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Research Article

Extraction, Purification, Characterization And Anticancer Activity Of L-Asparaginase From Novel Plant Source: *Caryota Urens* (Shivjata)

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ABSTRACT

L-asparaginase was screened for during the current study in several *Caryota urens* plant sections. One possible source for L-asparaginase synthesis was identified as the unripe fruit with the highest enzyme activity (925U/ml). The discovery of L-asparaginase in *Caryota urens* is a first. The enzyme was 38% pure, with an 85-fold purification factor, and 3325U/ml of total enzyme activity after purification. It was discovered that the enzyme's kinetic parameters, *Km* and *Vmax*, were 13.8 mM and 100µM/ml, respectively. The partly purified enzyme was shown to be a single protein with a molecular weight of 47 kDa by SDS-PAGE electrophoresis. The cytotoxic effects of L-asparaginase against the K562 and PBMC (peripheral blood mononuclear cell) cell line were investigated; the results showed 26% and 96% (IC50 23.03µg/ml) of cytotoxicity, respectively. Future research can examine the effectiveness of L-asparaginase from *Caryota urens* L., as it exhibits little glutaminase activity and reduced toxicity toward PBMC.

Keywords: *Caryota urens*; L-asparaginase; Purification; Characterization; Anticancer activity

INTRODUCTION

Leukemia is an infrequent hematological disease characterized by abnormal cell growth and the progression of leukocytes and their precursors in the blood and bone marrow (Downing *et al*., 1999). Leukemia is the 10th most familiar cancer in men and 12th most common in women and constitutes 3% of the global cancer burden (Parkin *et al*., 1993). L-asparaginase has persisted as an enthralling research topic after its introduction in the 1960s as an effective anti-leukemic drug (Egler *et al*., 2016). Asparaginase market expected to grow from 564 Million USD in 2021 to 1675 Million USD by 2028. It is estimated to grow at a CAGR of 16.1% from 2022 to 2028 (The insight partner). L-asparaginase (L-asparagine amidohydrolase E.C. 3.5.1.1) is an effective chemotherapeutic agent to treat hematopoietic malignancies such as acute lymphoblastic leukemia, acute myelocytic leukemia, acute myelomonocytic leukemia, lymphosarcoma, reticulosarcoma, chronic lymphocytic leukemia, melanosarcoma, Hodgkin and non-hodgkin lymphoma (Verma *et al*., 2007; Stecher *et al*., 1999). L-asparaginase has numerous applications in pharmaceutical and food industries (Gummadi *et al*., 2018).
L-asparaginase converts L-asparagine to L-aspartic acid and ammonia. Asparagine is synthesized by asparagine synthetase and the gene responsible for encoding is located on chromosome 7q21.3 (Andrulis *et al*., 1990). Norrmal cells have asparagine synthetase which allows them to synthesize asparagines but tumor cellsdeprived of it. Protein and RNA synthesis is inhibited in the absence of asparagines (Goody *et al*., 1975) and as a consequence cell cycle arrest and apoptosis are induced in leukaemia cell lines (Ueno *et al*., 1997).
L-asparaginase is mainly available in bacteria, fungi, algae, plants and animals. Rodents also possess asparaginase in their blood serum but humans have an insufficiency of this enzyme (Cachumba *et al*., 2016; Deshpande *et al*., 2014). L-asparaginase is produced by different sources: *P. aeruginosa 50071* (Eibessoumy *et al*., 2004) , *E. coli* (Sajitha *et al*., 2015), *E. carotovora* (Faret *et al*., 2019), *S. griseoluteus* (Kumar *et al*.,

2011), *Streptomyces ginsengisoli* (Deshpande *et al.,* 2014), *S. karnatakensis*, *S. venezuelae* (Mostafa, 1979), *Vigna unguiculata* (Ali *et al*., 2009), *Lupinus polyphyllus* (Lea *et al*., 1984), *Withania somnifera* (Oza *et al*., 2010) *Phaseolus vulgaris* (Alzobaidy *et al*., 2016) and guinea pig liver (Matthews *et al*., 1974).

