3.1 Introduction:

The enzyme L-asparaginase (ASN) (EC.3.5.1.1; L-asparagine aminohydrolase) catalyzes the deamination of L-asparagine (Asn) to L-aspartate and ammonia. The enzyme has been isolated from a variety of sources: animals and plant cells, yeast, fungi, and bacteria. It is identified as an effective agent in the therapy of certain types of lymphoma and leukemia. ASN can induce complete remission in up to 80% of patients suffering from acute lymphoblastic leukemia. The principal sources of asparaginase used extensively are from the bacteria *E coli* or *Erwinia carotovora* (Cammack et al. 1972). Using amino acid sequences and biochemical properties as criteria, enzymes with asparaginase activity can be divided into several families (Borek et al. 2001).

Two largest and best-characterized families include bacterial and plant-type asparaginases. The bacterial-type enzymes have been studied for over 40 years (Lough et al. 1992); their homologs are found in some mammals and in fungi (Bonthron et al. 1997). In particular, enzymes such as glutamin-(asparagin)-ases (EC 3.5.1.38) (Ortlund et al. 2000), lysophospholipases (EC 3.1.1.5) (Sugimoto et al. 1998), and the α-subunit of Glu-tRNA amidotransferase (EC.3.5.-) (Tumbula et al. 2000) can also be considered part of the bacterial asparaginase family. It has been shown on the basis of kinetic and structural studies that two conserved amino acid motifs are responsible for the activity of the abovementioned proteins (Lubkowski et al. 2003).

Though l-asparaginase has been reported in many higher plants, little work has been carried out on the characterization of l-asparaginase from...
higher plants. The presence of an amidase in barley roots capable of hydrolyzing l-asparagine had been reported earlier, and the distribution of l-asparaginase in *Lupinus luteus* and *Dolichos lablab* seedlings has also been reported (Lough et al. 1992b). The plant-type enzymes have been studied less thoroughly. In plants, L-asparagine is the major nitrogen storage and transport compound, and it may also accumulate under stress conditions (Bruneau et al. 2006). Asparagine divided into aspartate and ammonia and the asparagine is necessary for protein synthesis. There are two groups of such proteins, called potassium-dependent and potassium-independent asparaginases (Sodek et al. 1980; Sieciechowicz et al. 1988). Both enzymes have significant levels of sequence similarity. The plant asparaginase amino acid sequences did not have any significant homology with microbial asparaginase but was 23% identical and 66% similar to a human glycosylasparaginase (Lough et al. 1992b). The l-asparaginases of *Erwinia* and *E. coli* have been reported for many years as effective drugs in the treatment of acute lymphoblastic leukemia. Their main side effects are anaphylaxis, pancreatitis, diabetes, leucopenia, neurological seizures, and coagulation abnormalities.

*Withania somnifera* (L.) Dunal was considered a rasayana herb, which works on a nonspecific basis to increase health and longevity. The species name somnifera means “sleep-making” in Latin, indicating its attributed sedating properties. Extracts from the fruits, leaves, and seeds of *W. somnifera* L. were traditionally used for the Ayurvedic system as aphrodisiacs, diuretics, and for treating memory loss. *W. somnifera* L. is reported to have
anticarcinogenic effects in animal and cell cultures by decreasing the expression of nuclear factor-kappaB, suppressing intercellular tumor necrosis factor, and potentiating apoptotic signaling in cancerous cell lines (Ichikawa et al. 2006). Since W. somnifera was found to be a novel source of L-asparaginase and the enzyme could be obtained in a pure form, the enzyme was characterized for various respects such as molecular weight, pI value, sequence, optimum pH and temperature, enzyme kinetics and antitumor activity.

3.2 Materials and Methods:

3.2.1 Determination of molecular weight:

The molecular size of purified native L-asparaginase was estimated by chromatography on Sephacryl S-400 HR (1.6 × 25.5 cm). The column was equilibrated with 0.01 M sodium borate buffer, pH 8.6. The proteins were eluted with the equilibration buffer at a flow rate of 20 mL/h. The column was calibrated with urease pyruvate kinase (237 kDa), aldolase (158 kDa) and BSA (67 kDa) as protein standards.

3.2.2 Determination of pI value:

Sample preparation:

Purified L-asparaginase protein was precipitated through acetone precipitation method. The pellet was mixed with 100 µl of a solution containing Urea (8 M), CHAPS (4 % w/v), DTT (65 mM), Tris (40 mM) and a trace of bromophenol blue. 100 µl of the final diluted sample was loaded onto the IPG gel strip.

