 × 104 cells/well 24 h after plating. After 24 h of cells incubation, the medium was replaced with 100 µl medium containing partially purified sample at different concentrations (10–200 µg/ well) and incubated for 24 h.[18] Untreated cells served as control and received only 0.1% DMSO in which the fraction was prepared. At the end of treatment period, media from control and drug-treated cells were discarded and 50 µl of MTT (5 mg/ml phosphate-buffered saline [PBS]) was added to each well. Cells were then incubated for 4 h at 37°C in $CO_2$ incubator. MTT was then discarded and the colored crystals of produced formazan were dissolved in 150 µl of DMSO and mixed effectively by pipetting up and down. Spectrophotometrical

absorbance of the purple blue formazan dye was measured using an ELISA reader (BIORAD) at 570 nm. Optical density of each sample was compared with control optical density and graphs were plotted.

### Ethidium Bromide/Acridine Orange (Dual Staining)

Ethidium bromide/acridine orange staining was carried out by the method of Gohel *et al*. A549 cells were plated at a density of 5 × $10^4$ in 6-well plates. They were allowed to grow at 37°C in a humidified $CO_2$ incubator until they were 70–80% confluent. Then, cells were treated with 80 µg/ml and 160 µg/ml of partially purified sample (selected based on the $IC_{50}$ concentration) for 24 h. The culture medium was aspirated from each well and cells were gently rinsed twice with PBS at room temperature. Then, equal volumes of cells from control and partially purified sample treated were mixed with 100 µl of dye mixture (1:1) of ethidium bromide and acridine orange and viewed immediately under Nikon inverted fluorescence microscope (Ti series) at ×10. A minimum of 300 cells was counted in each sample at two different fields.

The percentage of apoptotic cells was determined by [% of apoptotic cells = (Total number of apoptotic cells/Total number of cells counted) × 100].

### Determination of Intracellular ROS Generation

The formation of ROS was measured using a non-fluorescent probe, 2,7-diacetyl dichlorofluorescein that can penetrate into the intracellular matrix of cells, where it is oxidized by ROS to form fluorescent dichlorofluorescein (DCF). The percentage of ROS was estimated in the control, and partially purified enzyme treated A549 cells. The results were expressed as percentage; increase in fluorescence was calculated using the formula ([*Ft*30−*Ft*0]/[*Ft*0×100]), and the fluorescence intensities at 0 and 30 min were measured.

### Flow Cytometry

A549 cells (1 × $10^5$ cells/ml) were treated with 80 µg/ml and 160 µg/ml of partially purified sample for 24 h. The treated cells were harvested, washed with PBS and fixed in 75% ethanol at 4°C overnight. After washing twice with cold PBS, cells were suspended in PBS containing 40 µg/ml propidium iodide (PI) and 0.1 mg/ml RNase A followed by shaking at 37°C for 30 min. The stained cells were analyzed with flow cytometer (Becton-Dickinson San Jose, CA, USA) and the data were consequently calculated using WinMDI 2.9 software (TSRI, La Jolla, CA, USA).[20]

Data were expressed as mean ± S.E.M and analyzed by Tukey's test to determine the significance of differences between groups. A *P*-value lower than 0.05, 0.01, or/and 0.001 was considered to be significant.

## RESULTS AND DISCUSSION

### Isolation of Endophytic Fungi

In the present study, endophytic fungi were isolated from leave segment of *J. adhatoda*, a plant of medicinal importance [Figure 1]. Conventionally, plants have served as a source of medicinal bioactive compounds against numerous ailments. Several studies have indicated the high therapeutic potential of endophytes associated with the medicinally important plants.[21] The isolated fungi used in the present study were regularly sub cultured on PDA slants and maintained at 28°C.

### Molecular Characterization

DNA from the isolated fungal culture was successfully extracted and amplified using ITS primer. The BLAST results revealed the species identity of the unknown fungi to be *Aspergillus nomius* [Figure 2].