Based on amino acid sequence, biochemical properties, structural and functional homologies L-asparaginase has been partitioned into three families: bacterial type (type I & II) asparaginase, plant type (type III) asparaginase and rhizobial type asparaginase (Muller *et al*., 1998; Borek *et al*., 2001; Queshmi *et al*., 2018). Plant-type L-asparaginase belongs to the Ntn hydrolase family and usually has dual iso-aspartyl aminopeptidase/l-asparaginase activity. Regarding amino acid sequence, plant asparaginase differs from microbial asparaginase yet is 23% identical and 66% similar to human glycosyl asparaginase (Lough *et al*., 1992). Compared to bacterial L-asparaginase, plant asparaginase is less toxic (Oza *et al*., 2010). Plant asparaginase is required for protein synthesis and nitrogen fixation, which is significant transport and storage compound for nitrogen (Lough *et al*., 1992). Based on potassium requirement for activation, plant-type asparaginase diversified into K^+-dependent and K^+-independent forms; both are immunologically distinct (Sodek *et al*., 1980). A K^+-independent cast was announced in *Lupinus arboreus (*Lough *et al.,* 1992; Chang *et al.,* 1981*), Lupinus polyphyllus* (Lea *et al*., 1984) and a K^+-dependent form in *Pisum sativum* and many other species (Sodek *et al*., 1980). In plants, seed germination and viability are maintained by plant asparaginases which cleave abnormal iso aspartyl peptide bonds (Michalska *et al*., 2006).

Clinically available asparaginase derived from *E.coli* and *Erwinia chrysanthemi* is currently used in chemotherapy. But, these enzymes exhibit low substrate specificity and high glutaminase activity (Al Zobaidy *et al.,* 2016). Thus, necessary to discover novel source of asparaginase having improved stability, lower glutaminase activity, high substrate affinity, and low *Km* values for therapeutics purpose.

Caryota urens L (family: Arecaceae) is an underutilized multipurpose palm species which is indigenous to a low land forest of tropical Asia including, India, Malaysia, Indonesia, Burma, Philippines and Srilanka (Dallibard, 1999). They are commonly called fishtail palm, toddy palm, wine palm, jaggery palm, elephant's palm, and Indian sago palm. Its familiar name in Srilanka is kithul. The epithet **urens** is Latin for "stinging" alluding to the chemicals in the fruit. The fruit of *Caryota urens* possesses calcium oxalate which causes skin and membrane irritation. *C.urens* is an important medicinal plant which possess analgesic, anti-inflammatory, anti-diabetic, anti-rheumatic, anti-malarial, antioxidant, antimicrobial and larvicidal activity (Chauhan *et al*., 2018).

Very less research data has been reported for plant asparaginase in the treatment of acute lymphoblastic leukemia. Hence, we have tried to explore *C.urens* as a novel source for this enzyme extraction.

MATERIALS AND METHODS

Plant materials

The plant parts (leaves, ripe fruits, unripe fruits, and dry seeds) of *Caryota urens* were collected from the Maroli sugar factory, Navsari, Gujarat (India) into a sterile polythene bag with ice packs and transferred to laboratory for further work. The taxonomic authentication of the plant was done by Prof Dr. Farzin parabiya at the department of Bioscience, VNSGU, Surat. The voucher number assigned to the plant specimen is “VNSGU/BVBRC/2020/09/TC-03”.

Extraction of L-asparaginase from *C.urens*

Distilled water was used after tap water to clean healthy fresh plant components, eliminating any foreign elements or surface dust. Powder was made by crushing dry seeds with a pestle and mortar and then sieving them. Plant asparaginase was extracted at 4^oC using fresh plant parts and dried seed powder. Using a pestle and mortar, homogenize with three volumes of 0.1M Tris-HCl buffer (pH 8.0) containing 50 mM potassium chloride, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA (ethylenediaminetetraacetic acid), and 10% (w/v) glycerol to remove cell debris. This allowed for the extraction of L-asparaginase from the various plant parts. After centrifuging the homogenate sample for 20 minutes at 8000 rpm, the supernatant was gathered and given the designation "crude extract." Every stage was carried out at 4^oC (Abdulla *et al*., 2012).

Optimization of different parameters for extraction of L-asparaginase

Extraction of L-asparaginase was increased by optimizing various parameters like plant parts (leaves, unripe fruits, ripe fruits and dry seeds), buffer types (potassium phosphate buffer, Tris-HCl buffer) and buffer concentrations (0.1M, 0.05M, 0.02M, 0.01M).

