Nature had always been an inspiration and guidance for human existence from time immemorial. Natural products which were derived from higher plants, animals or microbes were the main remedies to fight and control diseases. These medicinal preparations included the fresh / dried plants or an extract of the plants. Our country has a wide topography and agro climatic conditions permitting the growth of an estimated number of 20,000 plant species of which about 2500 are of known medicinal value.

Research on medicinal plants is an important area of biomedical research in India. With the advent of European Scientific methods many of the reputed medicinal plants came under chemical scrutiny, leading to the isolation of the active principles. Beginning with AD 1500 there was a continuous activity in this area and many of the well known medicinal plants were chemically analyzed and characterized for their active principles. Soon after their isolation the characterized extracts became part of pharmacopoeias of several countries.

1.1 CANCER:

Roughly one person in five in the prosperous countries of the world is at a risk of dying of cancer. Cancer is the disease which comes next to heart diseases in fatality count or mortality. When Richard M. Nixon signed the National cancer Act in 1971, he committed the US to a war on cancer. Since then, the battle has been waged around the world laboratories, hospitals and homes. As a result, death toll from some of the greatest cancers has begun to come down, at least in some segments of population. Some researchers have started to view cancer as a disease that might be managed over the long term,
as it cannot be cured. Eradicating every cell from a cancer patient’s body is a difficult goal and in many cases it may not be possible or necessary. The day of complete cancer management may not be here in the near future, but tools that medicine has now are a start. The ultimate goal remains however unchanged, “We have to keep our eyes on the better sources, which are to kill the cancer with very less toxicity.”

Cancer is the pathological condition which is completely incurable even now. The Allopathic medical system which is the more accepted system of medicine possesses more side effects when compared with that of other two medical systems - Homeopathy and Ayurveda. In both the medicinal systems plants are used as a raw material for the drug preparation. Ayurveda is still widely practiced in India and plants form an important part of Ayurvedic pharmacopoeia. ‘Charaka Samhitha’ was one of the earliest treatises of Ayurveda (600BC) which lists a total of 341 plants and plant products for use in health management. All these interesting aspects about medicinal plants lead us to study their anticancerous property.

Indians and Egyptians were known to have been affected by some malignant growth presumably cancer, over four thousand years ago. These rapidly growing swelling were named ‘Karkinomas’ by Hippocrates, the father of modern medicine, around 400 BC. And it is from this (Karkinoma) that the modern term ‘Carcinoma’ has originated. The term literally means ‘a crab’. He observed over 1,800 years ago that just as a crab’s feet are extended from every part of its body, so is this disease.
1.1.1 Types of Cancer:

Cancer is not a single disease. There are over hundred types of cancer classified according to their site of origin and their appearance. All cancers are classified into four subgroups, each indicating the type of body tissue from which the cancer originated.

1. Carcinoma, a malignant tumor of epithelial or lining tissue (Skin, various membranes and glandular tissues).
2. Sarcoma, a malignant tumor of connective tissue (Bone muscle and other ‘Supportive’ tissues).
3. Lymphoma, a malignant tumor of lymphatic tissue (Hodgkin’s disease and Lymphosarcoma).
4. Leukemia, a malignant disease of the Blood- forming tissues (often referred to as the cancer of the blood).

1.1.2. Treatment of Cancer:

Till about the middle of the nineteenth century, virtually no treatment for cancer existed. It was when general anesthesia came into use that surgical treatment for cancer came to be widely used. The surgery and radiation treatment for cancer were conventional forms of therapy that offer significant levels of cure which have been successfully used. Surgical treatment involves the excision of the tumor and of the tissues surrounding it which may have been affected by the disease. Sometimes surgical removal of tumors may involve the removal of all malignant tissue.

This may result in the removal of all malignant tissue which may result in the alteration of normal body functions. Radiation is the form of treatment
used to destroy the cancer tissues. Sometimes the adjacent normal tissues can be destroyed and devitalized due to exposure to radiation. Both these forms of treatment emphasize an early diagnosis and are effective only if the malignant tissue/organ is removed or destroyed which otherwise spreads to other parts of the body.