### L-asparaginase Assay

Primary screening of endophytic fungi was done for L-asparaginase production using modified protocol as previously described by Patii *et al*. Selected fungi showed the ability of changing the MCD agar plate from red to pink indicating the accumulation of ammonia from conversion of L-asparagine to L-aspartic acid [Figure 3].

**Figure 1:** Growth of endophytic fungi on potato dextrose agar plates

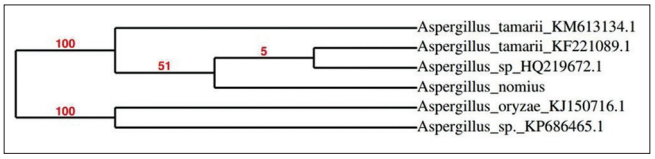


**Figure 2:** Phylogeny tree of *Aspergillus nomius*

The search of alternative sources for L asparaginase has been a priority, due to the disadvantages with the therapeutic bacterial asparaginases. The large scale extracellular production and the eukaryotic nature of the fungal source have remained attractive. Ashok *et al*.[22] work suggest that isolation of L asparaginase producing fungi from extreme environments such as Antarctica may lead to an important advancement in therapeutic applications with fewer side effects. Several species of endophytic fungi have been reported to be producers of L-asparaginase by Hatamzadeh *et al*.[23] L-asparaginase activities were between 0.019 and 0.492 unit/mL with *Fusarium proliferatum* being the most potent. L-asparaginase-producing endophytes were identified as species of *Plectosphaerella*, *Fusarium*, *Stemphylium*, *Septoria*, *Alternaria, Didymella*, *Phoma*, *Chaetosphaeronema*, *Sarocladium*, *Nemania*, *Epicoccum*, *Ulocladium,* and *Cladosporium*.

Shake flask culture assay was carried out in which the cultures selected after preliminary screening[19] were subjected for further confirmation of L-asparaginase production. These cultures were checked for production of secondary metabolites in L-asparagine-glucose medium so as to evaluate amido-hydrolytic activities of L-asparaginase. Protein precipitation and estimation were carried out to the crude protein amply recovered from cell free filtrate of selected isolates by cold acetone precipitation method. The total protein content obtained per milliliter of culture filtrate was 1.82 (mg\ml). The activity of L-asparaginase was evaluated by direct nesslerization and was found to be 1.8916 Units/min/ml. One international unit of enzyme is defined as the amount of enzyme needed to liberate 1 µmole of ammonia from L-asparaginase under the conditions of assay (pSSSSEDialysis was used to remove small molecules from a crude sample which purifies the sample partially which are further used to study the anticancer activities. SDS-PAGE was done in which the proteins fractionated into bands. Molecular weight of asparaginase was determined by 10% SDS-PAGE. The standard protein marker (5–100 KDa) was run on Lane 1. The peptides separated and formed bands with respect to their molecular weight. Lane 2, in which the crude sample separated in 4 bands at about 12, kDa 29 kDa, 45 kDa, and 66 kDa. The sample obtained from dialysis was run in lane 3. A single faint band was observed at about 66 KDa, suggesting the presence of L-asparaginase [Figure 4]. The L-asparaginase enzyme to homogeneity from *Penicillium* sp. showed 13.97 IU/mg specific activity and 36.204% yield. The polyacrylamide gel electrophoresis of the pure enzyme exhibited one protein of 66 kDa.[24] Similarly purified L-asparaginase obtained from *Streptomyces brollosae* had a molecular weight of 67 kDa.[25] The L-asparaginase variability could be attributed to genetic differences.[26]

The endophytic fungi of genus *Aspergillus* are predominant producers of L-asparaginase. *Aspergillus fumigatus* was a