Estimation of enzyme activity and protein concentration

The assay used 0.5 ml of crude enzyme extract, 0.5 ml of 50 mM Tris-HCl buffer (pH 8.6), and 0.5 ml of 0.04 mM asparagine. These were thoroughly mixed and incubated for 30 minutes at 37^oC. 0.5ml of 1.5M trichloroacetic acid was applied to halt the reaction after incubation. Supernatant was recovered after centrifuging the mixture. The amount of ammonia released during L-asparagine's catalysis was calculated using Nessler's technique. A test tube containing 0.1 ml of supernatant, 3.7 ml of distilled water, and 0.2 ml of Nessler's reagent was filled. A spectrophotometer was used to assess the color intensity of the reaction mixture at 425 nm after it had been incubated for 20 minutes at 37^oC. For the L-asparaginase test, Nessler's method—

which estimates the amount of ammonia generated during the enzyme's breakdown of asparagine—was used. The result of the reaction between ammonia and Nessler's reagent is a light yellow tint. The amount of ammonia present is directly correlated with color intensity. The ammonia that was liberated was estimated using the standard calibration curve of ammonium sulfate. The asparaginase activity is indicated in international units (IU) (Imada *et al*., 1973). The amount of L-asparaginase that releases one µmol of ammonia in one minute at 37 degrees Celsius is known as one international unit (IU). Bovine serum albumin (BSA) was used as the standard to measure the total protein (Lowry *et al*., 1951).

Purification of L-asparaginase

Every stage of purification was carried out at 4°C. The crude protein fraction was separated using ammonium sulfate saturation at varying degrees, ranging from 20% to 80%. Following each saturation interval, the pellets were collected by centrifugation at 8000 rpm for 30 minutes at 4°C. They were then dissolved in an appropriate volume of phosphate-buffered saline (pH 7.4) and the concentration of proteins and enzymes was assessed. The sample with the maximum enzyme activity was put onto a 1.5 cm × 15 cm Sephadex G-25 column with an eluant of 0.05 M Tris HCl buffer, pH 8.6. Using an ion exchange chromatography column (1.5 cm × 15.0 cm) equilibrated with 0.05 M Tris HCl buffer at pH 8.6, further purification was accomplished. From Sephadex G-25 gel filtration, fractions exhibiting the highest level of enzyme activity were collected. Then put back into a DEAE cellulose 52 column. After that, unattached proteins—that is, positively charged and uncharged proteins—from the partially purified sample were removed from the column using an equal amount of 0.05 M Tris buffer solution (pH 8.6). Negatively charged bound proteins were eluted using linear gradient concentrations of sodium chloride, which ranged from 0.1 to 0.5 Molar. By measuring each eluted fraction's absorbance at 280 nm, protein peaks in the elution steps were found. For characterization, the fractions exhibiting L-asparaginase activity were combined and utilized.

Molecular weight determination by SDS PAGE

To verify the enzymes' molecular weight and purity, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used. The Laemmli method was used for performing SDS-PAGE, using 12% (w/v) acrylamide gel for separation and 4% (w/v) stacking gel containing 10% (w/v) SDS. According to Laemmli (1970), standard protein markers ranged in molecular weight from 18.4 to 97.4 kD.

Kinetic Characterization of purified L-asparaginase

The effect of temperature, pH, and substrate concentration on the rate of reaction was investigated by analyzing the kinetics of L-asparaginase. The following buffers (0.05M) were employed in an enzyme experiment at 37°C to determine the ideal pH: potassium phosphate (pH 5-7), tris-HCl (pH 8.0–9.0), and glycine-NaOH buffer (pH 10–11).

To find out how temperature affected the reaction rate, the reaction mixture was incubated for 15 minutes at various temperatures (25–60°C) in 0.05M Tris buffer (pH 8.6). Different L-asparagine concentrations (0.01 to 0.1M) were used to examine the influence of substrate concentration using a Lineweaver-Burk plot.

In vitro anticancer activity of L-asparaginase

The anticancer activity of pure enzyme was measured against peripheral blood mononuclear cells (PBMC) and chronic myelogenous leukemia (K562) at different concentrations (ranging from 5 to 100 µg/ml). Cell viability was calculated as [(sample - blank)/(control - blank) × 100%] using the colorimetric dye reduction method of [3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl-tetrazolium bromide] (MTT) (Alley *et al*., 1983; Mosman *et al*., 1988). The design was determined how much enzyme (IC50) inhibited 50% of viability. As a control, the viability of the cells in the presence of DMSO rather than enzyme was assessed.