Immobilized pH gradient (IPG) as first dimension:
A non-linear immobilized pH gradient (3.5-10.0 NL IPG 7.0 cm) was used as the first dimension. The strips were 3 mm wide and 7.0 cm long.

Rehydration of IPG gel strips:

Hydration was performed overnight in Biorad reswelling cassette with 25 ml of a solution containing urea (8 M), CHAPS (2% w/v), DTE (10 mM), resolyte pH 3.5-10 (2% v/v) and a trace of bromophenol Blue.

Sample application:

When the rehydration cassette had been thoroughly emptied and opened, the strips were transferred to Biorad strip tray. After placing IPG strips, humid electrode wicks, electrodes and sample cups in position, the strips and cups were covered with low viscosity paraffin oil. Samples were applied at the cathodic end of the IPG strips in a slow and continuous manner, without touching the gels.

Running conditions:

The voltage was linearly increased from 300 to 3500 V during 3 hours, followed by 3 additional hours at 3500 V, whereupon the voltage was increased to 5000 V. A total volt hour product of 100 kv/h was used in an overnight run.

IPG gel strips equilibration:

After the first dimension run, the strips were equilibrated in order to resolubilize the proteins and to reduce -S-S- bonds. The strips were equilibrated within the strip tray with 100 ml of a solution containing Tris-HCl (50 mM) pH 8.4, Urea (6 M), glycerol (30% v/v), SDS (2% w/v) and DTE (2% w/v) for 12 min. -SH groups were subsequently blocked with 100 ml of a
solution containing Tris-HCl (50 mM) pH 6.8, urea (6 M), glycerol (30% v/v), SDS (2% w/v), iodoacetamide (2.5% w/v) and a trace of bromophenol blue for 5 min.

**SDS-PAGE as second dimension:**

After equilibration, the strip was transferred into 10 % SDS PAGE electrophoresis and covered with agarose containing bromophenol blue.

**Protein detection through colloidal coomassie stain:**

For preparing colloidal CBB, (A) To 16ml of Conc. H$_2$SO$_4$ in 768 ml water, 80 gm (NH$_4$)$_2$SO$_4$; (B) 5% CBB G-250 was added in 16 ml in solution ‘A’ and 200ml methanol was added when used it. The gel was kept in staining solution overnight for staining and destaining was carried out with double distilled water. All processes are done as per Bjellqvist et al. 1993 and Goerg et al. 1988.

### 3.2.3 Sequence characterization:

**N-terminal sequencing:**

First of all 10% SDS-PAGE gel was run in normal conditions and prepared electro blotting buffer (100 ml 10X CHAPS buffer with 100 mL of MetOH and 800 ml of de-ionized H$_2$O). The PVDF membrane was wetted with MetOH for a few seconds, and placed them in a tray containing blotting buffer. After SDS PAGE, the gel was removed from the electrophoresis cell and soaked in electro blotting buffer for 5 min. The gel and membrane were assembled as per trans blotting sandwich manner and electro blot was run at 100 V (2 hrs) with a cooling unit (for mini-gel).
The membrane was removed and rinsed with de-ionized H$_2$O prior to the staining. Coomassie blue staining procedures was used for visualized blotted protein onto PVDF membrane. The membrane was stained for 5 min in coomassie blue R-250** (0.1 % solution in 40% MetOH/10% acetic acid), followed by de-staining (5 - 10 min) in a 50% MetOH. The stained bands was removed with used of sharp razor blade. The membrane pieces were rinsed extensively with de-ionized water and store in a labeled micro centrifuge tube.

The degradation method developed by Pehr Edman (1950) was used for determine the amino acid sequence. In the first step, the free N-terminal amino acids were converted by phenyl iso thio cyanate to its phenyl thio carbamoyl derivative under mild alkaline conditions. The converted free N-terminal amino acids were cleaved as a thiazolinone derivative under acidic conditions. The thiazolinone amino acids were extracted and further converted the more stable phenyl thio hydantoin amino acid derivative. The phenyl thio hydantoin amino acid derivative were used for identified through chromatography. A standard mixture of 19 amino acids were also injected into the column for separation (usually as the first cycle of the sequencing run). The each edman degradation cycle chromatogram was compared with standard retention times of the standard amino acids.

**Peptide Mass Fingerprinting:**

The target gel piece was cut form normal SDS PAGE gel with a scalpel blade and placed in an eppendorf tube. The band was crushed it into 3-4 smaller pieces for better result.
A. Destain: The gel pieces were destained with 200µl of 50mM ABC (ammonium bicarbonate) and 200µl of 50% CAN (acetonitrile).