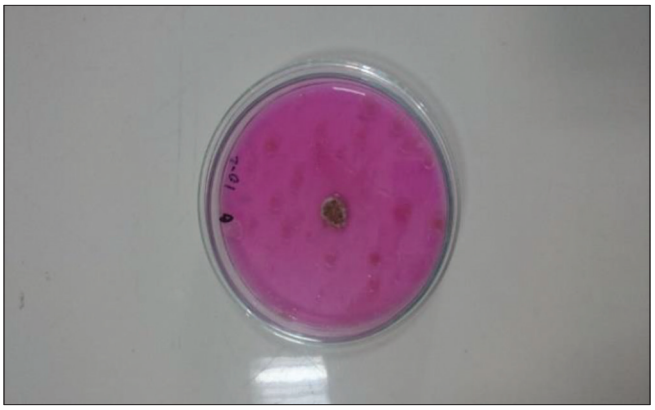

**Figure 3:** Modified Czapek-Dox agar plate showing L- asparaginase production by endophyte

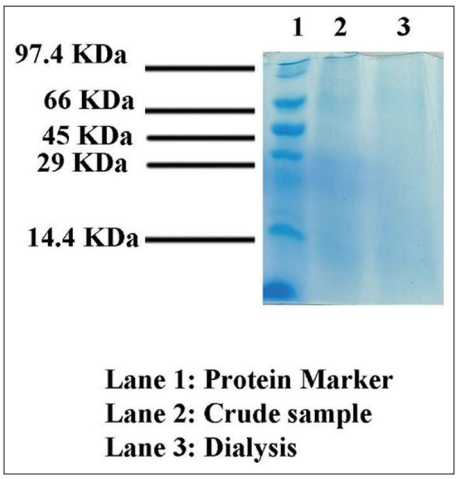


**Figure 4:** SDS PAGE analysis of the isolated protein and dialyzed sample

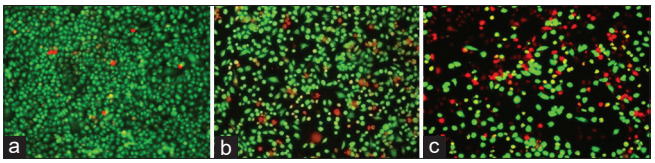


**Figure 5:** AO/EtBr staining of A549 cell line treated with varying concentrations of the enzyme. (a) Control, (b) 80 µg/ml of enzyme, (c) 160 µg/ml of enzyme

prominent endophytic fungi in red fruit of chili.[27] The study revealed in 7 days old culture, the relationship between biomass production and L-asparaginase production in individual isolate was highly specific. The varying enzyme activity has been reported from various endophytic fungi in qualitative and quantitative assays. This could be attributed to the growth conditions in the culture. *Fusarium* sp. LCJ273 was isolated from the medicinal plant *Adhatoda vasica*, showed maximum production of L-asparaginase using an optimized medium containing dextrose, ammonium sulfate along with natural inducer wheat bran. In this study, L-asparaginase production was 2 times higher compared to the original screening medium.[28]

## Cytotoxicity Studies

### *Inhibition of growth and proliferation of human A549 lung adenocarcinoma cells*

The % inhibition of cell growth against A549 lung adenocarcinoma cell lines showed that the enzyme was able to inhibit the cell growth. Maximum inhibition was found to be 85% at a concentration of 200 µg/ml. The $IC_{50}$ was calculated by linear regression analysis. The $IC_{50}$ of the drug was 160.62 µg/ml. Hence, further assays were carried out with 80 µg/ml and 160 µg/ml [Table 1].