Another form of treatment which has been recently discovered and has been effective in treatment of cancer and in relieving pain and management of symptoms is chemotherapy. Many of the drugs used in this form of therapy can bring serious side effects and no single drug is consistently reliable in putting a break in the advancing cancer growth.

With chemotherapy, a search for weaknesses in cancer cells is made to attack them where they are most vulnerable. Since cancer cells are dividing at an abnormally fast rate, many drugs target the processes of cell division. Cancer cells often communicate (or do not communicate) in odd ways, which provides other sensitive spots for treatment. The best drugs are those that attack features that are unique to cancer cells. Among the many chemotherapy agents used as a treatment for cancer an enzyme called L-asparaginase was more efficient and reliable for treatment of blood cancer.

1.2. **L-asparaginase:**

The enzyme L-asparaginase has been a clinically acceptable anti tumor agent for the effective treatment of acute lymphoblastic leukemia and lymphosarcoma. L-asparaginase (L-asparagine amino hydrolase, E.C.3.5.1.1) catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia. L-asparagine is a major requirement by the cells for the production of proteins.
It can be produced within the cell by an enzyme called asparagine synthetase or can be absorbed from outside (consumed in the diet, absorbed into the body and made available to the body’s cells). Tumor cells, more specifically lymphatic tumor cells, require large quantities of asparagine to keep up with their rapid malignant growth. Thus, the asparagine from the diet as well as what can be made by themselves (which is limited) is utilized by them to satisfy their large asparagine demand. Therefore L-asparagine is an essential amino acid for the growth of tumor cells, whereas the growth of normal cells is not dependent on its requirement as it can be synthesized in amounts sufficient for their metabolic needs with their own enzyme L-asparagin synthetase. The presence of L-asparaginase deprives tumor cells of an important growth factor and they fail to survive.

1.2.1 Action of L-asparaginase:

The effective depletion of L-asparagine results in cytotoxicity for leukemic cells (Figure 1). ELSPAR, ONCASPAR, ERWINASE and KIDROLASE are the brand names of L-asparaginase currently in use for the treatment of leukemia. The FDA has approved L-asparaginase for the effective treatment of acute lymphoblastic leukemia (ALL) and lymphosarcoma.

![FIGURE 1 Schematic Illustration of the reaction mechanism of L-asparaginases. The proposed covalent intermediate is formed through nucleophilic attack by the enzyme. Bold arrows indicate nucleophilic attack (Sanson and Jeskolski, 2004).]
1.3 Sources of L-asparaginase

A wide range of microorganisms such as bacteria, fungi, yeast, actinomycetes, algae and plants have proven to be proficient sources of this enzyme.

1.3.1 Bacterial Sources:

Bacterial asparaginases have been the subject of considerable medical interest and are being employed in the therapy of acute lymphoblastic leukemia. L-asparaginase from *Erwinia carotovora* is serologically and biochemically distinct from the L-asparaginase of *E. coli*, although its antineoplastic activity and toxicity is similar. L-asparaginase production has been reported in *Pseudomonas fluorescens* by Mardashev *et al.* (1975). *Mycobacterium phlei* has also been found to be a good L-asparaginase source by Pastuszak *et al.* (1976). L-asparaginase production in *Staphylococci* has been described by Mickucki *et al.* (1977). In *Tetrahymena pyriformis*, maximum activity of the enzyme has been found at the stationary phase of growth and mostly activity has been associated with the endoplasmic reticulum (Triantafolliou *et al.*, 1988). Curran *et al.* (1985) have obtained a specific L-asparaginase that has been further characterized from the thermophilic bacteria *Thermus aquaticus*. Production of L-asparaginase by marine luminous bacteria had been reported by Ramaiah *et al.* (1992). L-asparaginase from *Thermus thermophilus* has been reported as not hydrolyzing L-glutamine by Pritsa and Kyriakidis (2001). L-asparaginase from a new *Erwinia sp.* has been reported by Borkotaky and Bezbaruah (2002).
1.3.2 Yeast Sources:

The production of a new asparaginase from a *Rhodotorula* sp., red imperfect yeast, was reported by Foda, Zedan and Hashem (1980). A homodimer L-asparaginase from *Rhodosporidium toruloides* has been reported by Ramakrishan *et al.* (1996).

1.3.3 Actinomycetes Sources:

Gunasekaran *et al.* (1995) reported L-asparaginase production by *Nocardia* sp. Production of intracellular and extracellular asparaginase from *Streptomyces longsporusflavus* (F-15) has been described by Abdel- Fatah *et al.* (1998). *Streptomyces* sp. isolated from the gut of the fish *Therampon jarbua* and *Villorita cyprinoids* has L-asparaginase activity (Dhevendran and Anithakumari, 2002).

1.3.4 Plant Sources:

Two isoforms of the enzyme have been obtained from the Bryophyte *Sphagnum fallax* by Heeshen *et al.* (1996). Among plants, L-asparaginase has been reported to be produced from *Lupin arboreus* and *Lupin angustiplius* (Borek *et al.*, 1999). Borek *et al.* (2004) reported the expression, purification and catalytic activity of *Lupinus luteus* asparagine beta-amidohydrolase and its homologue in *Escherichia coli*. L-asparaginase activity has also been reportedly found in the soil of roots of *Pinus pinaster* and *Pinus radiata* due to ectomycorrhizal fungi in the wheat belt of Western Australia by Bell and Adams (2004). A K⁺-dependent L-asparaginase from *Arabidopsis*, At3g16150, has been characterized by Bruneau, Chapman and Marsolais (2006).
So far, the nucleotide and deduced amino-acid sequences of four plants L-asparaginase have been reported. They all belong to the potassium-independent family. Comparisons of the sequences from the above plants at nucleotide level showed 63-68% homology. The results for the lupine sequences alone are, respectively, 87-95% with higher plants. The plant asparaginase amino acid sequence did not have any significant homology with microbial asparaginases but was 23% identical and 66% similar to a human glycosylasparaginase (Lough et al., 1992b). A superposition of the active sites of four l-asparaginase from diverse organisms including Human showed a striking similarity indicating to the conservation of the active site in the enzyme.

Figure 2: Superposition of active site of *Lupinus luteus* L-asparaginase (Green) *E.coli* (Blue), Human L-asparaginase (Pink) and Threonine aspartase (Orange).
1.4 **Method for assay:**

Many attempts have been made by researchers for the assay of L-asparaginase and for monitoring its activity. Also, efficient methods for the assay of L-asparagine have been developed. Methods showing the substrate-enzyme relationship have been devised as well. An online gas analyzer for automated enzymatic analysis with potentiometer ammonia detection has been described by Fraticelli and Meyerhoff (1983) where an ammonia electrode was incorporated in conjunction with a predialysis unit. The direct measurement of L-asparagine in human plasma samples through the use of *E. coli* Lasparaginase in the soluble form has been a major clinical application of this system. A multi-analyte miniature conductance biosensor using enzymes such as urease and L-asparaginase and a three-enzyme system consisting of urease, creatinase and creatininase for determining urea, L-asparagine and creatinine, respectively, has been described by Cullen, Sethi and Lowe (1990). The device responded to changes in electrode double layer capacitance, as the ionic strength is increased by the enzyme-catalyzed generation of changed reaction products. An enzymatic method has been developed for the kinetic measurement of L-asparaginase activity and L-asparagine with an ammonia gas-sensing electrode. This method is based upon the deamination of L-asparagine by L-asparaginase from *E. coli* resulting in the formation of ammonia (Tagami and Mastsuda, 1990). A specific quantitative colorimetric assay for L-asparagine by mixing it with dilute ethanolic ninhydrin solution and noting its absorption (max) at 340–350 nm has been reported by Sheng *et al.* (1993). Both L-asparaginase and asparagine synthetase activities can be
measured by this procedure, followed by HPLC amino acid analysis. Determination of L-asparagine using a garlic tissue electrode has been reported by Kim et al. (1995). Garlic tissue cells were employed for conversion of L-asparagine into ammonia. An ammonium gas electrode (ISE) was used as the detector. The combination of L-asparaginase in garlic tissue cells and gas electrode responds linearly to L-asparagine concentration. L-asparaginase from *Erwinia chrysanthemi* was assayed fluorometrically by incubating it with beta L-aspartic acid and measuring the release of 7-amino-4-methylcoumarin (Ylikangas and Mononen, 2000). Rapid analysis of L-asparaginase activity was made by this assay and it can be used for monitoring L-asparaginase activity in the serum of ALL patients during L-asparaginase therapy. A thermostable recombinant asparaginase from *Archaeoglobus fulgidus* was cloned and expressed in *E. coli* as a fusion protein. It was later purified by an immobilized metal ion affinity chromatography method and its activity was determined by monitoring the change in the ammonia concentration in solution. The enzyme was immobilized and used with an ammonium selective electrode (ISE) to develop a biosensor for L-asparaginase (Wang and Bachas, 2002).

An automated kinetic enzymatic method for monitoring plasmatic L-asparaginase activity during therapy of acute lymphoblastic leukemia has been described by Orsonneau et al. (2004). The method is easy and requires less time to perform along with having better specificity and precision than the Nessler method. Tsurusawa et al. (2004) demonstrated a highly sensitive enzyme coupling method to determine the minimum levels of L-asparaginase activity necessary for maintaining asparagine depletion under L-asparaginase
treatment in acute lymphoblastic leukemia. It was shown that asparagine levels are strongly correlated with plasma L-asparaginase activity even at low enzyme activities (50 IU/ml).

1.5 **Properties of L-asparaginases obtained from different sources:**

Effect of oxygen absorption rates on L-asparaginase- II production by *E. coli* 3003C cells was studied by Zubanovic and Johanides (1975) and it was shown that a definite O₂ absorption rate of 0.4 mm/l per min resulted in good growth and high enzyme content. In *Bacillus mesentericus* 43A, the biosynthesis of L-asparaginase was inhibited by the addition of L-aspartic acid in the growing cultures (Eremenko *et al.*, 1976). Comparative experimental evaluation of immuno-depressive and toxic effects of L-asparaginase from *E. coli* and from *Erwinia carotovora* showed that L-asparaginase from *E. coli* is more immuno-depressive and immune toxic than that from *E. carotovora* (Cavanna *et al.*, 1976). Sulphydryl groups (–SH gps) of L-asparaginase from *P. flourescens* were reported to be essential for enzyme activity (Sokolov and Nikolaev, 1976). *E. coli* L-asparaginase has been shown to inhibit the growth of cultured pancreatic cells by Wu *et al.* (1978). A novel L-asparaginase has been characterized by the red imperfect yeast *Rhodotorula rubra* by growth on media supplemented with L-asparagine, L-glutamine or L-alanine as carbon and nitrogen sources. Enzyme activity was highest during the exponential phase of growth. Optimum pH of the enzyme was 7 and it was activated by Mg²⁺ and inhibited by Fe²⁺ and Pb²⁺ (Foda *et al.*, 1980). L-asparaginase of *Aspergillus nidulans* showed the clearest evidence of O₂ repression under N₂ metabolite derepressed conditions (Shaffer *et al.*, 1988).
Four forms of L-asparaginase were isolated from *Tetrahymena pyriformis* (Tsirka and Kyriakidis, 1989) and regulation of enzyme activity was studied. Lipids activated the enzyme and phospholipase-C inactivated the enzyme. L-asparaginase from *Tetrahymena pyriformis* was found in microsomal membranes. The enzyme exhibited an intrinsic phosphorylation activity with a Km value of 0.5 mM for ATP (Tsrika and Kyriakidis, 1990). The pH optima of purified *Staphylococcal* L-asparaginase were found to be between 8.6 and 8.8 while the temperature optima were between 30–32°C. The Km of the enzyme was found from the Lineweaver–Burk plot and was 3–71 times 10–2 M (Sobis and Mickucki, 1991). Two forms of L-asparaginase, L-asparaginase-I and L-asparaginase-II, were extracted and purified from *Thermus thermophilus* by Tsavdaridis, Triantafillou and Kyriakidis (1994). The two forms acted optimally at pH 8.6. An extracellular asparaginase from *Rhodosporidium toruloides* has been reported to be a homodimer having pH optimum of 6.35 and temperature optimum of 37°C (Ramakrishnan et al. 1996).

