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Antitumor activity of *L-asparaginase* produced from *E.coli* .

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Abstract:

.Extra and intra-cellular L-asparaginase were produced from E.coli, purified by ammonium sulphate precipitation, ion exchange chromatography and gel filtration chromatography and tested for their anti-tumor activities against four human tumor cell lines include MCF-7 , PC3 , A549 and Hep-G2 using MTT and (HCS) array scan. The MTT assay showed that the enzymes exhibited antiproliferative activity in different cell lines growth, PC3 cell line were more sensitive to extra and intracellular L-asparaginase as it gave a lower IC50 value (27.41±2.95 and 70.12±2.53) for extra and intracellular respectively, extracellular L-asparaginase treatment increases nuclear condensation, plasma membrane permeability, MMP attenuation and increased cytochrome C in the cystol compared to control. We suggest the extra-cellular Lasparaginase of E.coli was a therapeutic agent against human PC3 cell line.

Keywords: L-asparaginase, E.coli , PC3, Apoptosis, HCS.

Introduction:

L-asparaginase catalysis the hydrolysis of L-asparagine to L-aspartic acid and ammonia. (Fig.1) This is an essential enzyme for the synthesis of various nitrogenous metabolic intermediates and the enzyme plays a major role in the nitrogen metabolism of both prokaryotes and eukaryotes (1).

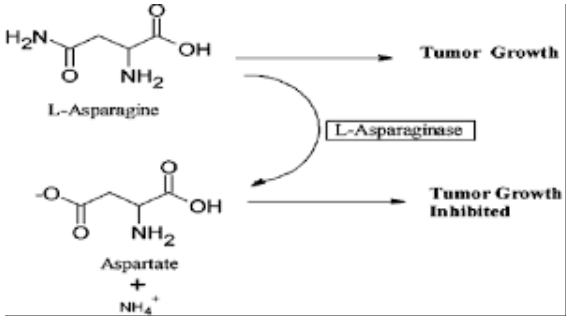

Fig.1 Illustrated L-Asparaginase Activity.

It is widely distributed in plants, animal tissues and microorganism including bacteria, fungi and yeast (2). Interest on amidohydrolases started with the discovery of their antitumor properties (3; 4; 5; 6) and since then, a lot of efforts have gone into extensive studies on microbial L-asparaginase with the intention of developing them as antitumor agents (7). Cancer cells are often referred to as being "asparagine addicted", as they typically are extremely sensitive to asparaginase deprivation and hence cannot proliferate in cell culture without it (8). asparagine metabolism in the tumor cells has been found to be considerably faster when compared with that in non-transformed cells of the same origin, this is true for human hepatocytes and hepatoma cells Cancer cells require a robust supply of reduced nitrogen to produce nucleotides, non-essential amino acids and a high cellular redox activity, asparagine provides a major substrate for respiration as well as nitrogen for the production of proteins, hexosamines, and macromolecules therefore, asparagine is one of key molecules in cancer metabolism during cell proliferation (9). The L-asparaginase causes selective death of asparagine dependent tumor cells by blocking these cells of asparagine. The present study deals with the purification of intra and extracellular L- asparaginase from E.coli, the study will be extended to evaluate the antitumor activity of the purified enzymes against different tumor human cell lines using MTT and High Content Screening (HCS) analyses.

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Materials and Methods:

Bacterial strain

The strain E.coli used in this study was isolated from soil

during a screening study for L-asparaginase producing bacteria, the culture was maintained on nutrient agar slant at 4° C.

Asparaginase extraction

The samples were taken out and centrifuged at 10000 rpm for 10 min in cold centrifuge, the supernatant was collected and used as a crude extract for extracellular enzyme assay. While the intracellular enzyme was extracted by destroying the cell in the bottom with 1ml NaOH and 3 ml distilled water using ceramic morter. Disrupt thus obtained was centrifuged at 3000 rpm for 30 min at 4°C , the cell free supernatant was subjected to intracellular enzyme assay immediately .(10)

Assay of L-asparaginase Activity

Assay of L-asparaginase activity was carried out as (10) . 0.5 ml of 0.2 M asparaginase was taken in a test tube, to which 1 ml of 0.2M of Tris-HCl buffer pH 8.4 and 0.5 ml of enzyme preparation was added and the reaction mixture was incubated for 15-20 min . After the incubation period the reaction was stopped by adding 1 ml of 10% TCA (Trichloroacetic acid) . 1 ml was taken from the above reaction mixture to this 3.7 ml distilled water and 0.2 ml Nessler's reagent was added and incubated for 15-20 min . The absorbance was measured at 450 nm against suitable blank . The enzyme activity was expressed in international units, One IU of L-asparaginase is the amount of enzyme which liberates 1ɯmole of ammonia under optimum condition . The enzyme yield was expressed as unit/ml (U/ml).

