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# Regulation of Operative Biomarkers Production by Treating with Marine Actinomycetes L-Asparaginase in HepG2 Cell Line

**Senthil Kumar. M[1,*], Selvam. K[2], Singaravel. R[3], Krishnaveni. N[4]**

[1]Research and development centre, Bharathiar University, Coimbatore, India
[2]Department of Botany, Periyar University, Salem, India
[3]Central Institute of Brackish water Aquaculture (CIBA), Chennai, India
[4]Department of Microbiology, PSG college of Arts and Science, Coimbatore, India
*Corresponding author: pearlsen78@gmail.com

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**Abstract** A unique novel extracelluar glutaminase free L-asparaginase obtained from marine *Streptomyces radiopugnans* MS1 is an important therapeutic enzyme used in the treatment of Hepatocellular carcinoma. A full-length gene of L-asparaginase was cloned from *Streptomyces radiopugnans* MS1 and over expressed in *Escherichia coli* BL21 (DE3). The recombinant L-asparaginase from *E.coli* was purified by Model 491 Prep Cell which gave 136.8% higher yield with final specific activity of 742.8 IU/mg than the indigenous L-asparaginase from *S. radiopugnans* MS1. The molecular weight of L-asparaginase was found to be approximately 33.3 kDa by SDS–PAGE analysis. L-asparaginase was tested for its efficacy and cytotoxicity to HepG2 cell line with $IC_{50}$ 0.45 IU/mg and selectivity index (SI) at 10.8 which is about eleven time's higher selectivity over the lymphocyte cells. HepG2 cells were the most sensitive, showing apoptosis with a higher incidence subsequent to L-asparaginase treatment. A multiplexed flow cytometric bead-based assay to analyze the release of cytokines and quantitative real-time PCR (qRT-PCR) to evaluate gene expression were performed. The obtained cytokine pattern showed that, at the increasing rate of two molecules concentrations, two pro-inflammatory cytokines such as VEGF and IL-8 were decreased whereas the anti-inflammatory cytokine such as IL-4 and IL-10 were increased. This is the first report of the cloning and functional expression of a glutaminase free L-asparaginase gene from noval marine Actinomycetes species. This study has endowed confirmation for the mechanism of L-asparaginase anticancer activity against Hepatocellular carcinoma which was compared with the commercial L-asparaginase. Results indicated that L-asparaginase could be utilized as an effective influential substance for cancer treatment.

***Keywords:*** *glutaminase free L-asparaginase, Streptomyces radiopugnans MS1, HepG2 cells and cytokines*

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## 1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common and the second deadliest cancer, with approximately one million patients falling victim due to HCC each year [1]. HCC has a high incidence rate in underdeveloped and developing countries where patients are often diagnosed with infiltrative or massive tumors [2]. Furthermore; the incidence of HCC in developed countries has been increasing in recent years.

In spite of advances in surgical and nonsurgical therapies for hepatocellular cancer, yet there is no satisfactory technies for improving the overall survival rate of affected patients. Although the major etiological agents have been identified, the molecular reaction for pathogenesis of HCC remains unclear [3]. Further study on the molecular mechanisms underlying HCC as well as identification of new targets of molecular therapy is critically needed.

During the last decade, a significant amount of research has been focused on apoptosis of cancer cell. Apoptosis/programmed cell death is a key control mechanism by which cells die if DNA damage is not repaired [4]. Apoptosis is an essential and highly regulated physiological process required for normal development and tissue homeostasis in all multicellular organisms [5]. Novel targeted therapies based on apoptosis principles can induce cancer cell death or sensitize them to conventional cytotoxic agents and radiation therapies [6]. Ideally, an effective way to augment anticancer strategies using the developing knowledge of apoptosis could provide more targeted anti-tumor therapy for HCC and other cancers. Two provocative approaches, the receptor-dependent (extrinsic) and the mitochondrial-dependent (intrinsic) pathway, can induce tumor-selective apoptosis [7].

Generally, the initiation and progression of apoptotic pathways are associated with multiple changes at the mRNA and protein levels. In both cases, pathways are controlled by various complex proteins activated by different triggers which operate in sequential signaling modules. Several proteins important in apoptosis regulation have been systematically explored; protein changes during apoptosis that are not completely elucidated include; altered expression, differential protein alterations, specific activity modifications, and aberrant localization. Hence, a novel, feasible method is necessary to resolve whole cell protein expressions and modifications during apoptosis process.