B. Reduction and alkylation: All the buffer and destain solution was removed from the gel piece and 40 uL of 10mM DTT solution was added into the tube for reduced the disulphide bonds on cys residues. The tube was incubated at 60°C for 30 min. DTT solution was removed and 40µL of 55mM iodoacetamide was added into the tube to alkylate the free cys residues. The tube was incubated at room temperature for 30 min in dark condition. Iodoacetamide solution was removed completely and 100µL of 50mM ABC was added and vortex 1-2 min for two times, to get rid of the excess IAA, as it can create adducts on your proteins. The gel pieces were dehydrated with 100% ACN and gel pieces were turned to white colour.

C. Enzymatic digestion: The gel pieces were rehydrated with 8µl trypsin (10ng/µl in 50mM ABC) for 10-20min at 4°C. After that an additional 6µl-16µl 50mM ABC buffer was added and kept the tube overnight at 37°C.

D. Peptide extraction: 50µl 50% ACN and 0.1% TFA (trifluoroacetic acid) was added into gel pieces. The tube was sonicated for 10 min and afterword spin it briefly and transferred supernatant to a new tube. The gel pieces were again shocked with 50µl of 50% ACN and 0.1% TFA. Sonicated again and pooled with 1st round of supernatant. The excreted peptide were lyophilised through speed vac and resuspended in 2µl 50% ACN and 0.1% TFA. Take 0.5µl of resuspended peptides was spoted at MALDI-TOF target plate with 0.5µl CHCA (α-cyano-4-hydroxycinnamic acid) matrix
(10mg/ml in 50% CAN; 0.1% TFA; 25mM diammonium citrate). The plate was kept for dry and installed into voyager 2000 for PMF.

3.2.4 Optimum temperature and pH:

The purified enzyme was incubated for 1 h at different temperatures from 4°C to 70°C in 0.01 M sodium borate buffer pH 8.6. The treated enzyme was used for activity assay.

To determine the optimum pH, the enzyme was assayed in the following buffers, all at 0.05 M final concentration at 37°C: 0.01 M sodium acetate (pH 3.5–5.5), 0.01 M Na₂HPO₄/NaH₂PO₄ (pH 6.0–7.0) and 0.01 M sodium borate buffer (pH 7.5–9.5).

3.2.5 Enzyme kinetics:

The determination of apparent \( K_m \), \( k_{cat} \), \( k_{cat}/K_m \) and \( V_{max} \) were carried out according to Lineweaver-Burk’s method (Lineweaver et al. 1934); using different L-asparagine concentrations (1.0 to 5.0mmol/L).

3.2.6 Antitumor activity:

_Culture of leukemic cell_

Primary leukemia cells from patients were obtained from peripheral blood, leukapheresis or bone marrow specimens under an IRB-approved protocol. All samples were obtained from patients newly diagnosed with leukemia prior to the administration of chemotherapy from Shree Krishna Hospital, Karamshad, Gujarat (India). Lymphoblasts were isolated following the method of Moriji Miura (1970) and cultured in RPMI-1640 supplemented with 20% fetal calf serum in a humidified atmosphere containing 5% CO₂.
Antitumor activity

Initially, the leukocyte culture was prepared in triplicate for further experiments. The experiment was designed for Normal leukocyte culture; Normal leukocyte culture + l-asparaginase (2 IU); Normal leukocyte culture + 1% PHA + l-asparaginase (2 IU); Patient leukocyte culture; Patient leukocyte culture + l-asparaginase (2 IU); Patient leukocyte culture + 1% PHA + l-asparaginase (2 IU) and Patient leukocyte culture + 1% PHA + l-asparaginase (2 IU) (E. coli commercial). After 48 and 72 h of incubation, cellular viability was determined by direct cell counts using a hemocytometer.