Determination of protein concentration

Protein concentration was determined (11) using bovine serum albumin as the standard.

Asparaginase purification

The intra and extracellular Asparaginase were purified as following:

Ammonium sulphate precipitation

Ammonium sulphate precipitation was carried out at 4°C in ice bath, the enzymes were precipitated from the supernatant by the gradual addition of solid ammonium sulphate with gentle stirring to 60% saturation and precipitate was collected by centrifugation at 7500 rpm for 30 min. Then, the precipitate was dissolved in a minimum volume of Tris-HCl buffer (0.02 M, pH 8.0), the fraction obtained were pooled and dialysed against the same buffer for 24 h at 4°C with continuous stirring and occasional changes of the buffer.

Ion exchange chromatography

The most active partially purified enzyme fraction from the previous step was applied on DEAE- Cellulose column (1.5 x 80 cm) that was pre-equilibrated with a 0.01 M Tris HCl pH 8.0 at a flow rate of 1 ml/min. The fractions were collected and examined for enzyme activity and protein content. The most active fractions were pooled together and concentrated.

Gel filtration chromatography

The purified fraction obtained from the previous step was loaded onto the pre-equilibrated Sephadex G-300 column (42 x 1.5 cm) a 0.01 M Tris HCl pH 8.0 at a flow rate of 0.6 ml/min. The fractions were collected and examined for enzyme activity and protein content. The most active fractions were pooled together, concentrated by lyophilization and stored at -20°C.

Antitumor Activity:

All steps of this Experiments was Executed in UTM in Malaysia include:

MTT cytotoxicity assay

PC3 cells were plated at 1 x 104 cells/well by adding 200 µL of a 5 x 104 cells/mL suspension to each well of a 96-well tissue culture plate. The plate was incubated for a sufficient time to assure attachment. The media was aspirated off and replaced with fresh media (200 µL) containing extra and intracellular Asparaginase of different concentration (100, 50, 12.5, 6.25, 3.125) µg/mL, the last row left as an untreated control. The plates were incubated at 37°C, 5% CO2, for 48 h. After incubation the media was aspirated off and replaced with fresh media, then MTT solution 20 µL for a total volume of 200 µL was added in every well and incubated for 2 h at 37°C with 5% CO2, supernatant was discarded gently and DMSO (200 µL/ well) to ensure total solubility of formazan crystals. The plates were read on microtiter plate reader at 570 nm. The IC50 (The enzyme concentration that reduced the viability of cells by 50% was generated from the dose-response curves for each cell line .

Multiple cytotoxicity assay

Cellomics Multiparameter Cytotoxicity 3 Kit (thermo Scientific) was used. Cells were plated at 1x104 cells per well on 96-well plate overnight. DMSO (solvent) or the enzymes were added at various concentration and further incubated for 24 h. MMP dye and the cell permeability dye were added to live cells and incubated for 1 h. Cells were fixed with 4% formaldehyde for 15 min, fixed cells were permeabilized with 1% Triton X-100 in phosphate buffer saline (PBS), sample were blocked with 3% bovine serum albumin and incubated with cytochrome c primary mouse antibody for 1 h. Samples were washed three times with wash buffer (1 x PBS) before addition of goat anti-mouse secondary antibodies conjugated with DyLightTM 649. Cells were

Results and Discussion:

The production of L-asparaginase by the bacterial cells may be endo- or exo-enzymes, which was based on the metabolic activity of bacterial cells (8). The results showed that E.coli have the ability to produce intra- and extra-cellular Asparaginase and the extra-cellular Asparaginase was higher than the intra- cellular. (12) reported the presence of intra- and extra cellular Asparaginase in Pseudomonas fluorescens, Vibrio costicola and V.cholerae and the extracellular secretion is about 2.6 to 6.8 times higher than intracellular production. L-asparaginase is secreted extracellulary also by strains of Bacillus subtilis and B. licheniformis (13), and Debaryomyces sp.(14).

