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Molecular investigation and anticancer properties of purified L-Asparaginase from *E. coli* isolate against, CaCo2, MCF7 and PC3 cell lines

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ABSTRACT

A clinical bacterial isolate from a patient urine sample in Kasr El-Aini was identified by biochemical and molecular means to be *E. coli* . This isolate was optimized for production of L-asparaginase (L-asparagine amidohydrolase), a relatively widespread enzyme found in bacteria, eukaryote and mammals but not man. This enzyme catalyzes the deamidation of L-asparagine to L-aspartic acid and ammonia. The production of L-asparaginase was achieved through optimization of fermentation parameters and it showed 6.05 IU of enzyme activity. The produced L-asparaginase was then purified by means of chromatography techniques and tested against three different cell lines for its anticancerous activity, human colon cancer CACO-2, Human breast Cancer MCF-7 and Human cancer prostate PC-3. The expression for the regulatory genes BAX, P53 and BCL2, was analyzed by RT-PCR and it was clear that L-Asparaginase enzyme shows anticancer activity against (MCF-7) and (PC-3), where it was non-effective to the cell line (CACO-2). It was also noticed that BAX and P53 genes were upregulated under the effect of Asparaginase enzyme and that BcL2 gene was down-regulated in Human Breast and prostate Cancer cell line while Human colon cell line was not.

Keywords: L-asparaginase, Purification, *E.coli*, Antitumor activity, Expression genes

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INTRODUCTION

L-asparaginase(L-asparagine amidohydrolase; EC.3.5.1.1) catalyzes the deamidation of L-asparagine to L-aspartic acid and ammonia, is relatively wide spread enzyme found in many tissues, bacteria, plant and in the serum of certain rodents, not of man. The microbial sources are very common for L-asparaginase, because they can be easily cultured and extraction, purification of L-asparaginase from them is also convenient, facilitating for the Industrial scale production. The most commonly used microorganism to produce L-asparaginase are *Erwinia caratovora*, *Bacillus sp*. *Corynebacterium glutamicum*, *Pseudomonas stutzeri* and *E. coli*[1]. L-asparaginase from *E .coli* has excellent power to inhibit the activity of tumour cells, and that from *E. chrysanthemi* is also pharmacologically active[2]. However, L-Asparaginase from bacterial sources causes hypersensitivity in the long term leading to allergic reactions and anaphylaxis.[3]

The anti-tumour activity of the enzyme is based on the dependence of certain tumour cells on an extracellular supply of l-Asn. Unlike normal cells, malignant cells can only synthesize l-Asn slowly, due to their deficiency in l-asparagine synthetase.[4,5] Thus, depletion of the circulating pools of l-Asn by l-ASNase leads to the destruction of the tumour cells, since they are unable to complete protein synthesis. The functional form of l-ASNase exists as a tetramer of identical subunits, with molecular mass in the range of 140–160 kDa [6; 7, 8; 9]. Each of the four active sites is located between the N- and C-terminal domains of two adjacent monomers. Thus, the l-ASNase tetramer can be treated as a dimer of dimers. Despite this fact, the active enzyme is always a tetramer.[10,11]

l-ASNases from *Erwinia chrysanthemi* and *Escherichia coli* are currently in clinical use as effective drugs in the treatment of Hodgkin's disease, acute myelocytic leukaemia, acute myelomonocytic leukaemia, chronic lymphocytic leukaemia, lymphosarcoma, reticulosarcoma and melanosarcoma[12; 13]. Unfortunately, despite evidence of toxicity, the main side effects of l-ASNases are: liver dysfunction, pancreatitis, diabetes, leucopoenia, neurological seizures, and coagulation abnormalities that may lead to intracranial thrombosis or haemorrhage [13]. Another limiting factor of l-ASNase treatment is the development of hypersensitivity, which ranges from mild allergic reactions to anaphylactic shock.[5] Because the l-ASNases from *E. coli* and *Er. chrysanthemi* are immunologically distinct, they provide an important alternative therapy to patients who become hypersensitive to one of the enzymes[5]. With a view to characterize enzymes with less toxic side effects, several members of a larger family of homologous l-ASNases have been thoroughly investigated over many years[14,15]. In addition, because the anti-

tumour activity of l-ASNase is also a function of its half life in the blood [16], attempts have been made to increase the half-life, for example by entrapment of the enzyme in liposomes [17] and by covalent coupling to macromolecules such as dextran [18] albumin[19] or monomethoxypolyethylene glycol (mPEG) [20] which is on the market. Unfortunately, none of these approaches have managed to eliminate the disadvantages of l-ASNase treatment, leaving scientists with the need to identify and characterize new enzymes with better properties.