Cytotoxicity assay

The growth inhibition effect of l-asparaginase was assessed using the [3-(4, 5-dimethylthiazol- 2yl)-2, 5-diphenyl-tetrazolium bromide] (MTT) colorimetric dye reduction method (Carmichael et al. 1987; Mosmann et al. 1983). One hundred thirty-five microliters of exponentially growing lymphoblasts at 48 hrs was plated at a density of 0.5×10^4 to 1.0×10^4 cells/well in a 96-well microtiter plate with l-asparaginase added to each well at concentrations specified below. After 24 h of continuous drug exposure, 15μl of MTT (final concentration 0.5 mg/ml) was added to each well, the plates were incubated for 4 hrs at 37 °C, and the absorbance was measured at 550 nm using a spectrophotometer. Cell survival was calculated by subtracting the background absorbance of media alone and then dividing the absorbance of test wells by the absorbance of the control (untreated) wells. For single drug assays, replicates of three wells were used for each drug concentration. Combination effects were analyzed using nonconstant drug combination
ratios at single drug 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 3, and 5 IU drug concentrations. After subtraction of blank values, the leukemic cell survival (LCS) was calculated by the equation:

\[
LCS = \frac{\text{OD treated well}}{\text{mean OD control wells}} \times 100\%
\]

Drug sensitivity was assessed by the LC50, the drug concentration lethal to 50% of the cells.

**Statistical analysis**

Statistical calculations were carried out with the SPSS 10.0 for Windows software package (Statistica). Statistical significances were considered at P values less than 0.05. The percentages of cell viability were presented graphically in the form of histograms, using Microsoft Excel computer program.

**3.3 Results:**

**3.3.1 Molecular weight:**

The molecular weight was determined to be approximately 72,000 ± 500 Da through chromatography on Sephacryl S-400 HR (1.6 × 25.5 cm) where urease pyruvate kinase (237 kDa), aldolase (158 kDa) and BSA (67 kDa) were used as a protein standards. The native gel electrophoresis also showed a similar result, while molecular weight of enzyme was found to be 36,000 ± 500 Da in 10% SDS PAGE (Figure 1), indicating that the enzyme is a dimer with each subunit having a molecular weight of approx 36 KD.
3.3.2 Determination of pI Value:

The purified l-asparaginase protein pI value was characterized through 3.0 to 10.0 non linear gradients Biorad IPG strip and the gel was stained through Silverstain. The result showed a band near 5.0 to 5.5.
Figure 2: The purified enzyme showed a band near to 5.0 to 5.5 pI points on 3.0 to 10.0 pH non linear gradients Biorad IPG strip.

3.3.3 Sequence characterization:

N-terminal sequencing:

The purified protein was subjected to N-terminal sequencing. However, since the protein n-terminal part was might be blocked, the sequence could not be obtained.

Peptide mass fingerprint (PMF):

Data obtained form MS-PMF was analyzed with DataExplorer. Monoisotopic peak list was first processed with PeakErazor to remove any contaminant peaks, then searched in MASCOT against all species, using trypsin as enzyme, 2 mis-cleavages, 0.5Da tolerance, carbamidomethylation (fixed) and methionine oxidation (variable) modifications. Two protein sequences were obtained and the data were directly compared using the FindMod tool in Expasy.
3.3.4 Optimum temperature and pH:

The pH effect on the activity l-asparaginase enzyme was studied at 37°C. The results are presented in Figure 4. The pH for maximum activity was determined as 8.5. The change in pH affects the ionization of essential active
site amino acid residues, which are involved in substrate binding and catalysis (i.e. breakdown of substrate into product).

**Figure 4.** Effect of pH on activity of l-asparaginase from purified enzyme of *Withania somnifera*

![Figure 4](image)

**Figure 5.** Effect of temperature on activity of l-asparaginase from purified enzyme of *Withania somnifera*.

![Figure 5](image)

L-asparaginase was assayed at different temperatures (4–70°C) at pH 8.5. Figure 5 shows the effect of temperature on the catalytic activity of L-asparaginase. In the bell shaped curve, the maximum activity of the enzyme was at 37°C.
3.3.5 Enzyme kinetics:

Kinetic Parameters were determined using different concentrations of L-asparagine as substrate. A Km value of $6.1 \times 10^{-2}$ mM was obtained by means of the double-reciprocal Lineweaver-Burk plots (Fig. 6). Different kinetic parameters like $K_m$, $k_{cat}$, $k_{cat}/K_m$ and $V_{max}$ of the enzyme from *Withania somnifera* were also analyzed. The values are as follows: $k_{cat}$: 178.57 sec$^{-1}$, $k_{cat}/K_m$: 2927.37 sec$^{-1}$/mM, $V_{max}$: 714.28 µmoles/min/mg.

These values clearly show the efficiency of the enzyme l-asparaginase from *Withania somnifera*.

**Figure 6:** Lineweaver -Burk Plots for the determination of Km and Vmax for l-asparaginase from *Withania somnifera* L.