Enzymes purification

Extracellular Asparaginase:

The sequential multi-steps purification procedure was summarized in Table 1. After ammonium sulphate precipitation the enzyme attained 1.29 purification fold and 21.8% yield

with specific activity of 10.6 U/mg. The precipitation fraction by 60% ammonium sulphate was applied to anion exchange chromatography (DEAE –cellulose). Fig.2 shows the elution profile of the enzyme , the most active fraction(F31-F41) for enzyme activity with specific activity 7.48 U/mg protein, purification fold of 0.9 and 18.1% recovery yield were pooled together.

Table 1. Sequential multi-steps process for purification of Extra-cellular L-asparaginase from E.coli.

Purification step	Total activity (U)	Total Protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
Culture supernatant	3200	390	8.2	100	1
Ammonium sulphate (80%)	700	66	10.6	21.8	1.29
DEAE cellulose	579.8	77.48	7.48	18.1	0.9
Sephadex G-300	465	40.3	11.53	14.5	1.4

The elution profile of the most active fractions collected from DEAE –cellulose and loaded on Sephadex G-300 column is illustrated in Fig.3. A distinctive peak of extracellular Asparaginase activity which fits with only one protein peak was observed. The most active fractions (F22-F32) with specific activity of 11.53 U/mg and 1.4 purification fold and 14.5% enzyme recovery were pooled together, concentrated with lyophilizer and stored at -20°C.

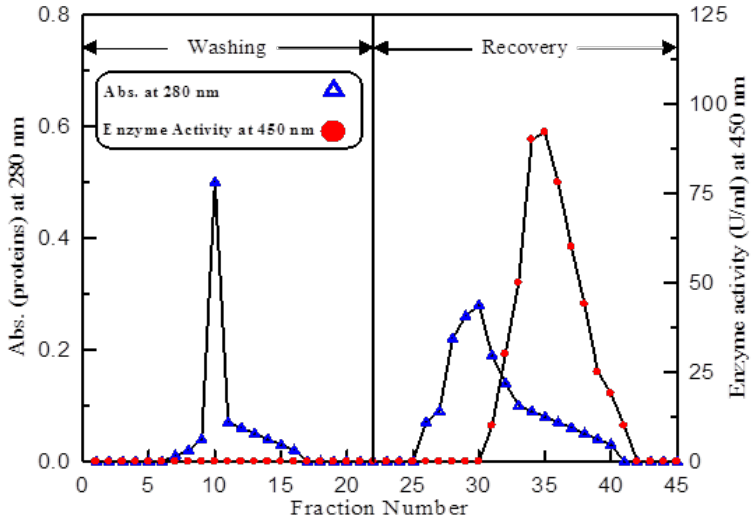

Fig.2: Ion exchange of Extra-cellular L-asparaginase of E.coli . from DEAE-cellulose

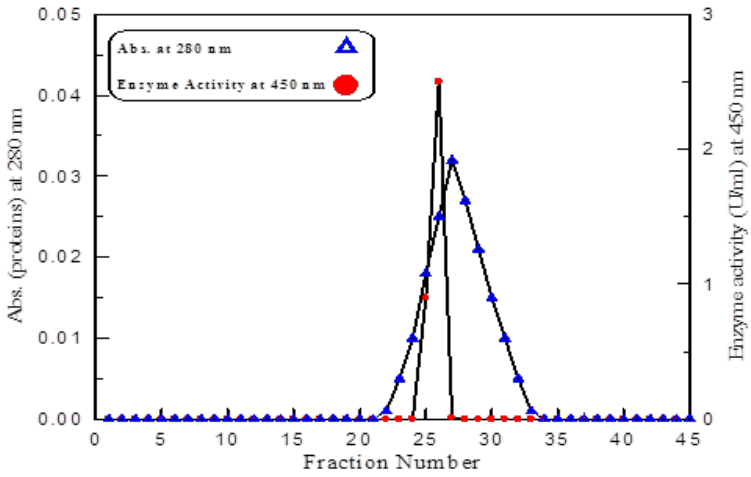

Fig.3: Gel filtration of Extra-cellular L-asparaginase of E.coli . from Sephadex G-300

Intracellular Asparaginase:

The details of purification steps of this enzyme were given in Table 2. After precipitation by 60% ammonium sulphate, the specific activity increased to 7.63 U/mg with purification fold and yield (2.57 and 36.9%) respectively.

Table 2. purification table for of Intra-cellular L-asparaginase from E.coli.