L-Asparagine is a non-essential amino acid used by immature lymphocytes for their proliferation. It's biosynthetic pathway involves the conversion of oxaloacetate by transaminase enzyme to aspartate followed by transfer of amino group from glutamate to oxaloacetate producing α- ketoglutarate and aspartate. The enzyme, Human asparagine synthetase in healthy cell converts aspartate to asparagine by using ATP as energy source. Tumor cells have an unusually high requirement for the amino acid asparagine and cannot synthesize sufficient endogenous L-asparagine due to very low levels of L-asparagine synthetase and therefore are dependent on serum levels of asparagine for their proliferation and survival [21] or one more attributed reason is the inability of these cells to increase L-asparagine synthetase activity after L-asparaginase administration So they use both asparagine from the diet (blood serum) as well as what they make themselves (which is limited) to satisfy their large L-asparagine demand. Thus, administration of L-asparaginase deprives dependent tumor cells of their extracellular source of L-asparagine and lead to apoptosis.

MATERIALS AND METHODS

Microorganism: Clinical isolate from a urine sample collected from kasr eleini, Cairo, Egypt.

Chemicals and Buffers

Anhydrous L-asparagine, trichloroacetic acid, Nessler`s reagent chemicals, bovine serum albumin and reagents for electrophoresis were obtained from Sigma chemical CO. Sephadex G-100 and Sephadex G-200 for chromatography were obtained from Sigma chemical CO. Molecular weight markers for SDS-polyacrylamide gel electrophoresis were purchased from Fermentas CO. All other chemicals were of the best analytical grade and of high purity. Buffers were prepared according to Gomori, (1955) [22.]

Cell lines:

Three cell lines;Caco-2,Mcf-7 and Pc-3 were obtained through the American Type Culture Collection (ATCC; Manassas, VA, USA).

Identification of bacterial isolate:

Different morphological, cultural and physiological characteristics of the bacterial isolate were studied for identification purpose and compared with standard description of Bergey's Manual of Determinative Bacteriology and the use of biolog kit.

Analysis of 16S rRNA Gene Sequence:

The purified bacterial isolate was cultured in nutrient broth. After centrifugation at 4500×*g*, 10 min, at 4°C, and twice washing with distilled water, the pellets were selected for DNA extraction and PCR amplification. Bacterial DNA was extracted by heat extraction method. The 16S rRNA gene was amplified by PCR, using the universal prokaryotic primers 5′-ACGGGCGGTGTGTAC-3′ and 5′-CAGCCGCGGTAATAC-3′, which amplify a ~800-bp region of the 16S rRNA gene. PCR was performed in a final volume of 50 µl containing PCR amplification buffer (1×), *Taq* DNA polymerase (2.5 U), dNTPs (4 mM), primers (0.4 µM) and template DNA (4 ng). Amplification conditions were initial denaturation at 94°C for 5 min, 10 cycles at; 94°C for 30 s, 50°C for 30 s and 72°C for 2 min. 20 cycles at; 92°C for 30 s, 50°C for 30 s, and 72°C for 2.5 min with a final extension of 72°C for 5 min. *Taq* polymerase was added to the reaction after initial denaturation. The lower denaturation temperature (92°C) during the 20 cycle step was used to avoid loss of enzyme activity[9] The samples were electrophoresed in a 1% (w/v) agarose gel, using TBE buffer containing ethidium bromide (1 µg/ml). A single ~800 bp DNA fragment was cut and extracted from the gel, using a Core Bio Gel Extraction Kit. The sequence was determined by the CinnaGen Company, Germany.

Construction of phylogenetic tree:

The retrieved gene sequence was compared with other bacterial sequences by using NCBI BLAST search that is available from the National Centre for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and GeneDoc software, version 2.6.002.for their pair wise identities. Multiple sequence alignment and the phylogenetic tree were constructed with MEGA 4.0 software (http://www.megasoftware.net) by using the neighbor joining (NJ) method with 100 replicates as bootstrap value and NJ belongs to the distance-matrix method.

Inoculum preparation and Production of L-asparaginase

The isolated colonies were transferred to 250 ml Erlenmeyer flasks with 50 ml broth medium containing; KH_2PO_4 (0.75 g), NaCl (0.5 g), l-asparagine (10 g), and glucose (1 g) [23] and incubated in a shaker incubator (150 rpm, 37°C) for 48 h.

After incubation, the cells were removed by centrifugation at 4000 × g for 5 min. The supernatant was used to assay extracellular l-asparaginase activity. For determination of the intracellular l-asparaginase, bacterial pellets were freeze-dried and 0.1 g of dry cell weight was

resuspended in 80 µl sonication buffer (50 mm Tris and 10 mM EDTA, pH 7.5). The suspension was transferred to a 1.5 ml thin wall micro tube. Then, the tube was placed in water and ice mixture and treated with sonicator. Six treatments of one min each at an amplitude setting were performed. The disrupted cells were then brought down to the bottom of the tube by centrifugation at 13000×g for 20 min. The supernatant was used as enzyme solution for intracellular l-asparaginase activity assay [24].

Assay of L-asparaginase:

L-Asparaginase activity was measured by Nessler's reaction. The assay procedure is based on direct Nesslerization of ammonia. Enzyme solution (30 µl) was added to Tris–HCl (pH 8.5, 50 mM) in a final volume of 1.5 ml. The reaction was started with addition of 0.5 ml l-asparagine solution (10 mM, in 50 mM Tris–HCl, pH 8.5) and incubation in 37°C water bath for 20 min. The reaction was terminated with addition of 0.5 ml trichloroacetic acid 15% (w/v) and the volume was adjusted to 4.5 ml with distilled water. Nessler's reagent (0.5 ml, 45.5 g HgI2 and 35.0 g KI in 1 liter distilled water containing 112 g of KOH) was added and the tubes were incubated at room temperature for 15 min. After vortexing, the absorbance was measured at 500 nm, using visible spectrophotometer [25] A standard curve was drawn with various concentrations of ammonia.

Purification of L-asparaginase

The purification was carried out using crude enzyme extract [26]. The enzyme was purified by the following steps at 0-4ºC, unless otherwise mentioned.

Finely powdered ammonium sulfate was added to the crude extract. The L-asparaginase activity was associated with the fraction precipitated at 40-60% saturation. The precipitate was collected by centrifugation at 9,000 g for 15 min, dissolved in 50mM Tris-HCl buffer pH 8.6 and dialyzed against the same buffer. The dialyzed fraction was applied to a Sephacryl S-200 column (1cm x 50cm) that was pre-equilibrated with Tris-HCl buffer pH 8.6. The protein elution was done with the same buffer at a flow rate of 5ml/30 min. The active fractions were pooled, dialyzed and concentrated. The concentrated enzyme solution was applied to the column of CM Sephadex C-50 that was pre-equilibrated with 50mM Tris-HCl buffer pH 8.6. It was eluted with NaCl gradient (0.1-0.5 M) and 0.1 M borate buffer pH 7.0. The active fractions were collected, dialyzed and concentrated. The L-asparaginase was assayed by the direct Nesslerization method as described earlier. Protein estimation was done with Folin-Phenol reagent using BSA as a standard.

Characterization of the purified enzyme

Effect of temperature

The enzyme was assayed in the reaction mixture containing 2.0ml of 0.5% casein solution in 0.1M carbonate buffer (pH 9.3) and 0.1ml of enzyme solution in the total volume of 2.1 ml. After incubation at 4^{o}C, 37^{o}C, 55^{o}C and 80^{o}C for 5mins, the reaction was stopped by adding 3.0 ml of 10% ice cold TCA and centrifuged at 10,000 rpm for 5 to 7 min.. The reading was taken at 660 nm in UV- Spectrophotometer to monitor the enzyme activity.

Effect of pH

The above method was repeated using 0.1M carbonate buffer of different pH (2, 4…10) and the enzyme activity was monitored spectrophotometrically with a separating acrylamide gel of 10% and stacking gel 5% containing 0.1% SDS. The gel was stained with coomassie brilliant blue R-250 and destained with a solution of methanol, acetic acid and water in the ratio of 4:1:5.

Determination of the molecular weight of the purified enzyme by SDS-PAGE

The molecular weight of the extracted enzyme was determined by performing SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (1970) [27]

CYTOTOXICITY ASSAY:

MTT method:

Each of the three cell lines, CaCo-2, MCF-7 and PC3 were seeded at 20,000 cells/well (80% cell confluence) in tissue culture plates and incubated at 37ºC for 28 hrs. For background absorption, some wells were remained cell-free, i.e. as blank control. The L-Asparagine was added to the cells at serial concentrations of 20, 10, 5, 2.5 and 1.25µg/mL, the control was also included (without L-Asparagine). The final volume was adjusted to 100µL/well. The plate was incubated overnight at 37ºC, 5%CO_2. 30µL of (1 mg/mL). MTT stain was added to each well and the plate was incubated at 37ºC for 4 h. 100µL of Dimethyl sulfoxide (DMSO) stop solution was added to each well. The plate was shaken at room temperature for 10 to 20 minutes. The plate was then read using ELIZA Micro plate reader at 570 nm. The percentage of viable cells was calculated from the formula: Survival fraction=OD of treatment cell/OD of control cells. The IC_{50} was calculated by fitting the survival curve using graphpad, Prizm software in corporate [28.]

RT PCR for BAX, P53 and BCL2 genes:

Evaluating the expression of pro-apoptotic genes (p53 and Bax) and antiapoptotic gene (Bcl-2) was carried out using the newly synthesized cDNA as templates for PCR. cDNA was amplified from total RNA (1µgne-Step RT-PCR System with Platinum Taq (Life Technologies). 25 µl dream Taq green master mix, 4 µl cDNA, 2 µl forward primer (10 picomole / µl), 2 µl reverse

primer (10 picomole / µl) and 17 µl nuclease free water were pre-denaturated at 94°C for 3 minutes. Amplification was performed (35 cycles) with each cycle consisting of denaturation at 94°C for 30 sec, annealing at 59°C (p53), 55°C (Bax) and 58°C (Bcl-2) for 30 sec followed by extension at 72°C for 45 sec. Reactions were terminated by heating at 72°C for 5 minutes. Non-reverse transcribed RNAs were included to confirm the absence of genomic DNA. Negative control without adding template was also included to assess for reagent contamination. PCR product as well as DNA Ladder were loaded as 10 µl on 1.5 % agarose gel at 100 volts for 20-30 minutes, visualized using UV transillumiator and photographed after staining with ethidium bromide. Information for primers used for the amplification of the three expression genes is summarized in table 1**.**

Table 1: Primer sequences of apoptosis related genes:

Gene	Primer sequences	Size of PCR product (bp)	References
P53	F: 5' TCA GAT CCT AGC GTC GAG CCC 3' R: 5' GGG TGT GGA ATC AAC CCA CAG 3'	438	[29]
Bax	F: 5' ATG GAC GG TCC GGG GAG CA 3' R: 5'CCC AGT TGA AGT TGC CGT 3'	322	[30]
Bcl-2	F: 5' GTG AAC TGG GGG AGG ATT GT 3' R: 5' GGA GAA ATC AAA CAG AGG CC 3'	216	[30]

RESULTS AND DISCUSSION

Isolated bacterial strain:

The bacterial isolate was identified by physiological characteristics to be *E.coli*.

rRNA Gene Sequence Analysis

The PCR amplification of 16S rRNA gene revealed efficient amplification; a single band of amplified DNA product of ~800-bp was recorded (Figure 1). The result of PCR blasted with other bacterial sequences in NCBI showed similarity to the 16S small subunit rRNA. Edited sequences were used as queries in BLASTN searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi), to determine the nearest identifiable match present in the complete GenBank nucleotide database. A bioinformatic tool, GeneDoc software, version 2.6.002, was used for more 16S rRNA gene sequence investigation. A total of 802 nucleotides of the partial sequence of *E. coli* isolate was 99% similar to the 16S ribosomal rRNA genes in other recorded strains of *E. coli* in National

Centre for Biotechnology Information (NCBI) (Figure 2).

ACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC

GGAGGGTGCAAGCGATAATCGGAATTACTGGGCGTAAAGCGCACGCAGGC

GGTTTGTTAAGTCAGATGTGAAATCTCCGGGCTCAACCTGGGAACTGCAT

CTGATTCTGGCAAGCTGGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGT

AGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCC

CCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGG

ATTAGATACCCTGGTAGT

Figure 1: 16S rRNA gene sequence investigation of *E.coli* by bioinformatic tool, GeneDoc software, version 2.6.002

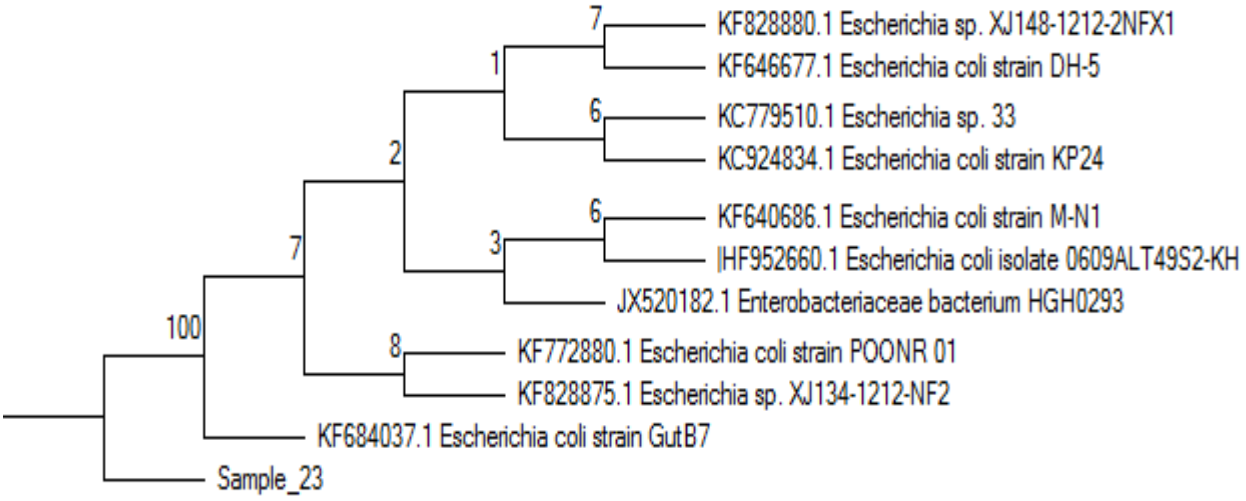


Figure 2: Phylogenetic analysis of *E.coli* strain by using the results of PCR amplification of the 16srRNA gene.

Molecular Weight Determination by Sodium Dodecyl SulphatePolyacrylamide Gel Electrophoresis (SDS–PAGE)

SDS–PAGE (of the enzyme preparation from different purification steps showed that the resolved electrophoretic bands were progressively improved from the crude extract to the final step of the Sephadex G-200 column. It revealed only a single distinctive protein band for the pure preparation of L-asparaginase with an apparent molecular weight of 33 kDa (Figure.3). In this respect, L-asparaginases purified from This *E. coli* isolate, agrees with the results previously shown in [31, 32] which indicated that *Thermusthermophilus* and *Escherichia coli* were with small Mr values ranging from 33-34 kDa.

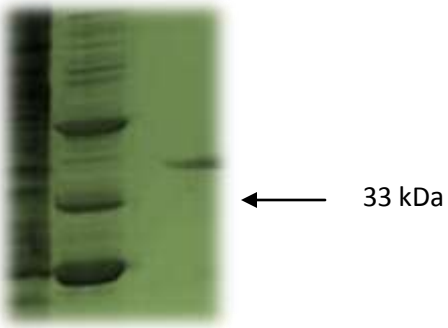


Figure 3: purification on S-Sepharose FF cation exchanger. Protein bands were stained with Coomassie Brilliant Blue R-250. Left lane, *E. coli* crude extract after induction with 1mM IPTG; middle lane, unbound protein fraction; right lane, l-ASNase eluted from S-Sepharose FF cation exchanger. The figure shows the presence of a single polypeptide chain at 33 KDa.

Antitumor activity of L-asparaginase

Using MTT assay, the in vitro bioassay cytotoxic effect of *E.coli* L-asparaginase enzyme on the growth of three human tumor cell lines namely Caco-2[Colon cell line], MCF-7[Breast cancer cell line], and PC-3[Human prostate cell line] showed that the L-asparagainase had anti-proliferative activity on the growth of Human breast Cancer(MCF7) and Human cancer prostate (PC3), where it was non effective to the human colon cancer cell line (CaCo2). (Figure 4).

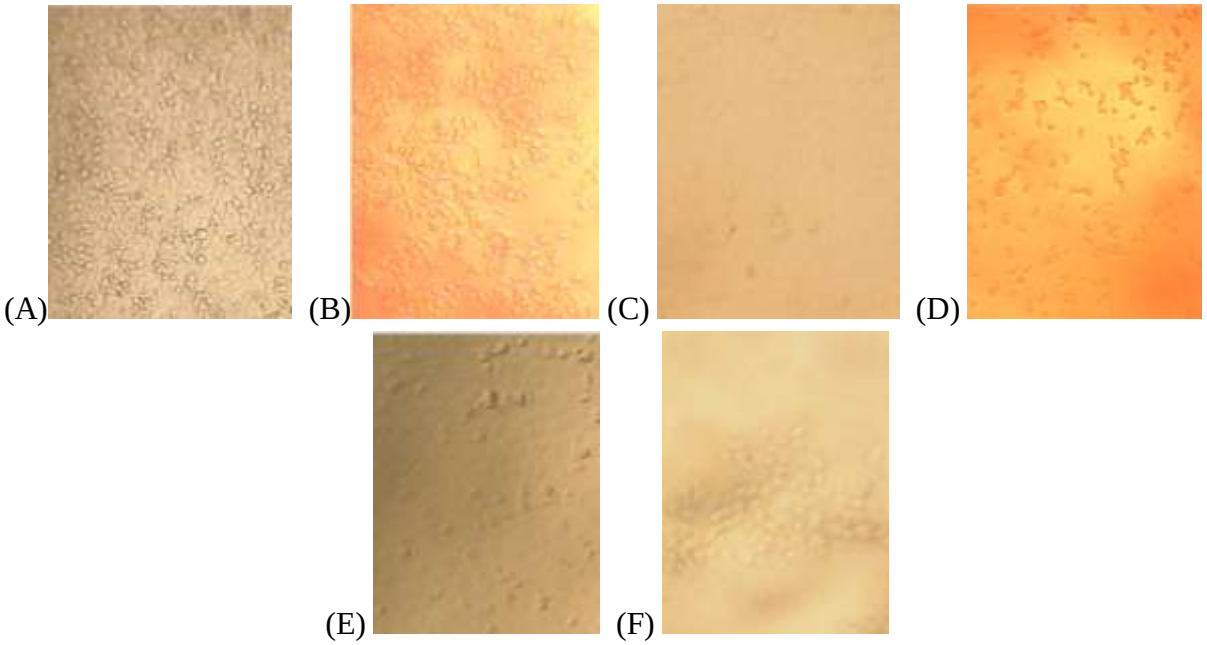
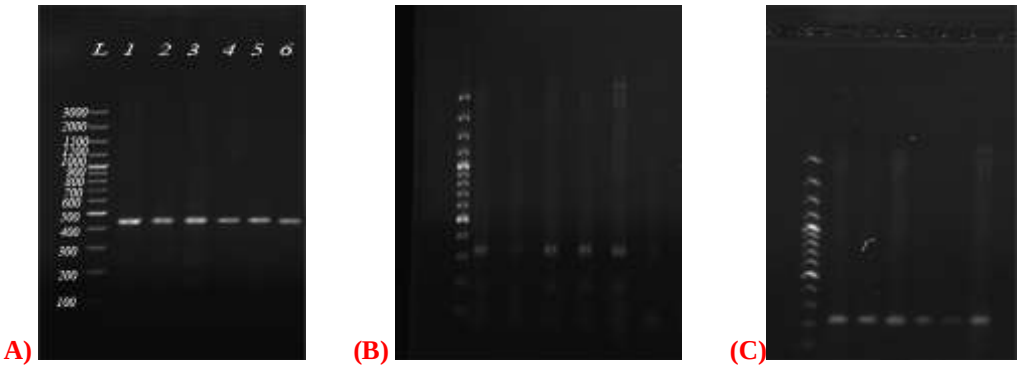


Figure 4: Photomicroscopy of Caco 2, MCF 7 and PC-3 cells stained with MTT. Cells were cultured for 3 days in 200 plmedium and subsequently incubated with MTT dye solution. A). Caco-2 control, (B) Caco-2 toxicity, (C) MCF-7 control, (D) MCF-7 toxicity, (E) PC-3 control, (F) PC-3 toxicity. note "hairy" extensions representing long-shaped formazan crystals with D and F. Scale bar = 25 pm.

The RT PCR gel electrophoresis

The RT PCR gel electrophoresis picture is shown in figure 6. It was noticed that BAX and P53 genes were up-regulated under the effect of Asparaginase enzyme while BcL2 gene was down regulated in Human Breast and prostate Cancer cell line while Human colon cell line was not when compared with the growth of untreated control cells.

The anticancer property of L-asparaginase against Caco-2 cell line revealed that;by the analysis of the concentration and viability of cells, the cytotoxicity index IC_{50} value was 82.647 µg/mL) at 28 h and it was 89.367µg/mL for PC3, while it was 56.567µg/mL for MCF-7 (Figure 5).



2	3	4	5	6	7
P53					
PC3 TREATED	PC3 UNTREATED	MCF7 TREATED	MCF7 UNTREATED	CACO2 TREATED	CACO2 UNTREATED
P	C	P	C	N	C
BAX					
PC3 TREATED	PC3 UNTREATED	CACO2 TREATED	CACO2 UNTREATED	MCF7 TREATED	MCF7 UNTREATED
P	C	N	C	P	C
BCL2					
CACO2 TREATED	CACO2 UNTREATED	PC3 UNTREATED	PC3 TREATED	MCF7 TREATED	MCF7 UNTREATED
N	C	C	P	P	C

Figure 5: RT PCR gel electrophoresis and table for layout of gel lanes from 2 to 7 showing effect of l-asparaginase on (A) P53 gene, (B) BAX gene and (C) BCL-2 gene. In all figures, a 3 kb DNA ladder marker was used in lane one. A band of 438 bp was obtained with P53gene, a band of 322 bp was obtained with BAX gene and a band of 216 was obtained with BCL-2 gene.

L-asparaginase producing *E.coli* was isolated from Kasr ELeini in Egypt and identified on the basis of biochemical and molecular technique (18sRNA) and the result was detailed in the figures above. The enzyme was extracted from the production media by centrifugation and further purified by salt precipitation, dialysis and ion exchange chromatography. The optimum

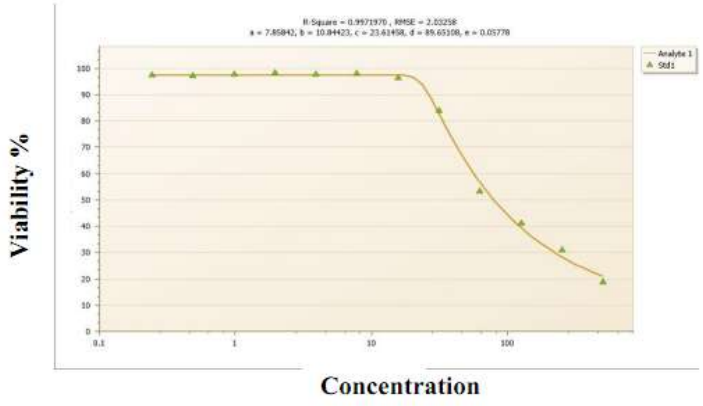
enzyme activity was determined at different temperature and pH. The optimum temperature for the enzyme activity was found to be at 55°C. The results indicate that at 30°C, L-asparaginase production was 5.4U/ml. The decrease in the yield of enzyme was observed when incubation temperature was higher or lower than optimum incubation temperature. The enzyme was found to be maximally active at 40°C. This optimum L-asparaginase activity at 40°C is similar to that of *Corynebacterium glutamicm* reported by Mesas *et al.*1990 regarding the thermal stability, at 80°C, the enzyme retained 55% of the activity. According to our results the enzyme was found to be maximally active at 55°C, so it is thermostable and the enzyme found to be retained 98% of its activities. The purified L-asparaginase was active at a pH range of 7.3–8.9. The maximum L-asparaginase activity was obtained at pH 8.5, and activity decreased significantly (50%) when the pH was decreased to 6.5. Although maximum activity at a physiological pH is one of the prerequisites of L-asparaginase for antitumor activity, the purified enzyme would useful because 75% of the enzyme activity was retained at pH 7.2. The enzyme showed stability at alkaline pH (pH 8.0– 10.0) as it retained 90% of its original activity when incubated up to 24 h. The molecular weight of the purified enzyme was determined by SDS-PAGE and found to be 33 KDa.

The antitumor activity of the purified L-asparaginase was analyzed by MTT method against three cell lines and the results was clear that the enzyme had antiproliferation effect on both MCF-7 and PC3 cell lines while had no effect on Caco-2 cell line.

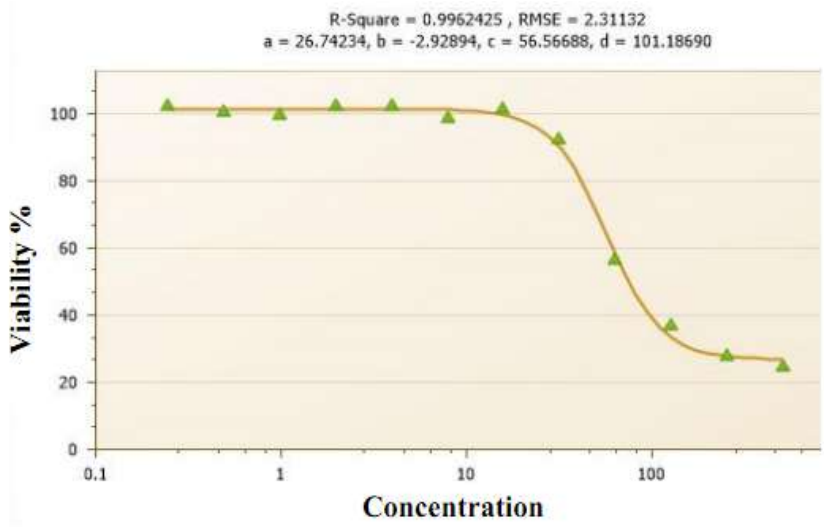
The level of expression for the three genes of expression BAX, P53 and BCL2 was measured using RT PCR and analyzed for the IC_{50} value of the three cell lines.

The anticancer property of L-asparaginase against Caco-2 cell line revealed that; by the analysis of the concentration and viability of cells, the cytotoxicity index IC_{50} value was 82.647 µg/mL) at 28 h and it was 89.367 µg/mL for PC3, while it was 56.567 µg/mL for MCF-7 (Fig 6).

(A)



(B)



(C)

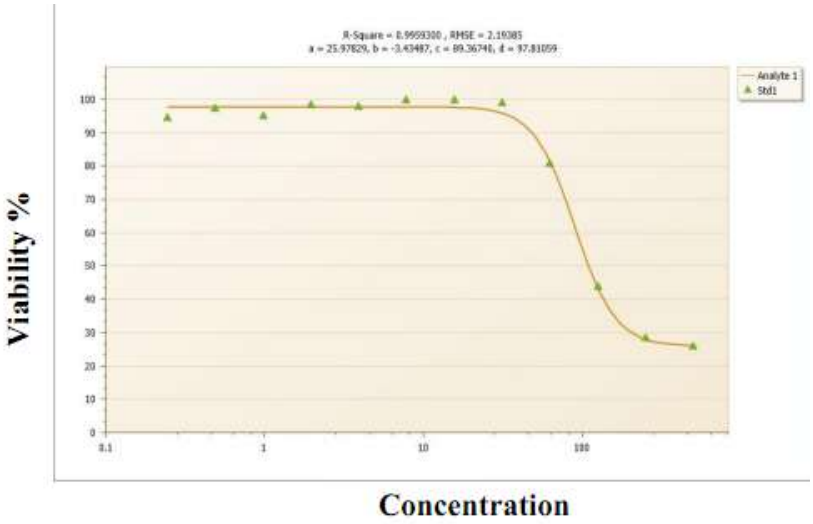


Figure 6: (A). The evaluation of Caco-2 cell viability relative to concentration was shown to give IC_{50}% of 82.647.

(B). The evaluation of MCF-7 cell viability relative to concentration was shown to give IC_{50} % of 56.567.

(C). The evaluation of PC-3 cell viability relative to concentration was shown to give IC_{50}% of 89.367.

CONCLUSION

Our aim was the production, purification and characterization of L-asparaginase from *E.coli.* A comparative study based on temperature, pH. *E. coli* was screened for the production of L-asparaginase. As the isolation and screening method for *E. coli* is very easy, and the enzyme that was extracted was found to be active at higher temperature (55^{o}C). Considering all these

characteristics, the production of L-asparaginase from this *E. coli* isolate may be recommended for industrial production of the said enzyme. The purified L-asparaginase from *E. coli* clinical isolate has a favorable activity over wide ranges of pH and temperature, In addition, anti-proliferative activity of the enzyme on different cell lines growth, especially the human Human breast Cancer MCF-7 and Human cancer prostate PC-3cell line, could be used to develop therapy ofdifferent types of tumors.

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