Proteomic analysis could easily characterize the qualitative alterations and quantitative protein expression level changes in response to varying conditions during apoptosis [8]. Traditional proteomics methods combining both two dimensional gel electrophoresis with mass spectrometry and database-analyzing techniques are used to observe the global changes in protein expression during the apoptotic process. However, the traditional methods are complex and time-consuming, precise protein spots are often unattainable and contamination is often faced in the whole process.

Apoptosis is a dynamic process where different conditions should be maintained and detected over time. A simple, easy and rapid method for proteomic analysis would greatly benefit this identification. In this work, apoptosis in hepatocellular carcinoma cell line, HepG2, was induced using an unique extracelluar glutaminase free L-asparaginase from novel marine Actinomycetes, which was isolated to perceptible homogeneity in agro industrial wastes. This L-asparaginase protein gene is cloned into *E.coli* DH5α and the L-asparaginase effects on HepG2 in terms of proliferation and apoptosis-induction were assessed as functions of concentration and exposure time.

This retarded tumor growth *in vitro* elicited by L-asparaginase was associated with direct suppression of hepatocellular carcinoma cell cycle and reduced expression of proangiogenic factors VEGF & IL-8 whereas the anti-inflammatory cytokine such as IL-4 and IL-10 increased leading to the inhibition of tumor angiogenesis. Thus, our results indicate that glutaminase free L-asparaginase, a novel antiangiogenic enzyme, may be valuable in antiangiogenic therapy for HCC.

# 2. Materials and Methods

## 2.1. Construction of Vector for L-Asparaginase Expression

The gene coding for the mature region of L-asparaginase II was PCR amplified from the genomic DNA of *Streptomyces radiopugnans* MS1 strain using primers Forward primer (5' -GGGTACCCCGGTACCATGACCTCGCAGAAC-3') and Reverse primer: (5'-GGAATTCCGAATTCTCAGAACGCCGC-3') kpn1 and EcoR1 restriction sites were incorporated in the primers to facilitate cloning of the structural asparaginase gene (without its native signal sequence) in the *E. coli* expression vector pET-32a for fusion with a histidine tag at the N-terminus. The resultant recombinant plasmid pET-32a -Asp was sequenced to confirm the asparaginase gene insert.

This resulted in a recombinant plasmid pET-32a that contained the desired N-terminal pelB leader peptide, a histidine tag, and the gene sequence coding for the mature L-asparaginase gene, downstream of the T7 promoter and Ampicillin Resistance. The resulting construction, pET-asp, was then introduced into *E.coli* DH5α cells by electroporation (Biorad, USA) and cultivated on LB agar plate containing 100 µg/ml ampicillin. The positive clone was verified by DNA sequencing.

Recombinant plasmids were extracted from the positive clones using plasmid extraction kit (Biobasic inc). Extracted recombinant plasmid of asp gene were transformed into an expression host *E.coli* BL21 (DE3) and the transformants were selected on the LB agar plates containing ampicillin (100 µg/ml).The cells containing the recombinant expression plasmid were cultured under vigorous shaking at 37°C.

## 2.2. Growth Media and Conditions for Expression Studies

The Recombinant cells were grown in 100 ml of Tapioca effluent medium [Tapioca effluent (75% v/v), Natural sea water (25% v/v), Corn steep liquor (2% w/v), Peptone (1% w/v), yeast extract (1.5% w/v), $KH_2PO_4$ (0.1% w/v), $MgSO_4$ (0.5% w/v), $CaCO_3$ (0.4% w/v) pH 7.2~7.8] each supplemented with ampicillin (100µg/ml), in 1L conical flasks at 37°C with shaking at 220 rpm.

It had been observed previously that in the case of asparaginase expression, induction with 0.1 mM IPTG was more favorable for secretion as well as for cell growth (data not shown), hence the cultures were induced with 0.1 mM IPTG at divergent cell densities. Samples were collected at different time points post-induction to determine the profile of extra cellular asparaginase expression, each supplemented with ampicillin (100 µg/ml), in 1 L conical flasks at 37°C with shaking at 250 rpm. When the culture reached an optical density of 1.0 unit at 600 nm, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 400 µM. The culture was further cultivated for 24 hr at 30°C with 250 rpm. Samples were collected at different time points post-induction to determine the profile of extra cellular asparaginase expression.

## 2.3. Purification of *E.coli* Recombinant L-Asparaginase

The culture supernatant was collected at different time points post-induction, by centrifuging the cells at 10000 rpm for 30 min at 4°C and this was used for purification of recombinant asparaginase by Ni–NTA affinity chromatography. A column packed with 8 ml of 50% Ni–NTA resin (Qiagen) was equilibrated with equilibration buffer (50 mM potassium phosphate, pH 7.8, 200 mM NaCl, 0.5 mM PMSF (phenylmethylsulfonylfluoride) and 20 mM imidazole). The culture supernatant was treated so as to contain 200 mM NaCl, 1 mM PMSF, and 20mM imidazole and the pH was adjusted to 7.8 by 1 M $K_2HPO_4$. One hundred milliliter of this treated culture supernatant was passed through 0.45 µm filter and loaded on to the Ni–NTA column. The column was washed with 60ml of

equilibration buffer (50 mM phosphate buffer, pH 7.8, 200 mM NaCl, 0.5 mM PMSF, and 20 mM imidazole) and the protein was eluted with elution buffer (50 mM phosphate buffer, pH 8.0, containing 250 mM imidazole and 1 mM PMSF).

Fractions containing recombinant asparaginase were pooled and dialyzed against 50 mM Tris–HCl, pH 8.0. The protein solution was concentrated by Ultra filtration using "Prep Cell system", Model 491(Bio-Rad, Hercules, CA).

## 2.4. Asparaginase Activity Assay and Protein Estimation

L-asparaginase activity was assayed by a modified method of Mashburn and Wriston [9]. A 0.1ml sample of cell suspension or purified enzyme solution, 0.9ml of 0.1M sodium borate buffer (0.1 M, pH 8.5) and 1ml L-asparagine (0.04 M) solution were combined and incubated at 37°C for 10 min. The reaction was stopped by adding up of 0.5 ml of 15% wt/vol trichloro acetic acid. The reaction mixture was centrifuged at for 20 min at 10,000 rpm. The supernatant was collected and about 0.2 ml supernatant was diluted with distilled water to make it 8 ml. The resulting contents were treated with 1.0 ml Nessler's reagent and 2.0 M NaOH of 1.0 ml volume. Appearance of color reaction was allowed to proceed for 15 min before the absorbance at 500 nm was determined (Xmark, Bio-Rad, Hercules, CA). The absorbance was then compared with a standard curve prepared from solutions of ammonium sulfate as the source of ammonia. One international unit of L-asparaginase is the amount of Enzyme which liberates 1 µmole of ammonia in 1 min at 37°C. Protein concentration was determined at 25°C by the method of Bradford [10] using bovine serum albumin (fraction V) as standard.

## 2.5. Exposure of Hepatocelluar Carcinoma to L-Asparaginase

### *2.5.1. InVitro* L-Asparaginase Cytotoxicity Assay

L-asparaginase was tested for cytotoxicity against Hepatocelluar carcinoma cell lines and compared with three potent chemotherapeutic drugs; Imported L-asparaginase, melphalan and 5-FU with 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Prayong et al. 2008 [11] and Mossman., 1983 [12]. Stock samples were diluted with RPMI medium to desired concentrations of L-asparaginase ranging from 0.01 to 5 IU/mg and also for L-asparaginase, melphalan and 5-FU ranging from 10 to 500 µg/mL. One hundred microliters of cells were seeded in 96 well at the density 5×105 cell/mL and incubated at 37°C in 5% $CO_2$, 95 % air for 24 hr. Then, cells were treated with various concentrations of samples in total volume (200 µL / well) for 24 hr. At 21 hrs, cell were centrifuged at 2,000 for 10 min and resuspended with 180 µL RPMI medium to rinse treated samples. Twenty µL of MTT solution (5 mg/mL) was added to each well and incubated at 37°C for another 3 hr. Then the medium was aspirated about 180 µL from each well. The formed formazan crystals were dissolved with 180 µL of DMSO. An optical density (OD) of formazan was detected by a dual wavelength UV spectrometer at 570 nm with 650 nm wavelength as reference. The percentage of cytotoxicity compared the untreated cell as a control was determined as equation below.

$$\%\text{cytotoxicity}=\frac{(\text{OD without treatment}-\text{OD treatment})}{(\text{OD without treatment})}\times 100$$

The plot of % cytotoxicity versus sample concentration was used to calculate the concentration lethal to 50% of the cells ($IC_{50}$) [11].

## 2.5. Isolation of Lymphocyte and Determination of Selectivity Index

Normal lymphocyte was used to determine the non-toxic dose of the tested sample. Lymphocytes were isolated from blood provided from blood bank, Chennai, India according to the method described by Alhilli [13] with some modification. Selectivity index (SI) was calculated from the $IC_{50}$ ratio in lymphocytes over the HepG2 cell. The SI value indicates selectivity of samples to the cell lines tested. More than 3.0 of SI value will be considered to be high selectivity.

## 2.6. Flow Cytometric Assays for Apoptosis

HepG2 cells ($1\times10^6$ in 100-mm dishes) were incubated with or without L-asparaginase enzyme at various concentrations (100 to 300 IU/mg). After treatment (24 h), the cells were collected and fixed in 70% cold ethanol (−20°C) overnight. The obtained cells were washed twice and were resuspended in PBS (10 mM phosphate and 150 mM NaCl, pH 7.0). The endogenous RNA in the cells was digested with RNase A (0.5 mg $ml^{-1}$) at 37°C for 1 hr. Finally, propidium iodide (PI, 2.5 µg $ml^{-1}$) is used to strain the cells. The cellular DNA content was then analyzed by a FACScalibur flow cytometer (Becton Dickinson, USA). All experiments were carried out in triplicates and the average values were reported.

**Table 1. Sequences of oligonucleotide primers, annealing temperatures (AT) and expected Reverse Transcription - Polymerase Chain Reaction product sizes**

| Primer | Sequence (5'-3') | Annealing temperature | Amplification length | Accession number | Calibrator gene |
|---|---|---|---|---|---|
| VEGF - F | GGGCAGAATCATCACGAAGTG | 58°C | 343bp | AL_136131 | GAPDH |
| VEGF - R | ATTGGATGG CAGTAGCTGCG | | | | |
| IL-4 - F | GGTCTCAGCCCCCACCTTGC | 55°C | 111bp | NM_201270.1 | GAPDH |
| IL-4 - R | CCGTGGTGTTCCTTGTTGCCGT | | | | |
| IL-8 - F | GACCACACTGCGCCAACA | 57°C | 231bp | AF_385628.2 | GAPDH |
| IL-8 - R | GCTCTCTTCCATCAGAAAGTTACATAATTT | | | | |
| IL-10 - F | TGCTACGACGTGGGCTACG | 55°C | 69bp | NM_012854.2 | GAPDH |
| IL-10 - R | TGCAGTCCAGTAGATGCCGGG | | | | |
| GAPDH - F | GTGGAGTCCACTGGCGTCTT | 55°C | 257bp | J0_4038 | |
| GAPDH - R | GCAAATGAGCCCAGCCTTC | | | | |

## 2.7. Expression Studies of Cytokines

### 2.7.1. Analysis of Cytokines MRNA Expression by Quantitative RT-PCR

Total RNA from HepG2 cells was isolated using AquaPure RNA Isolation Kit (Bio-Rad, Hercules, USA) [6] and subsequently purified by ethanol precipitation. Each of the RNA sample (1 µg aliquot) was reversely transcribed into cDNA using the iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad, Hercules, USA). VEGF, IL-4, IL-8 and IL-10 and the respective calibrator genes were amplified in separate tubes: primers were selected by Primer Express Software Applied Biosystems (Foster City, CA, USA) and are indicated in Table 1.

The cDNA (1 µl) was then amplified for 40 PCR cycles using the iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, USA) in CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, USA). The real-time PCR reactions were performed in duplicates for both target and normalizer genes [14]. Changes in IL-4, IL-8, IL-10 and VEGF- expression were determined after 72 hours exposure to L-asparaginase (0.5 IU/mg).

### 2.7.2. Multiplex immunoassay of cytokine proteins

In this study, we evaluated the cytokines production in HepG2 cellular supernatants after incubation with L-asparaginase after 48 and 72 hr by Bio-Plex assay (Bio-Rad Laboratories, Hercules, CA) [15] as described by the manufacturer. The supernatant was frozen at -80°C until simultaneously analyzed for the following seven cytokines: IL-1b, IL-6, IL-8, IL-10, VEGF, TNF-α and TRAIL. The Bio-Plex cytokine assay is designed for the multiplexed quantitative measurement of multiple cytokines in a single well using as little as 50 µl of sample.