RESULTS

Optimization of different parameters for extraction of L-asparaginase

In arecaceae family, *Caryota urens* plant was screened for L-asparaginase activity and protein content. Different parts of plant (leaves, unripe fruits, ripe fruits, and dry seeds), types of buffers (Tris HCl and Potassium phosphate) and buffer concentrations (0.01M, 0.02M, 0.05M, 0.1M) were optimized to isolate maximum amount of L-asparaginase. Maximum asparaginase activity was detected in the unripe fruit (308 U/ml), in comparison to dry seeds (52 U/ml) and leaves (51 U/ml) extracts respectively. Further, buffer types with various buffer concentrations were optimized in unripe fruit. Maximum enzyme activity was obtained at 0.1 M Tris-HCl buffer (242 U/ml).

Purification of L-asparaginase

L-asparaginase was purified from *C.urens*, using ammonium sulfate precipitation, Sephadex G25 column chromatography and DEAE cellulose ion exchange chromatography. The purification steps of L-asparaginase of *C.urens* seed is given in table 1. The specific activity of the enzyme increased with every step of purification with a minimum loss in quantity, giving a final recovery of 38%.

Table 1 Purification stages of l-asparaginase extracted from *Caryota urens*.

Purification steps	Enzyme activity(U/ml)	Protein concentration (mg)	Specific activity (U/mg)	Purification Fold	Yield (%)
Crude extract	111	5.44	20.36	1	100
Ammonium sulfate precipitation	262	4.3	60.93	2.99	94
Sephadex G-25 gel filtration	437	1.65	264.8	13	33
DEAE cellulose chromatography	665	0.38	1750	85	38

The enzyme precipitated out at 80% of ammonium sulfate saturation. Enzyme loss was minimal following the removal of salt from the enzyme using Sephadex G–25 filtrations. Sephadex G-25 chromatography peak fractions were combined and placed onto DEAE cellulose columns for purification. Enzyme activity analysis and protein estimation were then performed on the final collected fractions. 1.9 mg of total protein with a specific activity of 1750 U/mg and 3325 U total activity were recovered after purification. With 85-fold purification, the final enzyme recovery was 38%. By calculating the optical density at 280 nm, the protein in each fraction was periodically assessed. Fig 1 displays the profile of the protein and L-asparaginase elution from the DEAE cellulose 52, demonstrating the existence of a single peak. Following each purification process, the fractions were examined using SDS-PAGE to determine the molecular weight.

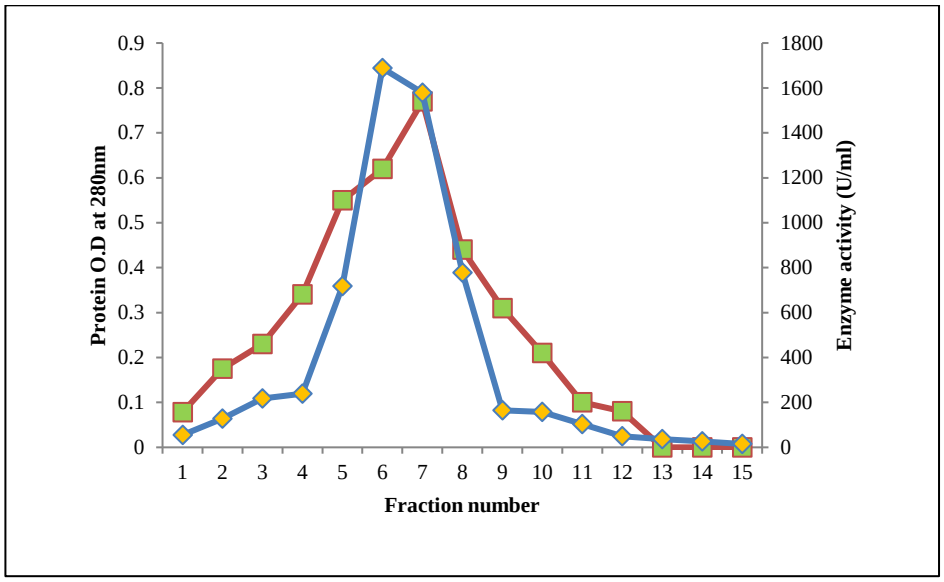

Fig 1 An elution profile for the chromatography of asparaginase on DEAE cellulose column.

Molecular mass determination of L-asparaginase from *Caryota urens*

L-asparaginase, with an approximate molecular weight of 47 kDa, was only visible as a single separate protein band on the enzyme's SDS–PAGE (Fig 2).