## Ethidium Bromide/Acridine Orange (Dual Staining)

To assess the type of cell death induced in A549 cells, the morphological changes after double staining with Acridine Orange/Ethidium Bromide (AO/EB) were investigated. AO/EB staining uses combination of two dyes to visualize cells with aberrant chromatin organization. The differential uptake of these two dyes allows the identification of viable and non-viable cells, where the second dye, EB emits red fluorescence in the cells with an altered cell membrane. Viable cells with intact DNA and nucleus gave green fluorescence. Early apoptotic cells had fragmented DNA which exhibited intense green colored nuclei. Late apoptotic and necrotic cell DNA were fragmented and stained orange and red. Besides, some cells exhibited typical characteristics of apoptotic cells such as plasma membrane blebbing. The enzyme was found to induce apoptosis and with increasing concentration of drug, the number of viable cells decreased tremendously. The percentage of apoptotic cells after treatment showed a drastic increase in apoptotic cells ($P < 0.001$) to 62% and 84%, respectively [Figure 5].

## Determination of Intracellular ROS Generation

The formation of intracellular ROS was measured in terms of fluorescence by DCF. Pre-treatment of the cells with enzyme of different concentrations (80 µg/ml and 160 µg/ml) showed significant damage to the A549 lung adenocarcinoma cell lines which is exhibited by increase in the fluorescence percentage [Table 2].

## Flow Cytometry

Flow cytometry analysis was performed to evaluate the cell cycle arrest phase induced by different concentrations. Incubation of fixed and permeabilized cells with fluorochrome PI results in quantitative PI binding with total cellular DNA, and the fluorescence intensity of partially purified sample PI-labeled cells was proportional to DNA contents. L-asparaginase selectively depletes the serum asparagine, which devoid the tumor cells of their only source and leads to their arrest in G1 phase which ultimately ends