This was achieved using a custom 7-plex fluid-phase immunoassay (Bio-Rad) run on a Bio-Plex Suspension Array System (Bio-Plex 200 System; Bio-Rad). Cytokine standards or samples (supernatants recovered from treated cells) (50 µl in both cases) were incubated with 50 µl of anti-cytokine conjugated beads in 96-well filter plates for 30 min at room temperature with shaking conditions. Plates were then washed by vacuum filtration with 100 µl of Bio-Plex wash buffer for three times, then about 25 µl of diluted detection antibody was added to the plates, and were incubated for 30 min with shaking at room temperature. After three filter washes, 50 µl of streptavidin–phycoerythrin was added, and the plates were incubated for 10 min at room temperature with shaking. Plates were washed by vacuum filtration three times finally, then the beads were suspended in Bio-Plex assay buffer, and samples were evaluated on a Bio-Rad 96-well plate reader by means of the Bio-Plex Suspension Array Systemand Bio-Plex Manager software (Bio-Rad Laboratories, Hercules, CA) [16].

# 3. Result and Discussion

The present work was performed in order to characterize a new recombinant L-asparaginase from *Streptomyces radiopugnans* MS1 (Figure 1) (Streptomyces radiopugnans strain MS1 L-asparaginase II gene, complete cds. ACCESSION JF799106).

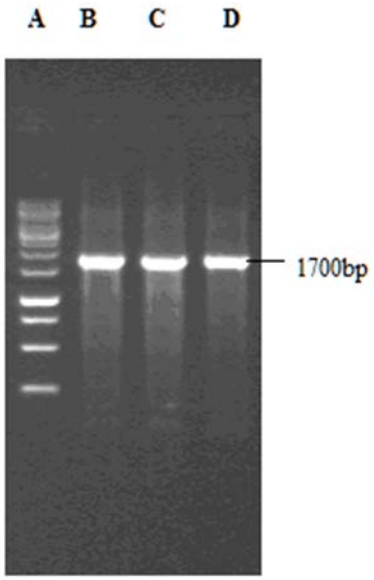


**Figure 1.** pET32a cloned L-asparaginase sequence

1% agarose gel electrophoresis showing screening of clones for the presence of L-asparaginase gene in pET32a vector using T7 promotor and T7 terminator primers from *E. coli* DH5α and BL21 *E. coli* expression host.
Lane A: 1 Kb DNA marker (250, 500, 750, 1000, 1500, 2000bp).
Lane B and C: PCR product confirming the presence as gene from *E. coli* DH5α.
Lane D: PCR product confirming the presence as gene from BL21 *E. coli* expression host.

The recombinant cells were grown to mid log phase in different media-LB, Czapekdox medium and Tapioca effluent medium supplemented with ampicillin and then induced with 0.1 mM IPTG. Post-induction, the cultures were grown for 24 hr, samples were collected at various time points, and the supernatants were analyzed for L-asparaginase activity and total protein content. It was observed that the secretion of recombinant L-asparaginase into the culture medium increased subsequently with the complexity of the media. The maximum Specific activity was observed in Tapioca effluent medium, which was 5.7 fold higher to LB and 3.2-fold higher than that of Czapekdox medium (data not shown). Therefore Tapioca effluent medium was chosen for further studies in order to improve secretion of recombinant L-asparaginase.

## 3.1. Purification of recombinant L-asparaginase enzyme

The recombinant L-asparaginase secreted into the culture supernatant was purified in a single step by Ni–NTA affinity chromatography. One hundred milliliter of the supernatant was collected and treated (Materials and methods) and then loaded on to the affinity column. After washing, the bound recombinant L-asparaginase was eluted with elution buffer. SDS-PAGE analysis of the purified recombinant L-asparaginase revealed a single protein band migrating at ~33.3 kDa (Figure 2). The specific activity of the recombinant asparaginase increased from 106.34U/mg to 742.85 IU/ mg a 6.9-fold increase during this purification step. Overall yield of 78000 IU/L, i.e., ~105mg/L of the purified recombinant L-asparaginase was achieved at the shake flask level. This corresponded to a recovery of 136% from the initial recombinant asparaginase present in the culture medium (Table 2).

**Table 2. Summary of steps employed in purification of recombinant L-asparaginase**

| Step | IU | Total Activity(IU) | Total Protein(mg) | Specific activity(IU mg) | Purification (Fold) | YIELD ( %) |
|---|---|---|---|---|---|---|
| Crude Extract | 5.7 | 5700 | 53.6 | 106.34 | | 100 |
| Prep cell | 97.5 | 7800 | 10.5 | 742.85 | 6.98 | 136.84 |