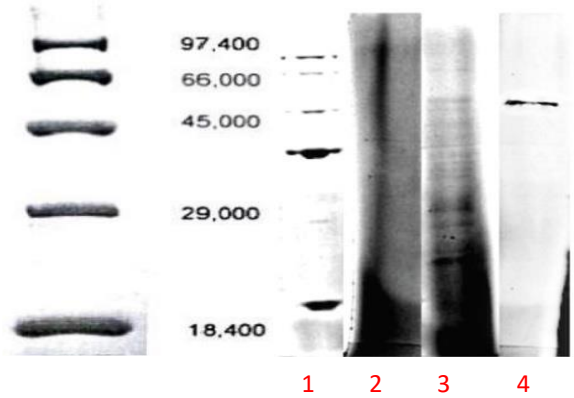

Fig 2 SDS-polyacrylamide gel electrophoresis of L-asparaginase during the purification stages. Lane 1, molecular weight marker; lane 2, crude enzyme; lane 3, ammonium sulfate precipitation and desalting; lane 4, ion exchange chromatography.

Characterization of L-asparaginase:

The activity of L-asparaginase in the plant was evaluated at different levels of pH, temperature, substrate specificity, substrates concentration and incubation time.

Optimum pH and temperature

It was discovered that 8.6 was the ideal pH for enzyme activity. Essential amino acid residues at the active site, which are involved in catalysis, are affected by pH changes in terms of ionization (Fig 3). At pH 8.6, the L-asparaginase assay was run at various temperatures (25–60°C). The bell-shaped curve in the figure indicates that the maximal enzyme activity was reached at 40°C.

Impact of substrate concentration on L-asparaginase activity

The effect of substrate concentration on L-asparaginase activity was examined in this experiment using a range of substrate concentrations, from 0.01 to 0.1 Molar. The data are shown in Fig 4, where an increasing rise in enzyme activity is shown with an increase in substrate concentration. The enzyme that was isolated was found to have kinetic values of 13.8 mM and 100 µM, respectively.

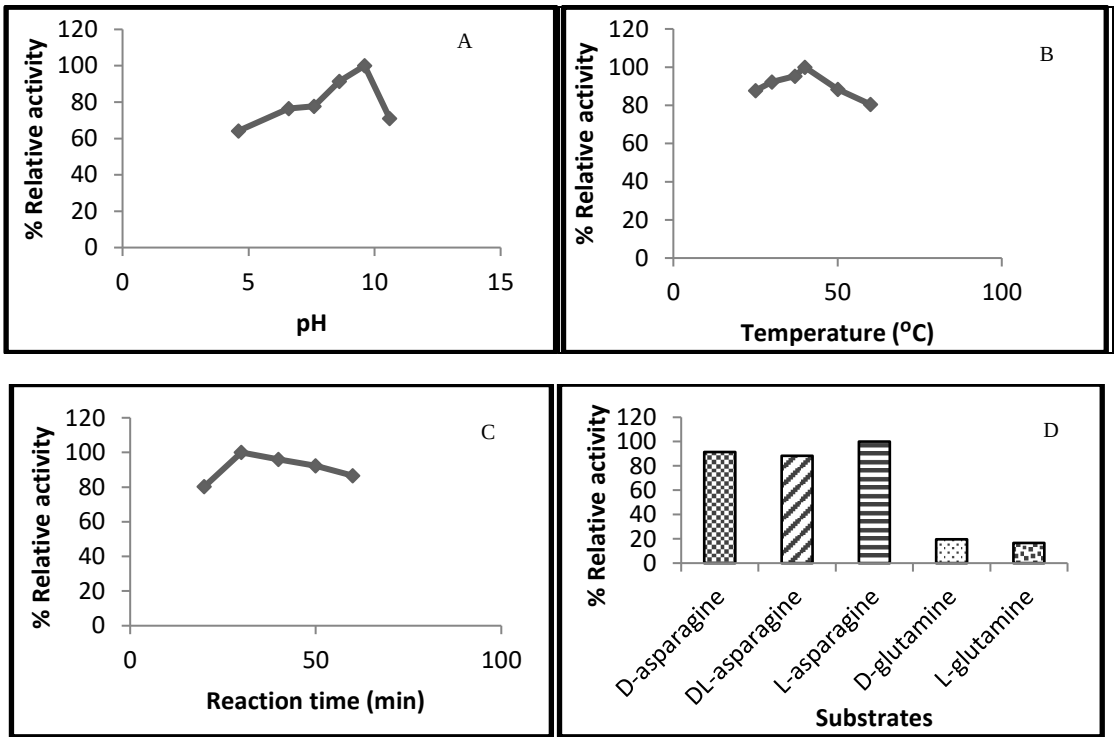

Fig 3 Effect of (A) pH, (B) Temperature, (C) Incubation time and (D) Substrates on the L-asparaginase activity