Purification step	Total activity (U)	Total Protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification fold
Culture supernatant	1176	396	2.96	100	1
Ammonium sulphate (80%)	435	57	7.63	36.9	2.57
DEAE cellulose	459	19.8	23.18	39	7.83
Sephadex G-300	244	20.2	12.07	20.7	4.07

Fig.4 shows the elution profile of the enzyme on DEAE-cellulose, the most active fractions(F22-F27) with specific activity, purification fold and yield(23.18 U/mg, 7.83 and 39%) respectively, were pooled together and loaded on Sephadex G-300 column, this yielded one peak for Asparaginase activity having 20.7% yield. The most active fractions (F22-F32) were pooled together, concentrated with lyophilizer and stored at -20°C.

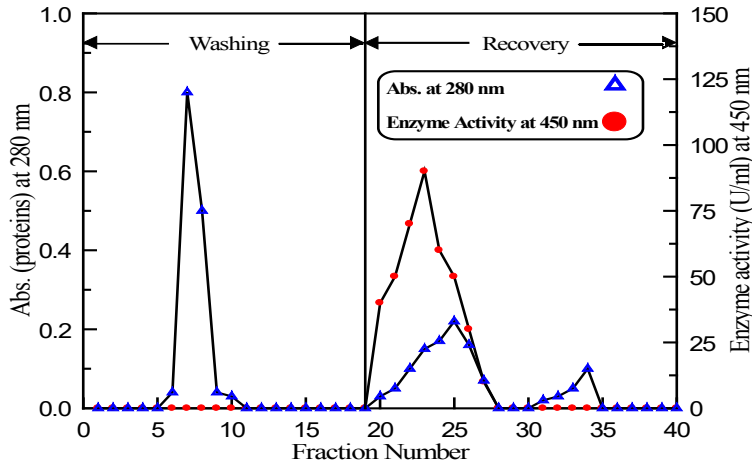

Fig.4: Ion exchange of Intra-cellular L-asparaginase of E.coli. from DEAE-cellulose

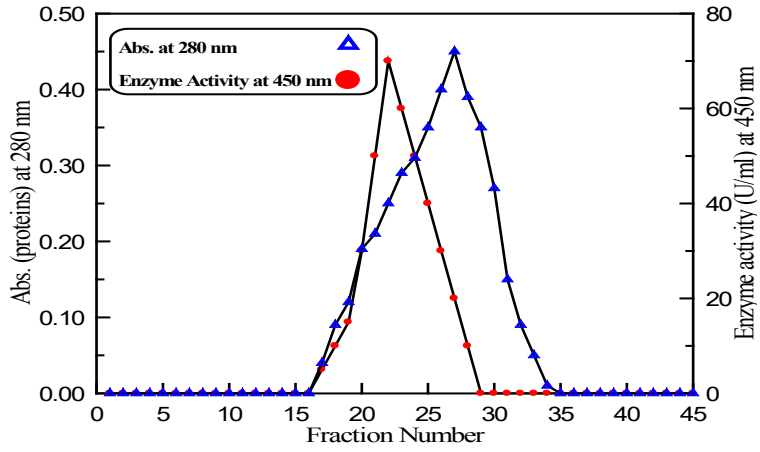

Fig.5 : Gel filtration of Intra-cellular L-asparaginase of E.coli. from Sephadex G-300

Antitumor activity

To evaluate the cytotoxic activity, extra and intracellular Asparaginase was tested with various doses (100, 50, 12.5, 6.25, 3.125 µg/mL) on the growth of four human tumor cell lines namely MCF-7 [Human breast cancer cells] PC3 [Human prostate cancer cells] A549 [Human lung adenocarcinoma epithelial cells] Hep-G2 [Liver hepatocellular carcinoma cells]. After 48 h, cell viability was analyzed using the end-point MTT assay. The enzymes exhibited antiproliferative activity in different cell lines growth, the highest concentration of extra and intracellular Asparaginase (100 µg/ml) showed the highest toxicity on four cell lines. Fig.6. and Fig.7. The plot of cell viability (%) versus sample concentration was used to calculate the concentration lethal to 50% of the cells (IC50), however, the highest antitumor was recorded towards PC3 cells with IC50 27.41±2.95 and 70.12±2.53 for extra and intracellular respectively. While the least activity were obtained towards Hep-G2 cells with IC50 45.08±6.32 and 94±8.45 for extra and intracellular respectively (Table3). In this connection, the cytotoxicity of L-asparaginase from Aspergillus flavus KUGF009 towards MCF-7 cell line by the MTT assay (IC50 250 µg/ml) was reported by (15) and (16) reported purified L-asparaginase IC50 63.3 µg/ml and partially purified L-asparaginase IC50 109.9 µg/ml from Penicillium brevicompactum NRC 829 towards Hep-G2 cell line. While the cytotoxicity of L-asparaginase from Aspergillus oryzae towards MCF-7 cell line by the MTT assay (IC50 283.288µg/ml) was reported by (17).