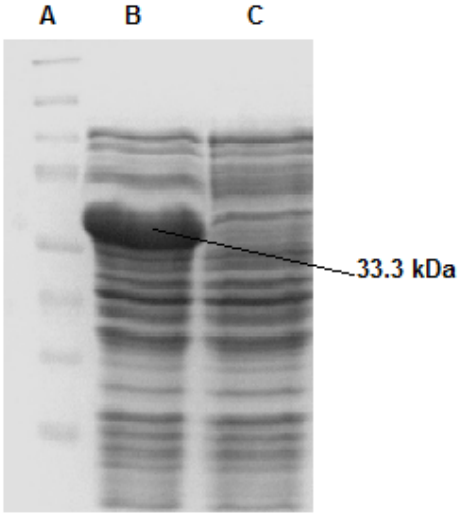


**Figure 2.** Expression comparison of recombinant L-asparaginase and normal L-asparaginase

SDS-PAGE profile of total lysate from samples after expression. Lane A: Molecular marker (6.5, 14.4, 21.5, 31, 45, 66.2, 97.4, 116.2, 200 kDa). Lane B: Total cell lysate from induced samples with 400 µM IPTG. Lane C: Total cell lysate of uninduced sample.

## 3.2. Cytotoxicity of L-Asparaginase in HepG2 Cell Line

Cytotoxicity results of recombinant L-asparaginase are summarized in Table 3. L-asparaginase showed positive activity and high selectivity against HepG2 cell line (SI = 10.86), when compared with commercially available L-asparaginase (6.99), melphalan (SI = 2.42) and 5-FU (SI = 1.61).The result illustrated high selectivity of L-asparaginase which indicated high specific toxicity of L-asparaginase only to the cancerous cells. $IC_{50}$ value was observed to be common to demonstrate the cytotoxic activity similar to previous studies [17]. In this study, not only $IC_{50}$ value but also SI data has been used to demonstrate the cytotoxic effect of L-asparaginase.

**Table 3. *In vitro* cytotoxicity activity of L-asparaginase, Commercially available – aspraginase, Melphalan and 5-FU on HepG2 cell line and Lymphocytes**

| Drug ($IC_{50}$) | Lymphocyte | HepG2 cell | SI |
|---|---|---|---|
| L-asparaginase (IU/mg) | 4.89 | 0.45 | 10.86 |
| Commerically available - aspraginase (IU/mg) | 22.58 | 3.23 | 6.99 |
| Melphalan (µg/ml) | 96.77 | 39.9 | 2.42 |
| 5-FU (µg/ml) | 109.15 | 67.42 | 1.61 |

## 3.3. Effects of Recombinant *L-Asparaginase* on DNA Synthesis in Tumor Cells

Results were processed with Cell fit software package. Compared with the control group, treatment with either recombinant or imported L-asparaginase s significantly increased the number of HepG2 cells at the G0/G1 stages. On the other hand, the number of cells at the S stage was dramatically decreased, indicative of reduction in DNA synthesis due to interception at the G0/G1 stages by L-asparaginase treatments. Meanwhile, the appearance of the sub-peak at the G1 stage before the G0/G1 stages is due to apoptosis. It suggested that the accelerated tumor cell death was achieved through cell apoptosis after treatment with L-asparaginase s (Table 4).

**Table 4. The flow cytometry analysis of different phases of tumor cell proliferation after treatment of recombinant L-asparaginase**

| Group | HepG2 cell (%) |
|---|---|
| Control | ° |
| G0/G1 | 0.35 |
| S | 8.5 |
| G2/M | 40.5 |
| Commercial L-asparaginase | ° |
| G0/G1 | 45.3 |
| S | 15.7 |
| G2/M | ° |
| Recombinant L-asparaginase | ° |
| G0/G1 | 65.4 |
| S | 27.6 |
| G2/M | ° |

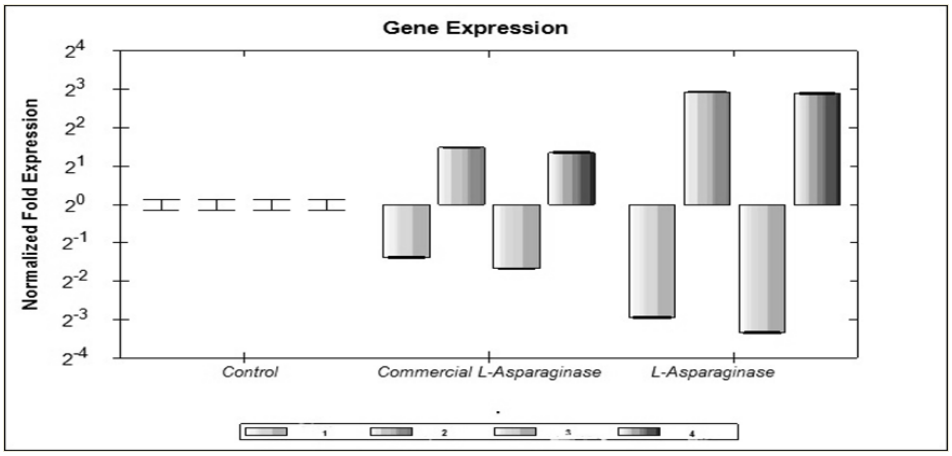


**Figure 3.** Effect of L-asparaginase on VEGF, IL-4, IL-8 and IL-10 expression in HepG2 cells by RT-PCR

1. VEGF; 2. IL-4; 3. IL-8; 4. IL-10.
Treatment with L-asparaginase down-regulated constitutive VEGF & IL-8 and up-regulate IL-4 & IL-10- mRNA levels in HCC cells (HepG2), as measured by real-time PCR. VEGF, IL-4, IL-8, and IL-10 mRNA expression is normalized to GAPDH.

### 3.4. Cytokines MRNA Expression of HepG2 Cells Treated with L-Asparaginase by RT-PCR

The cytokines secreted by tumor cells can promote immune suppression and angiogenesis, which eventually mediate tumor survival and metastasis. To evaluate the physiological and pathological roles of L-asparaginase in HepG2 cells, the HepG2 cells were treated with L-asparaginase for 72 hr, and then the supernatants were collected to facilitate determination of the levels of VEGF, IL-4, IL-8, and IL-10 by RT-PCR. The unstuminalted HepG2 cells (controls) were significantly express or secrete VEGF, IL-4, IL-8, and IL-10 and detected down-regulated mRNA expression for VEGF & IL-8 whereas up-regulated mRNA for IL-4 & IL-10 in HepG2 cells after L-asparaginase stimulation (Figure 3).

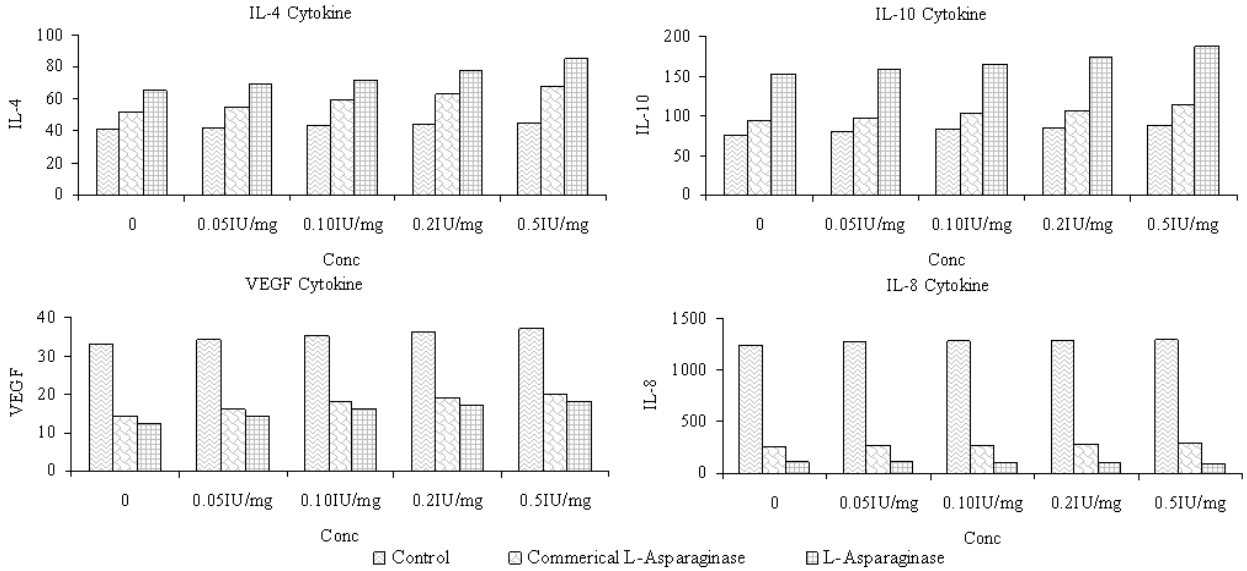


**Figure 4.** Cytokines levels in HepG2 cell line after 48 hr of treatment of Recombinant L-asparaginase

### 3.5. Cytokine Analyses Using Multiplexed Flow Cytometric Bead-Based Assays

The cytokine production in HepG2 cellular supernatants were evaluated after incubation with L-asparaginase at 48 and 72 h by Bio-Plex assay. The results obtained were compared with untreated cells used as control. These experiments showed that at the increasing of two molecules concentrations, two pro-inflammatory cytokines such as VEGF and IL-8 decreased whereas the anti-inflammatory cytokine such as IL-4 and IL-10 increased in statistically significant way at concentration increasing of L-asparaginase. However, HepG2 treated at 72 hr a significant reduction was observed only using 0.5 IU/mg in agreement with the results of cytotoxicity test Figure 4 (4a, 4b, 4c & 4d) – Figure 5 (5a, 5b, 5c & 5d).

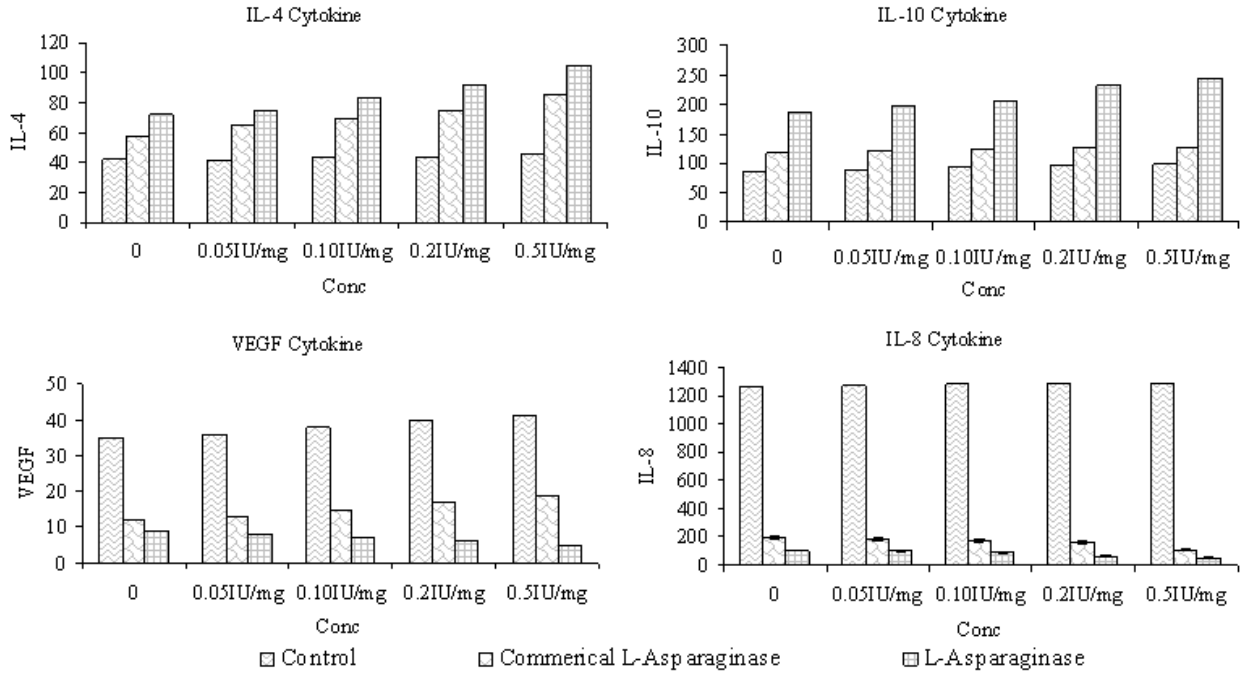


**Figure 5.** Cytokines levels in HepG2 cell line after 72 hr of treatment of Recombinant L-asparaginase

# 4. Conclusion

The present study indicates that the novel marine Actinomycetes strain *Streptomyces radiopugnans MS1* can be used as a potential source of L-asparaginase. Furthermore, the purified recombinant extracelluar glutaminase free L-asparaginase showed potential cancer therapeutic property against Hepatocellular carcinoma. Moreover, the cytokine concentrations have been evaluated in treated and untreated cellular supernatants in order to evaluate the proinflammatory and anti-inflammatory effects of four molecules. In conclusion the present research data has evidenced that L-asparaginase inhibited the tumor cell growth in dose and time dependent way, they decrease the VEGF & IL-8 whereas the anti-inflammatory cytokine such as IL4 and IL-10 increased. The Recombinant extracelluar glutaminase free L-asparaginase should be considered for further pharmaceutical use as an anti cancer candidate.

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