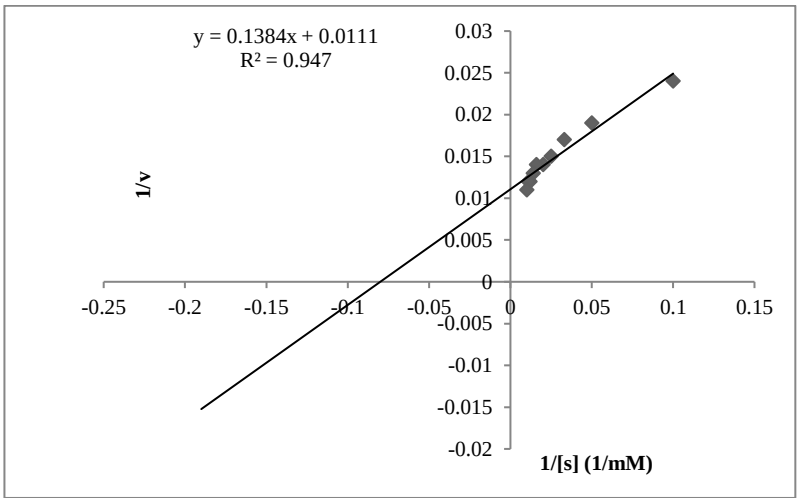

Fig 4 Lineweaver burk plot relating of L-asparaginase derived from *C.urens* to different asparagine concentrations.

In vitro anticancer activity of L-asparaginase

The anticancer activity of L-asparaginase was tested by standard MTT assay employing leukemic cancer cell lines such as K562 (chronic myelogenous leukemia) and normal cell line PBMC (peripheral blood mononuclear cell). The outcomes demonstrated that L-asparaginase's suppression of the tested cancer and normal cell line varied. By treating the K562 cell line with several concentrations of asparaginase for 24 hours, the percentage viability was determined, indicating the efficacy of the medication. Cell viability of PBMC cell line treated with L asparaginase extract was increased by the increase of enzyme concentration. At the concentrations examined, the pure asparaginase enzyme had minimal cytotoxic effects on the normal human cell line, which is not carcinogenic. A cytotoxic effect of 26% was noted for the normal PBMC cell line upon treatment with 100µg/ml of pure L-asparaginase.

The mean percentages of cell viability were 100±07, 92±03, 71±3.2, 46±03, 31±1.5, and 8.5±2.8 in 0, 5, 10, 25, 50, and 100 (µg/ml) of L-asparaginase, after a 24-hour exposure. Its low IC50 value of 23.03µg/ml indicates that the incubation of K562 cell line with increasing concentration of L-asparaginase enzyme effectively inhibited cell growth. With an increase in enzyme concentration, the cell line's sensitivity to L-asparaginase appeared to be dosage dependent, as seen by the marked decline in viable cells. The graph of concentration Vs percentage of cell cytotoxicity is shown in Fig 5. Table 2 shows the *in vitro* anticancer efficacy of PA on K562 cell line and PBMC.

Table 2 *In vitro* anticancer activity of plant asparaginase on K562 cell line and PBMC

		K562 cell line			PBMC		
Sample	Concentration (µg/ml)	% Cytotoxicity	% Viability	IC_{50}	% Cytotoxicity	% Viability	IC_{50}
	5	7.07	92.933083		4.73	95.27	
	10	28.24	71.76985		7.96	92.04	
Plant asparaginase	25	53.6	46.404003	23.0	15.48	84.52	N.A
	50	68.61	31.394622		20.86	79.14	
	100	91.42	8.5803627		26.24	73.76	