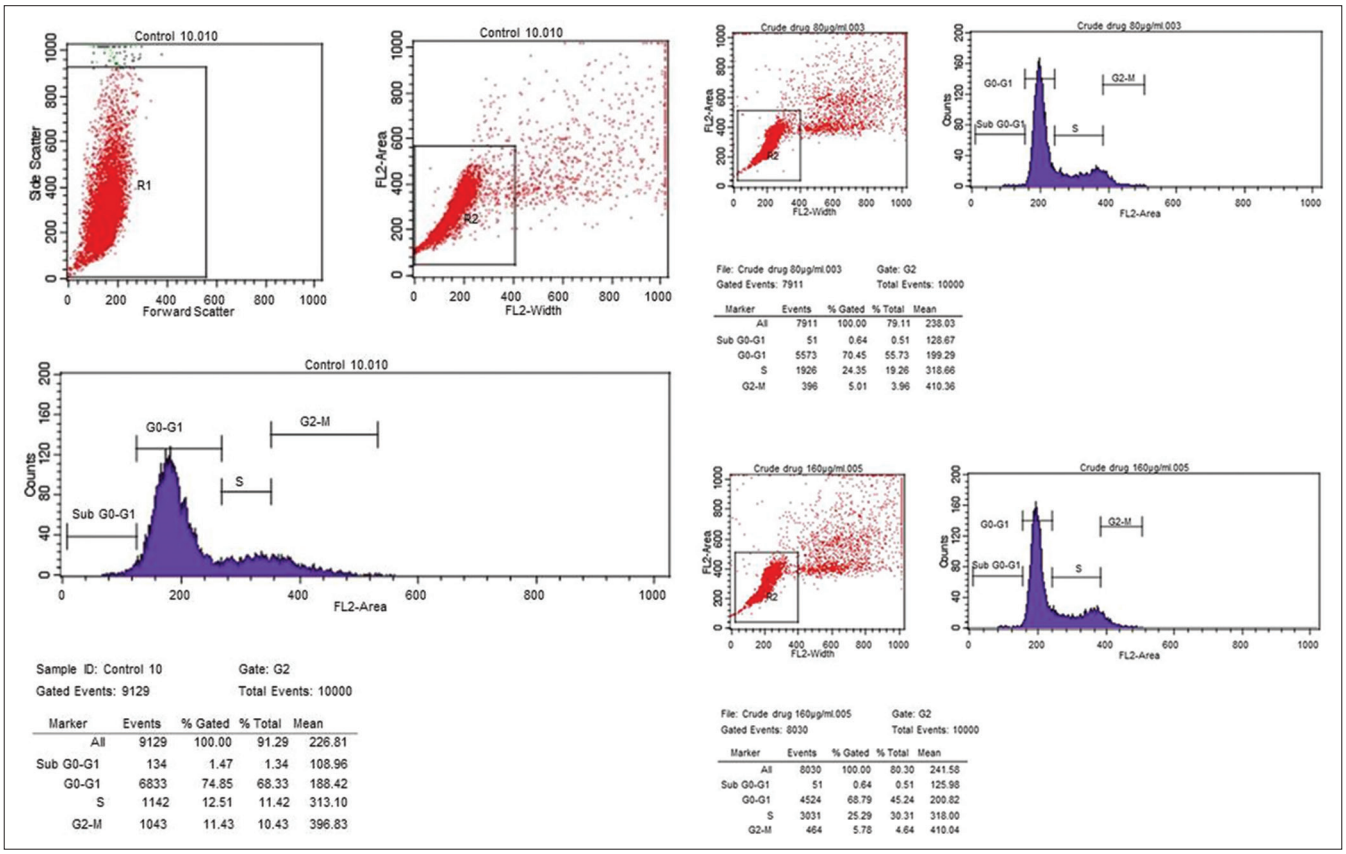


**Figure 6:** Flow cytometry of control and cell cycle arrest induced by different concentrations of enzyme

**Table 1:** Cytotoxic effect of the enzyme against A549 lung adenocarcinoma cells

| Concentration (µg/ml) | % Inhibition of cell growth enzyme |
|---|---|
| 10 | 18.34±0.6 |
| 20 | 25.23±0.71 |
| 40 | 33.18±0.56 |
| 80 | 39.97±0.56 |
| 120 | 43.77±3.04 |
| 160 | 49.59±0.05 |
| 200 | 68.54±0.15 |

**Table 2:** Intracellular ROS generation analysis by DCF – DA in treated and control A549 cell line

| Groups | Fluorescence intensity (%) |
|---|---|
| Control | 15±1.06 |
| 80 µg/ml | 40±1.24 |
| 160 µg/ml | 76±1.77 |

up with their apoptosis. The elimination of L-asparagine from the environment of cancer cells leads to the cell cycle arrest in S phase, inhibition of DNA and protein synthesis, induction of apoptosis and eventually, cell necrosis. At lower concentration (80 µg/ml) showed arrest at S phase with 19% cells accumulated. At higher concentration (160 µg/ml), it showed an increased cell population at S phase with 30% with concomitant alterations in the other phases of cell cycle [Figure 6].

A549 cells are sensitive to the treatment of asparaginase. Growth inhibitory effect of asparaginase was observed in a dose-dependent manner in these cell lines. The deprivation of asparagines activated apoptosis in A549 cells leading to cytotoxicity. ROS also plays an essential role in asparagine deprivation therapy induced by asparaginase. ROS was evaluated to be involved in autophagy and cytotoxicity induction when cells faced the deficiency of asparagine.[29] L-asparaginase had emerged as a potent health-care agent for the treatment of acute lymphocytic leukemia.[30] L-asparaginase produced by *A. fumigatus* performed as an anti-proliferative agent on MDA-MB-231 and suggested for the treatment of human breast cancer.[31] L-asparaginase from endophytic fungi of papaya deprives asparagine for *Plasmodium*, thus suggesting its use in malarial prophylaxis.[32]

## CONCLUSION

Our present study led to the conclusion that the L-asparaginase isolated from endophytic *A. nomius* exhibited a marked cytotoxic activity against A549 cell lines. Optimization of culture conditions would lead to extrapolation of the enzyme production. Future studies on animal models and toxicity assays would help to validate the use of this enzyme as an anticancer drug.

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