3.3.6 Antitumor activity:

**Table 1** shows the effect of l-asparaginase on lymphocyte cell culture when incubated with different combinations of normal blood with or without the enzyme or the enzyme with PHA same as it for patient blood and cell counting at 48 hr and 72 hr. A remarkable suppression of lymphocyte cell culture was observed on concentrations 2 IU/tube of l-asparaginase from *Withania somnifera*.
Table 1: Influence of L-asparaginase inhibition of PHA-induced lymphocyte cell culture

<table>
<thead>
<tr>
<th>Nos</th>
<th>Tube</th>
<th>Cell counting after 48hr</th>
<th>Cell counting after 72hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal Blood</td>
<td>24 x 10^3</td>
<td>47 x 10^3</td>
</tr>
<tr>
<td>2</td>
<td>Normal Blood + L-asparaginase</td>
<td>10 x 10^2</td>
<td>12 x 10^2</td>
</tr>
<tr>
<td>3</td>
<td>Normal Blood + 1% PHA + L-asparaginase</td>
<td>16 x 10^2</td>
<td>19 x 10^2</td>
</tr>
<tr>
<td>4</td>
<td>Patient Blood</td>
<td>48 x 10^5</td>
<td>05 x 10^6</td>
</tr>
<tr>
<td>5</td>
<td>Patient Blood + L-asparaginase</td>
<td>75 x 10^3</td>
<td>88 x 10^3</td>
</tr>
<tr>
<td>6</td>
<td>Patient Blood + 1% PHA + L-asparaginase</td>
<td>84 x 10^3</td>
<td>91 x 10^3</td>
</tr>
<tr>
<td>7</td>
<td>Patient Blood + 1% PHA + L-asparaginase (E coli)</td>
<td>64 x 10^3</td>
<td>69 x 10^3</td>
</tr>
</tbody>
</table>

The results of the cytotoxicity of L-asparaginase to human leukemia cells are presented in (Figure. 7). Data obtained from this assay indicated a strong dose response relationship with regard to the cytotoxic property of L-asparaginase from *Withania somnifera*, indicated in this figure, there was a gradual decrease in the viability of leukemia cells, with increasing doses of L-asparaginase. Upon 24 h of exposure, the mean percentages of cell viability were 100 ± 07; 98 ± 03; 98 ± 3.2; 96 ± 03; 94 ± 1.5; 88 ± 2.8; 74 ± 3.2; 25 ± 2.6; 16 ± 2.5 and 08 ± 0.2 in 0, 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 3 and 5 IU of L-asparaginase, respectively. The drug dose required to cause 50% reduction in cell viability was computed to be 1.45 ± 0.05 IU. These results showed that the enzyme induced a slight decrease in cell viability between 0 IU (control) and 0.5 IU, followed by a gradual decrease between 1 and 5 IU.
**Figure 7:** Cytotoxic effect of L-asparaginase on cell survival in leukemia cells. The cells were treated in triplicate with each concentration for 24 h and the cell viability was determined by the MTT assay.

3.4 **Discussion:**

The molecular weight of *Withania somnifera* l-asparaginase is approximately half to that of prokaryotic asparaginase (Lea et al. 1984). However the molecular weight of other higher plant asparaginase was similar to that of *Withania somnifera* L. The properties of L-asparaginase from *Withania somnifera* showed a number of similarities and some sharp differences when compared with the l-asparaginases from other sources. The molecular weight of the enzyme from *Withania somnifera* was found to be 72 ± 0.5 kDa; which sharply differs from the enzymes of guinea pig serum and *E. coli*, which have a molecular weight of around 130 kDa.

The enzyme showed some degree of thermal stability and showed activity over a wide range of pH values. The optimum pH of 8.5 for *Withania somnifera* enzyme resembled that of *E. coli* A which also had pH optima around 8.5; however *Withania somnifera* enzyme differed from the guinea pig
serum enzyme which showed a wide range of optimal pH from 7.5–8.5. The optimum temperature of 37° C of the enzyme from *Withania somnifera* is similar to all reported bacterial sources already used in the treatment of leukemia. The $K_m$ value for the *Withania somnifera* asparaginase enzyme is $6.1 \times 10^{-2}$ mM. This is considerably similar to the enzyme of *E.coli* and slightly lower than that of *Erwinia caratovora*. The L-asparaginase enzyme from *Withania somnifera* has striking similarities to the bacterial enzymes in pH, temperature and $K_m$, indicating its potential as a therapeutic protein. Findings from the present study clearly demonstrate that l-asparaginase is highly effective to leukemia cells, showing a 24 h-LD50 of $1.45 \pm 0.05$ IU.

### 3.5 References:


