

High Efficiency, Selectivity against Cancer Cell Line of Purified L-Asparaginase from Pathogenic *Escherichia coli*

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Abstract—L-asparaginase was extracted from pathogenic *Escherichia coli* which was isolated from urinary tract infection patients. L-asparaginase was purified 96-fold by ultrafiltration, ion exchange and gel filtration giving 39.19% yield with final specific activity of 178.57 IU/mg. L-asparaginase showed 138,356±1,000 Dalton molecular weight with 31024±100 Dalton molecular mass. Kinetic properties of enzyme resulting 1.25×10^{-5} mM Km and 2.5×10^{-3} M/min Vmax. L-asparaginase showed a maximum activity at pH 7.5 when incubated at 37 °C for 30 min and illustrated its full activity (100%) after 15 min incubation at 20-37 °C, while 70% of its activity was lost when incubated at 60 °C. L-asparaginase showed cytotoxicity to U937 cell line with IC₅₀ 0.5±0.19 IU/ml, and selectivity index (SI=7.6) about 8 time higher selectivity over the lymphocyte cells. Therefore, the local pathogenic *E. coli* strains may be used as a source of high yield of L-asparaginase to produce anti cancer agent with high selectivity.

Keywords—L-asparaginase, Purification, Cytotoxicity, selectivity index

I. INTRODUCTION

L-asparaginase enzyme (EC 3.5.1.1) specifically catalyzes L-asparagine to L-aspartate and ammonia [1] and play important roles both in the metabolism of all living organisms as well as in pharmacology [2]. L-Asparaginase enzyme maintains nitrogen balance and the level of amino acids within cells [3]. Asparaginases are widely distributed in nature from bacteria to mammals and play a central role in amino acid metabolism and utilization. In human body, L-aspartate plays an important role as a precursor of ornithine in the urea cycle and in transamination reactions forming oxaloacetate in the gluconeogenic pathway leading to glucose formation [4]. It was previously demonstrated that the main function of L-asparaginase is to make cell growth in ammonia-deficient media by regulation of asparagines usage as a nitrogen source for cell nutrition [5]. *Escherichia coli* (*E. coli*) is a source of two types of L-asparaginase. Type I *E. coli* L-asparaginase is located in the cytosol. Whereas, type II *E. coli* is located in the preplasmic region of the bacteria between plasma membrane and the cell envelope [3], possessing higher affinity for amino acid asparagine than type I *E. coli* L-asparaginase [6].

Initially, the interest in L-asparaginase was investigated by their antitumor activity [7]-[8]. L-asparaginase type II has antileukemic activity and was used for treatment of acute lymphoblastic leukemia [6]-[9]-[10]. This enzyme is effective against neoplasias that require asparagine from circulating pools, presumably because the cancer cells have diminished expression of asparagine synthetase. It is uncertain whether neoplastic cell death after asparaginase administration results directly from the depletion of circulating asparagine levels or, from some other metabolite of the asparaginase reaction [6]. Interest in L-asparaginase arose from the striking effect on the treatment of cancer, so this study was aimed to isolation, purification, characterization and determine *in vitro* cytotoxicity activity of L-asparaginase for further utilization on development of therapeutic agent against cancer cells.

II. MATERIALS AND METHODS

A. Materials

All materials and reagents were commercially purchased such as L-asparaginase (Bio Basic, Canada), L-asparagine, Melphalan, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), 5-FU, Nessler's Reagent (Sigma, Germany), Blue Dextran, Catalase, Ovalbumin Sephadex G-100, DEAE-cellulose (Pharmacia, Sweden).

B. Bacterial strains collection

The study has been approved by the Khon Kaen University Ethics Committee for Human Research (HE522136). One hundred and nine of clinical isolates of bacteria gained from urinary tract infection patients in the Central Health laboratory and Educational laboratory in Baghdad, Iraq. Bacterial identification was confirmed by microscopic, routine biochemical tests and API 20 E system (BioMerieux, France).