1.5 **Purification and characterization of L-asparaginase:**

L-asparaginase from *Mycobacterium phlei* was purified by fractionation with ammonium sulphate, absorption of contaminating proteins on calcium phosphate gel and chromatography on Sephadex G-150 and DEAE cellulose. The apparent Km for L-asparaginase was 0.7 mM and the energy of activation was 9800 cal/mol (Pastuszak and Szymona, 1976). Extracellular L-asparaginase from *Candida utilis* was partially purified by acetone and by column chromatography on DEAE, Sephadex A-50 and Sephadex G-200. Optimum pH was 6 and the enzyme was stable for 10 min. at 50°C. Metal
ions, -SH inhibitor and chelating agents did not show any inhibition or activation of the enzyme (Sakamoto et al., 1977). KSCN, NaClO4 and Triton X-100 have been used for the solubilization of enzyme purified from *T. pyriformis* (Triantafillou et al., 1988).

Asparagine catabolism in Bryophytes has been studied and purification and characterization of two forms of L-asparaginase, L-asparaginase-I and L-asparaginase - II, obtained from *Sphagnum fallax* was carried out by anion-exchange chromatography by Heeshen et al. (1996). They observed that the pH optimum of the enzyme was 8.2 and its mol. wt. was 126,000. It had characteristics that were intermediate between those from higher plants and those from microorganisms. L-Asparaginase from *Thermus thermophilus* showed a dual L-asparaginase/kinase activity. It was purified and its apparent molecular mass by SDS-PAGE was found to be 33 KDa by Pritsa and Kyriakidis (2001). Purification of the enzyme from *P. aeruginosa* by Sephadex G-100 gel filtration and SDS-PAGE analysis of the protein was performed by El-Bessoumy, Sarhan and Mansour (2004).