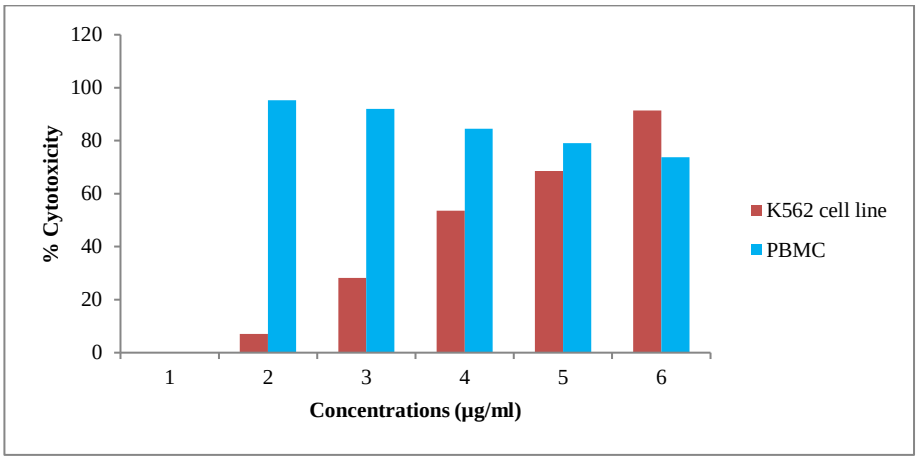

Fig 5 Graphical representations of Concentrations versus % Cytotoxicity

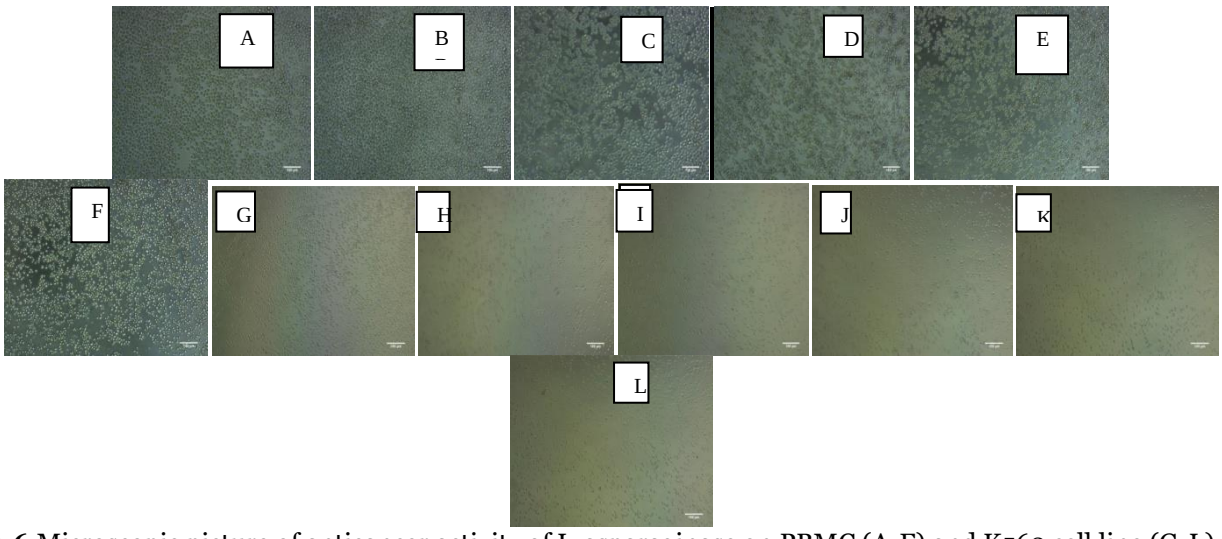

Fig 6 Microscopic picture of anticancer activity of L-asparaginase on PBMC (A-F) and K562 cell line (G-L) at different concentrations

DISCUSSION

The identification of unripe fruit as the most potent source of L-asparaginase underscores the importance of selecting the appropriate plant part for enzyme extraction. The significantly higher activity in unripe fruit extracts compared to dry seeds and leaves suggests that the enzyme's expression or accumulation may be higher in this developmental stage. Moreover, the optimization of buffer conditions further enhances the enzyme's activity, with 0.1 M Tris-HCl buffer emerging as the optimal choice. These findings provide valuable insights for scaling up enzyme production and industrial applications. The presence of L-asparaginase in *C.urens* has not been reported yet. A similar study was carried out for the optimization of extraction buffer and maximum activity of asparaginase in *Withania somnifera* by using Sodium borate buffer as the best extraction buffer (Oza *et al*., 2009) and 0.01M Tris-HCl buffer with pH 8.6 as the best for extraction of asparaginase from *Phaseolus Vulgaris* (Alzobaidy *et al*., 2016).