C. Screening of the ability of isolates to produce L-asparaginase

Enzyme activity of isolates strains was determined by both semi-quantitative and quantitative assay. The strain that showed high activity of L-asparaginase was subjected to further purification. Isolated strains were checked for their ability to produce L-asparaginase by Semi quantitative assay

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following methods of Cedar and Schwartz [11], by using L-Asparaginase quality detection solid medium (10 mM L-asparagine, 2gm agar (Hi-media, India) and 20 gm sucrose dissolved in 100 ml distal water). Single colony from each isolated strains was transferred (20-40 strains) to nitrocellulose membrane filters that were placed on the surface of nutrient agar (Hi-media, India), then the filters were incubated for 18 hr at 37 °C, and transferred to detection media and incubated for 6 hr at 37 °C. The nitrocellulose membranes bearing the colonies were removed and replaced with Whatman no. 1 filter paper for 1 min in order to absorb amino acids. After drying, papers were dipped in 0.5% ninhydrin in acetone. The conversion of asparagine to aspartic acid was indicated by a blue spot given by aspartic acid corresponding to the position of the positive colonies. While colonies with Low density or absence of a blue spot indicated the position of L-Asparaginase appeared with brownish background.

Moreover, quantitative assay was carried according to the method described by Mokran [12] by using direct Nesslerization method to determine the enzyme activity in bacteria. Growth cells were washed twice with 0.05M Tris-HCl buffer pH 7.4 and centrifuged at 6000 rpm, 4 °C, the pellet resuspended and incubated with slow shaking for 4 h at 37 °C, then centrifuged at 6000 rpm at 4 °C for 15 min. The liberated ammonia was determined in supernatant by Nesslerization methods. Briefly, the method was achieved to estimate ammonia concentration with the aid of standard curve of ammonia (plotted between the different concentrations of ammonium sulphate versus an absorbance at 436 nm). The mixture of 4.4 ml distilled water was prepared with 0.1 ml from each tested samples and added 0.5 ml Nessler's indicator. In the same method blank were prepared by mixed 4.5 ml of DW with 0.5 ml from Nessler's indicator. The enzyme activity was determined according to the following equation:

$$\text{Enzyme activity} = \frac{[\text{NH}_3] \text{ in micromole}}{\text{Reaction time (min)} \times 14}$$

Reaction time was estimated to be 240 min of enzyme activity in cells and 30 min in cell free extracts. International unit IU of L-asparaginase is calculated from the amount of enzyme which liberates 1 micromole of ammonia in 1 min at 37 °C. Protein content was determined by the method of Bradford [13].

D. L-asparaginase crude extracts preparation and purification

The crude extract of enzyme was prepared according to method described by Mokrane [12] with some modification. Selected local *E. coli* strain was cultured in 4 l of production medium consisting of basal synthetic medium (10.75 g NaHPO₄, 3.55 g KH₂PO₄, 0.025 g MgSO₄, 0.0025 g MnCL₂.4H₂O, 0.0027 g FeSO₄.7H₂O, 0.015 g CaCL₂.6H₂O in one litter DW), 0.6 % yeast extract and 0.2 % Trypticase soy broth in a shaker incubator for 4 hr at 37 °C. Then culture was

incubated for further 20 hr without shaking. Bacteria were collected after centrifugation at 2000 rpm, 4 °C. The supernatant was decanted, the pellet washed by sterilized DW, and resuspended in 70 ml of sucrose buffer, which was prepared from 13 g of sucrose in 100 ml Tris-HCL (25 mM, pH8). Lysozyme (biorad, England) and EDTA were then added to suspension and incubated at room temperature for 20 min to convert cells to spheroplasts, and then L-asparaginase crude extract (supernatant) was collected after 10 min of centrifugation at 12000 g, 37 C°.

L-asparaginase was purified after reducing the volume to 13 ml using the Amicon® ultrafiltration apparatus (Amicon, England). The enzyme activity was determined based on the ammonia releasing measured by the direct Nesslerization method as described previously. However, the method had minor modification by adjustment of the incubation time of the enzyme sample and substrate to 30 min. Estimation of protein concentration was determined as previously. After ultrafiltration, 10 ml of the concentrated crude extract was loaded slowly on an ion exchange chromatography column packed with DEAE-cellulose. The column was pre-equilibrated and washed with a 0.02 M of phosphate buffer, pH 8 to remove unbounded proteins. The enzyme crude extract was eluted by two mobile phase systems continuously with a linear gradient. The first mobile phase contained low salt (0.02 M of phosphate buffer, pH 8) and the second one contained high salt solution (0.2 M phosphate buffer, pH 6.5). The 3 ml of fractions were collected from 0.5 ml/min flow rate adjusting. The active fractions determined by direct Nesslerization method were collected and dialyzed against sucrose for 5 hr. The enzyme activity and the protein concentration were also determined for concentrated sample according to the previous method. The 5 ml of dialyzed sample was loaded slowly over the Sephadex G-100 column which was pre-equilibrated with a 0.02 M of Phosphate buffer pH 8. The protein was eluted from the column with the same buffer and the flow rate was adjusted to 20 ml/ hr and 3 ml for each fraction was collected. Then active fraction which showed the highest activity of the L-asparaginase against the amino acid L-asparagine measured by direct Nesslerization method was collected and dialyzed against sucrose. The enzyme activity and protein concentration were determined. The purified L-asparaginase enzyme was stored at -20 °C for further studies.

E. Characterization of purified L-asparaginase

E.1 Determination of L-asparaginase molecular weight

Molecular weight was determined by gel filtration chromatography according to Alazawe [14] by using Sephadex G-100. Briefly, void volume of the packed gel was estimated by using Blue Dextran. Three milliliters fraction with 600 nm absorbance was collected. The fraction number and optical density to obtain void volume of the packed gel was calculated according to the equation:

$$\text{Void volume (Vo)} = \text{fraction number on the peak} \times 3 \text{ ml}$$

Solutions of the standard proteins were loaded separately at the top of the gel and fractions were collected. An absorbance was read for each fraction at 280 nm. Elution curve was plotted between absorbance and fraction numbers. Standard curve was constructed between log of molecular weight of each standard protein and the ratio of V_e/V_o of each protein and L-asparaginase molecular weight was extrapolated from the plot by aid of standard proteins.

E.2 Determination of molecular masse and purity

Molecular masses and purity of L-asparaginase were determined by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Garfin [15]. The prestained protein markers (Biolabs, England), broad range 7 to 175 kDa were used in detection of molecular masses.

E.3 Optimum pH for L-asparaginase activity and stability

Fixed volume of 100 μ l of each purified enzyme solution was mixed with 100 μ l buffer of different pH from 4 to 9.5. The acetate buffer was used for pH range of 4 to 5.5. The phosphate buffer was used for pH range of 6 to 8. The glycine buffer was used for pH range of 8.5 to 9, respectively. Enzyme activity was measured directly based on the method mentioned above for all of the solutions in a sequenced manner without incubation. The optimum pH for L-asparaginase stability was studied as following. The solution mixtures of enzyme with various pH were incubated for 30 min in a water bath at 37 °C and were directly cooled in ice bath. Enzyme activity of each tube was measured simultaneously as mentioned above for all of the solutions.

E.4 Optimum temperature for L-asparaginase activity and stability

Fixed volume of 100 μ l of enzyme solution was incubated for 30 min with 100 μ l, 0.02 M of Phosphate buffer, pH 8 at different temperature ranging from 20 °C to 60 °C in 5 degree increments. After the end of the incubation periods, enzyme activity was measured as previously mentioned in optimum pH for L-asparaginase stability. Only modification of incubation temperature of the enzyme and substrate were made. The optimum temperature for L-asparaginase stability was studied as following. The solution mixtures of 100 μ l of enzyme solution and 100 μ l of 0.02 M of Phosphate buffer, pH 8 was incubated for 15 min at different degree of temperature varying from 20 °C to 60 °C in 5 ° increments and was directly cooled in ice bath. Enzyme activity of each tube was measured as previously described.

E.5 Determination of kinetic properties (K_m and V_{max})

The initial velocity of enzyme, L-asparagine reaction and the K_m , V_{max} values were determined from the Lineweaver-Burk [16].

F. Cytotoxicity assay of L-asparaginase

L-asparaginase were tested for cytotoxicity against leukemia cancer cell line, U937 and compared with two potent

chemotherapeutics drug (melphalan and 5-FU) by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Prayong *et al.*, and Mossman [17], [18]. Viable cells counting were performed before each cytotoxicity experiment. Drug Stocks were dissolved in DMSO (dimethyl sulfoxide) at a concentration 100 mg/ml. Stock samples were diluted with RPMI medium to desired concentrations of L-asparaginase ranging from 0.001 to 5 IU/ml and also for melphalan and 5-FU ranging from 10 to 500 μ g/ml. Experiments were done in duplicate. Hundred microliters of cells suspension were seeded in 96 well at the density of 5×10^5 cell/ml and incubated at 37 °C in 5 % CO_2 for 24 h. The cells were treated with various concentrations of samples in total volume (200 μ l/well) for 24 h. At 21 h, cells were centrifuged at 2000 rpm for 10 min and resuspended with 180 μ l of RPMI medium to rinse off the treated samples, and 20 μ l of MTT solution (5 mg/ml) was added to each well and incubated at 37 °C for another 3h. Then 180 μ l of the medium was discarded from each well. The formed formazan crystals were dissolved with 180 μ l of DMSO. An absorbance of formazan was detected by a dual wavelength UV spectrometer at 570 nm with 650 nm reference wavelength. The percentage of cytotoxicity compared the untreated cell as a control was determined. The plot of % cytotoxicity versus sample concentration was used to calculate the concentration which exhibited 50% cytotoxicity (IC_{50}).

G Lymphocyte isolated and selectivity index determination

Normal lymphocyte was used to determine the non-toxic dose of the tested sample. Lymphocyte was isolated from blood provided from blood center, Faculty of Medicine, Khon Kaen University, Thailand, according to the method Described by Alhilli [19] with some modification. Selectivity index (SI) was calculated from the IC_{50} ratio in lymphocytes over the U937 cells [17]. 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.

III. RESULTS AND DISCUSSION

A. Identification of Bacterial isolates

Eighty one isolates of *E. coli*, 13 *Proteus mirabilis*, 9 isolates *Proteus vulgaris* and 6 *Serratia marcescens* were isolated from the UTI patient's urine samples.

B. Screening of the ability of isolates strains to produce L-Asparaginase

Isolated species and strains were checked for their ability to production of L-asparaginase by Semi quantitative and quantitative assay. Generally, *E. coli* strains isolates showed the highest activity (2.1 U/mg) with more potent L-asparaginase production than other isolates. Therefore, *E. coli* strain no.3 was chosen as candidate for further extraction and purification.

C. Purification of L-asparaginase

The yield, activity, protein content and purification fold of the purified L-asparaginase from various purification steps are summarized in Table II. Spheroplasts method was achieved to prepare crude extract because it was more selective method to release L-asparaginase type II with greater affinity to L-asparagine that located in preplasmic in the bacterial cell. Reference [20] found that over 90% of L-asparaginase I remained within the spheroplasts and none was detected with intact cells. The partial purification of crude extract that was affected by ultrafiltration showed that most of enzyme activity was preserved in the concentrated crude extract. The method which was applied in this study was similar to the method of Mokran [12] in which the sample was concentrated by ultrafiltration before being exposed to ion exchange chromatograph. The results of the present study were also in agreement with their result which revealed that ultrafiltration step resulted in considerable loss of enzyme activity. Ten ml of crude extract was passed through the ion exchange column. The unbound protein was removed (Fig. 1) then the column was eluted.

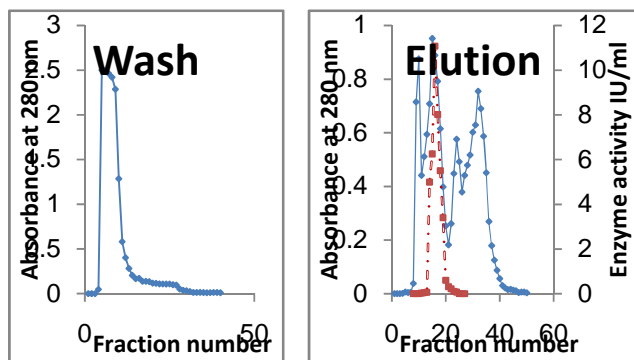


Fig. 1 Ion exchange chromatography for L-asparaginase through DEAE-Cellulose column (2.5×15) cm. The column was calibrated with phosphate buffer pH 7.5, flow rate 30 ml/hrs and 3 ml/fraction (Dashed line, enzyme activity).

The collected fractions with higher enzyme activity were dialyzed against sucrose and were applied to Sephadex G-100 column (Fig. 2).

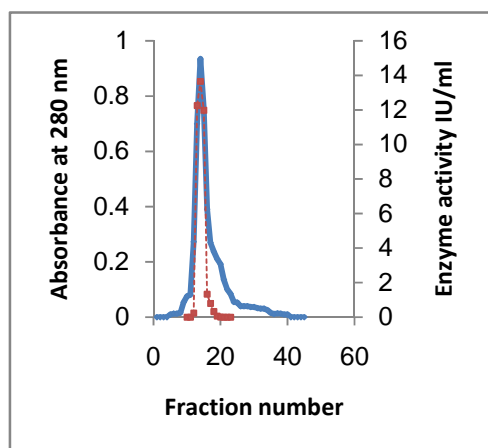


Fig. 2 Gel filtration chromatography for purification L-asparaginase by using Sephadex G-100 column (1.6×90) cm. The column

calibrated with 0.02 M Phosphate buffer, pH 8, flow rate 30 ml/hr and 3 ml/fraction (Dashed line, enzyme activity).

The specific activity increased to 178.57 U/mg and the enzyme was purified approximately 96 fold after ion exchange and gel filtration chromatograph (Table I). Two previous studies reported the purity of the L-asparaginase to 43.38 fold with specific activity of 22.68 U/mg protein [12] and purity of 1240-fold with the specific activity of 620 U/mg from 40 liter-crude extract [21]. Hence, our result, showed higher enzyme purification of 96 fold with specific activity 178.57 U/mg from one liter-crude extract compared to the previous studies.

E. Characterization of purified L-asparaginase

The molecular weight was determined by gel filtration in Sephadex G-100 based on the standard curve made by standard proteins (Lysozyme 14, 4 kDa, BSA 67 kDa, IgG 160 kDa and Catalase 232 kDa). The Blue Dextran 2000, which is the high molecular weight blue dye was used as a guide for assessing the correct column packaging and the estimation of void volume (V_0). The curve plotted between absorbance at 660 nm and fraction numbers were made and the resolution of one identical peak indicated the correct column packaging with resin without crack that causes error in elution sample in same time resulting more identical peak. Samples of standard proteins solutions were also loaded on to the column separately. Absorbance at 280 nm was used for all fractions to determine elution volume (V_e) for each measured standard proteins. A standard curve was plotted between the values of V_e/V_0 and the log values of the molecular weights of the standard proteins. The molecular weight of the enzyme was estimated with the aid of standard curve and its $138,356 \pm 1000$ Dalton. Our result was in agreement with the result of [22] which estimated the molecular weight of purified L-asparaginase from *E. coli* to be 139,000 Dalton. Reference [12] was also estimated the molecular weight of purified L-asparaginase from *E. coli* to be 151,365 Dalton. The molecular mass of purified L-asparaginase was also estimated by SDS-PAGE (Fig. 3) with the aid of migration distance of prestained standard proteins markers in the gel.

TABLE I THE YIELD, ACTIVITY, PROTEIN CONTENT AND PURIFICATION FOLD OF THE PURIFIED L-ASPARAGINASE FROM *E. coli*

Step	Activity U/ml	Specific activity U/mg	Total activity	Yield	Purification fold
Crude extract	4.10	1.86	287	100	1
Ion exchange	6.93	19.8	124.74	43.46	10.6
Gel filtration	12.5	178.57	112.5	39.19	96.0

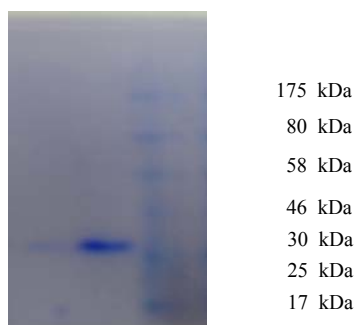


Fig. 3 SDS-PAGE Polyacrylamide gel electrophoresis of purified L-asparaginase from pathogenic *E. coli*.

Electrophoresis was carried out on a 10 % polyacrylamide gel containing 10 % SDS. Lane A, purified L-asparaginase; Lane B, commercial L-asparaginase; Lane C, prestained protein marker, Broad range (7-175 kDa).

Results showed that the molecular mass of purified L-asparaginase was $31,024 \pm 100$ Dalton and it was very close to the molecular mass of commercial *E. coli* L-asparaginase (31,731 Dalton) and in a good agreement with the result of Cedar and Schwartz [22] who found the molecular mass for purified L-asparaginase from *E. coli* to be $32,000 \pm 3,000$ Dalton. Mokran [12] was also estimated the molecular mass for purified L-asparaginase from *E. coli* to be 36,307 Dalton. The differences between value of the molecular weight and the value of the molecular mass suggesting that the enzyme is a tetramer formed of four identical subunits. Also purified L-asparaginase showed one band in the slab SDS-PAGE electrophoresis mini-gel (Fig. 3) in denaturation condition indicating the purity of our enzyme. The purified L-asparaginase showed the highest activity at pH 7.5 but with more stability at pH 8 (Fig. 4), which is closed to blood pH.

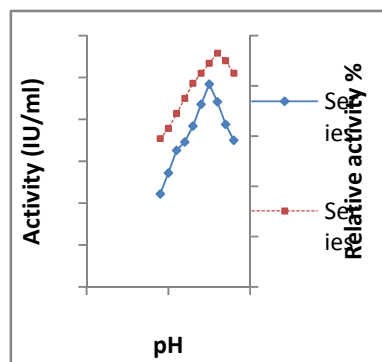


Fig. 4 Effect of pH on purified L-asparaginase activity and stability (-○- Series1, enzyme activity, -□- Series2, relative activity).

Also the purified enzyme exhibited relative its full activity when incubated for 15 min at temperatures ranging 20-37 °C. It was observed that 70 % of enzyme activity was lost when incubated at 60 °C (Fig. 5) and highest activity at 37 °C (Fig. 6).

The kinetic properties of L- asparaginase were obtained using L-asparagine as substrate in a Lineweaver-Burk plot (Fig. 7). The apparent K_m of purified L-asparaginase for L-asparagine was 1.25×10^{-5} mM and V_{max} was 2.5×10^{-3}

M/min. The K_m value of purified L-asparaginase is the same as the K_m values of L- asparaginase reported previously by Scheetz et al.

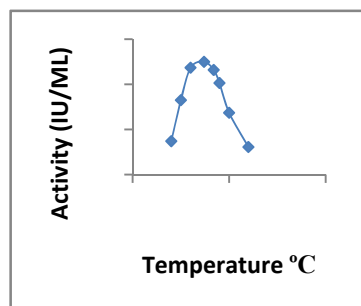


Fig. 5 Effect of temperature on purified L-asparaginase activity.

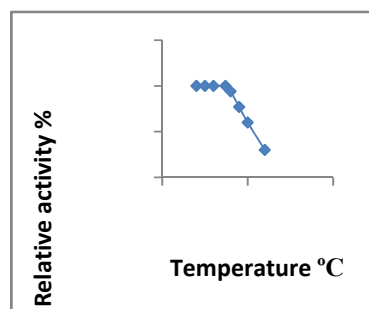


Fig. 6 Effect of temperature on purified L-asparaginase stability.

[23] which was 1.25×10^{-5} mM. Our data indicated that the obtained purified L-asparaginase was followed the Michaelis-menten Kinetics.

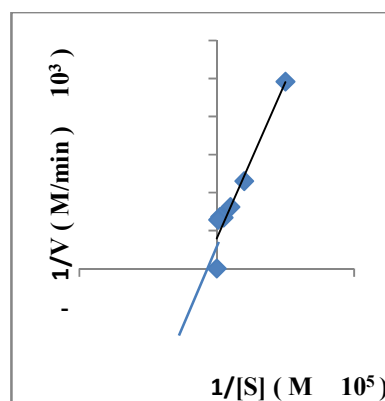


Fig. 7 Determination of k_m and V_{max} of purified L-asparaginase from pathogenic *E. coli* by Lineweaver-Burk plot when S means substrate concentration and V means velocity.

E Cytotoxicity of L-asparaginase in U937 cell line
 Cytotoxicity results of L-asparaginase are summarized in Table II. L-asparaginase showed positive activity and high selectivity against leukemia cell line U937 (SI = 7.56), when compared with melphalan (SI = 1.85) and 5-FU (SI = 1.26). The result illustrated high selectivity of L-asparaginase which indicated high specific toxicity of L-asparaginase only to the cancer cells. IC₅₀ value is common to demonstrate the cytotoxic activity in previous studies [24]. While this study, not only IC₅₀ value but also SI data was use to demonstrate

the selectivity of L-asparaginase to cancer cell lines over normal lymphocyte.

TABLE II *IN VITRO* CYTOTOXICITY ACTIVITY OF L-ASPARAGINASE, MELPHALAN, AND 5-FU ON U937 CELL LINE AND LYMPHOCYTE

Drug (IC ₅₀)	Lymphocyte	U937	SI
L-asparaginase (IU/ml)	3.9 ±0.35	0.5±0.19	7.56
Melphalan (µg/ml)	42.5±5.24	22.9±6.25	1.85
5-FU (µg/ml)	inactive*	395.35±34.60	1.26

* IC₅₀ > 500 µg/ml is considered to be inactive.

IV. CONCLUSION

Present study indicates that the local pathogenic *E. coli* strains can be used as a potential source of L-asparaginase. Furthermore, the purified L-asparaginase showed potential cancer therapeutic agent against U937 cell line with higher selectivity over the lymphocyte cells and should be considered for further pharmaceutical use as a anti cancer candidate.

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