1.6 **Recombinant l-asparaginase:**

Recombinant L-asparaginase has been produced by the use of molecular cloning and genetic engineering techniques. *E. coli* mutants resistant to substrate of L-asparaginase were studied by Spring et al. (1986). It was found that the genes encoding L-asparaginase-I and L-asparaginase-II were not sequence related. Cloning of *E. coli* gene ans B encoding L-asparaginase-II, using a strategy based on PCR, and sequencing the gene was discussed by Bonthron (1990). The amino acid sequence obtained differed at
11 positions from the data previously derived by direct amino acid sequencing. In plants, mainly *Lupin arbores*, isolation and characterization of a cDNA clone encoding L-asparaginase from the developing seeds was reported by Lough *et al.* (1992). Molecular cloning of the gene encoding developing seed L-asparaginase from *Lupinus angustifolius* has also been described by Dickson *et al.* (1992). Expression of L-asparaginase-II encoded by ans B in *Salmonella enterica* was found to be positively regulated by a cAMP receptor protein (cRP) and anaerobiosis (Jennings and Beecham, 1993). The trypsin sensitive L-asparaginase can be made trypsin resistant by genetically fusing its gene with that of a single chain antibody derived from preselected antibody capable of providing protection against trypsin (Newsted *et al.*, 1995). The formation of the fusion protein L-asparaginase ScFv expressed in *E. coli* in the form of inclusion bodies was reported by Guo *et al.* (2000). The fusion protein conferred steric hindrance, blocked cleavage sites and changed the electrostatic potential surface of the enzyme. Wang *et al.* (2002) discussed the cloning and expression of L-asparaginase in *E. coli*. Cloning was done as a DNA fragment generated by PCR. The recombinant plasmid PASN, containing ASN gene and expression vector PBV 220, was transformed in *E. coli* host strains. Greater enzyme activity was observed in recombinant enzymes. Guo, Wu and Chen (2002) reported that recombinant L-asparaginase has great potential as an antitumor agent. Comparisons of the anti-tumor effect of recombinant L-asparaginase with the wild type one *in vitro* and *in vivo*, has revealed this fact. The Lasparaginase gene of *E. coli* and alpha-acetyl actate decarboxylate gene (ALDC) of *B. bravis* were amplified by
PCR and cloned into new vectors transformed into *S. cerevisiae*. Most of the enzyme activities were secreted into the medium and the new vectors had excellent segregation stability (Zhao *et al.*, 2002). Recombinant *Erwinia carotovora* L-asparaginase was expressed in *E. coli* and was later purified as described by Borisova *et al.* (2003). Abundance of AS mRNA was measured by RQ-PCR as reported by Irino *et al.* (2004). The AS mRNA level paralleled the AS enzyme activity and the AS protein level. Cellular levels of AS synchronized with cellular resistance to L-asparaginase in cell lines. Recombinant L-asparaginase from *Erwinia carotovora* purified by 1-step chromatography, was described by Krasotkina *et al.* (2004). The kinetic properties showed that recombinant L-asparaginase combined the main advantages of *Erwinia chrysanthemi* and *E. coli* L-asparaginase- II. Recombinant L-asparaginase was developed by cloning L-asparaginase from *Erwinia carotovora*NCYC 1526 (Er A) and expressing in *E. coli*. The enzyme was purified by anion-exchange chromatography and affinity chromatography on immobilized asparagine (Kotzia and Labrou, 2005). The Km and Vmax of the enzyme were also estimated. Its activation energies were found to be dependent on the substrate. Recombinant human AS, that is C-terminally tagged has been prepared in a *Baculovirus* based expression system. The recombinant enzyme showed high catalytic activity and offers a major possibility in identifying and characterizing inhibitors that may be used to treat asparagine resistant cells (Ciustea *et al.*, 2005). Expression of recombinant L-asparaginase fused to pub leader sequence under the inducible T7 lac promoter in BLR (DE) host cells resulted in optimum extracellular
production in shake flasks. The enzyme had 80% activity of the native enzyme (Khushoo, Pal and Mukherjee, 2005). Mapping B-cell epitopes and ascertaining which of them are clinically relevant for triggering immune reactions, due to asparaginase-II of *E. coli* treatment of ALL, has been reported by Werner, Rohm and Muller (2005). L-asparaginase from *Erwinia chrysanthemi* 3937 (Erl-ASNase) has been expressed in *Escherichia coli* BL21 (DE3) pLysS (Kotzia and Labrou, 2006). The enzyme was later immobilized on epoxy-activated Sepharose CL-6B. The immobilized enzyme retained most of its activity (60%) and showed high stability at 4°C. Recently, studies have been carried out by Tang *et al.* (2006) for the development of a novel growth hormone releasing hormone (hGHRH) analog by using 127 amino acid residues of the C-terminus from L-asparaginase, which act as a fusion partner gene for recombination with the asp-pro-hGHRH(1-44) gene synthesized by the PCR method to form one kind of fusion protein with the unique acid labile linker Asp-pro.

Bacterial L-asparaginase has been a subject of intense research in spite of their undesirable side effects. *E. coli* enzyme has l-glutamine activity as well as endotoxins, which creat so many side effects. The protein produced in *E. coli* are not glycosylated. Glycosylation increases the stability of protein and influences reaction kinetics, solubility, serum half-life, thermal stability, *in vivo* activity, immunogenicity and receptor binding. The present investigation attempts identified a novel source of l-asparaginase from plant which contain less side effect as well as potential against l-asparaginase and the characterization and purification of the potential l-asparaginase.
1.7 **Reference:**