The successful purification of L-asparaginase with an 85-fold increase in specific activity demonstrates the effectiveness of the purification protocol employed. L-asparaginase is purified from *C.urens* which possesses good asparaginase activity and less glutaminase activity. It can lessen the likelihood that adverse consequences from anticancer therapy will occur. A related investigation using fresh *Pisum sativum* seeds with specific activity (226.5 U/mg) and enzyme activity (605.0 U) were conducted (Abdulla *et al*., 2012). Under ideal conditions, *Vigna unguiculata* produced the maximum asparaginase activity (886.4 U/ml) with a specific activity (1140.7 U/ml/mg), a 31-fold purification, and a 28% yield (Moharib, 2018).
The characterization of the purified enzyme, with a molecular weight of approximately 47 kDa as observed on SDS-PAGE, confirms its homogeneity and purity. The enzyme's stability and optimal pH and temperature for activity (pH 8.6, 40°C) further elucidate its biochemical properties, essential for understanding its function and potential applications. The determination of optimal pH and temperature for enzyme activity provides essential insights into the biochemical properties of L-asparaginase from *Caryota urens*. The observed optimal pH of 8.6 and temperature of 40°C suggest that the enzyme exhibits robust activity under slightly alkaline conditions and moderate temperatures. Understanding these optimal conditions is crucial for the development of enzyme-based processes and applications. The optimal pH for L-asparaginase stability (pH 8) is considered clinically important, because of its proximity to the pH of blood plasma in humans. A similar study published in *V.unguiculata* with optimum temperature and pH of asparaginase was 40^{0}C and 8.5 (Ali, 2009). The results are consistent with the 58 kDa molecular weight of L-asparaginase found in pea leaves (Sieciechowicz *et al*., 1989). The enzyme L-asparaginase was extracted from seeds of *Phaseolus vulgaris* (Mohamed *et al*., 2015) and Withania somnifera (Oza *et al*., 2010), exhibiting a molecular weight of 79 KDa.
The enzyme of *C.urens* have kinetic values of 13.8 mM and 100 µM, respectively. It was found that the Km values of the asparaginases from *L. arboreus* and *L. angustifolius* were the same—6.6 and 7.0 mM, respectively (Chang *et al*., 1981). The Km values of L-asparaginase from bacteria, specifically *Escherichia coli* and *Erwinia carotovora*, are 3.5 and 7.14 mM, respectively, according to Willis *et al*. (1974).

The observed cytotoxic effect of L-asparaginase on the PBMC cell line indicates its potential as an anticancer agent. The dose-dependent decrease in cell viability, with an IC_{50} value of 23.03µg/ml against the K562 cell line, highlights its ability to inhibit cancer cell growth effectively. These findings are particularly significant considering the critical role of asparagine metabolism in cancer cell proliferation and survival. The relatively low IC_{50} value underscores the enzyme's potency and suggests its potential as a therapeutic agent for leukemia treatment. Similar study was executed with *Withania somnifera* for finding anti leukemic activity of L-asparaginase toward human leukemia cells and L-asparaginase is highly effective against leukemia cells having LD_{50} of 1.45±0.05 IU (Oza *et al*., 2010). L-asparaginase of *Vigna unguiculata* exhibited higher cytotoxicity toward HEPG2 and HCT116 and lower cytotocity toward HELLA and MCF7 cell lines (Moharib, 2018).

A medicinal plant *Caryota urens* is a novel source of L-asparaginase that has gained industrial and pharmaceutical significance recently. The demonstrated anticancer activity of L-asparaginase from *Caryota urens* opens avenues for further research into its mechanism of action and potential synergistic effects with existing cancer therapies. Future studies may explore the enzyme's impact on other cancer cell lines and in vivo models to validate its therapeutic efficacy and safety profile. Additionally, investigations into the enzyme's mode of action and interactions with cellular pathways could provide insights for the development of novel cancer treatments targeting asparagine metabolism.

CONCLUSION

Shivjata, or *Caryota urens*, proved to be a productive source of L-asparaginase. For the purpose of extracting the L-asparaginase from *C.urens*, Tris HCl buffer worked well. This enzyme has many good qualities that make it a helpful tool for developing effective anti-cancer treatments, including strong substrate selectivity, catalytic activity across a pH and temperature range, and more. Because *C.urens* L-asparaginase has less glutaminase activity, it may be less likely to cause negative effects when used in anticancer treatments. Its lower toxicity

against anticoagulated blood further bolsters its efficacy as a therapeutic agent, and future research can explore its potential as a treatment for acute lymphoblastic leukemia and lymphoma.

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AUTHOR CONTRIBUTION
Dr. Gayatri Chauhan: Writing-original draft, Investigation, Visualization, Data curation
Dr. Rekha Gadhvi: Supervision, Conceptualization, Validation

CONFLICT OF INTEREST
The authors report no financial or any other conflicts of interest in this work.

DATA AVAILABILITY
Data are available on request to the corresponding author.

CONSENT TO PARTICIPATE
The authors give consent to participate.

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