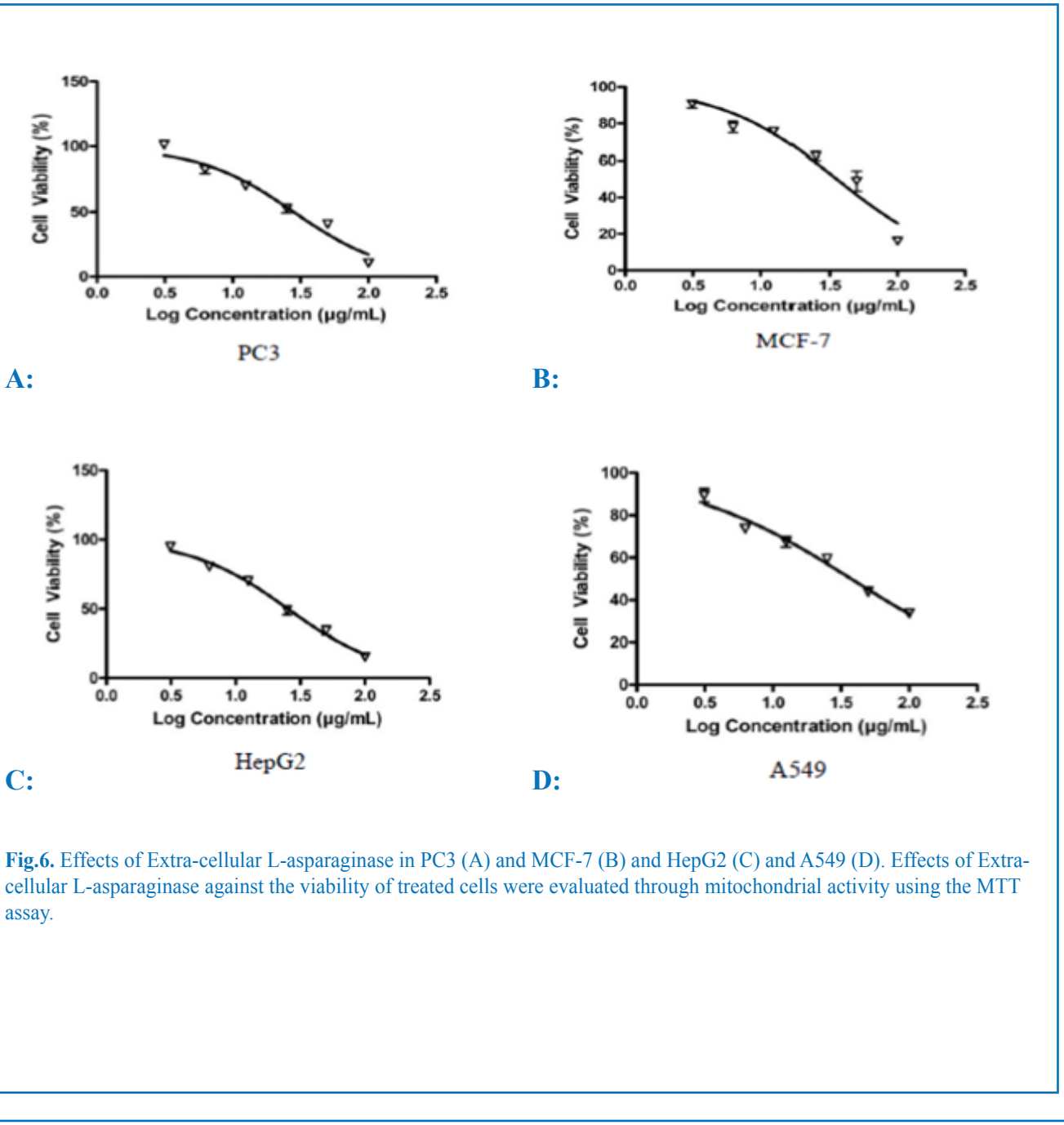

Fig.6. Effects of Extra-cellular L-asparaginase in PC3 (A) and MCF-7 (B) and HepG2 (C) and A549 (D). Effects of Extra-cellular L-asparaginase against the viability of treated cells were evaluated through mitochondrial activity using the MTT assay.

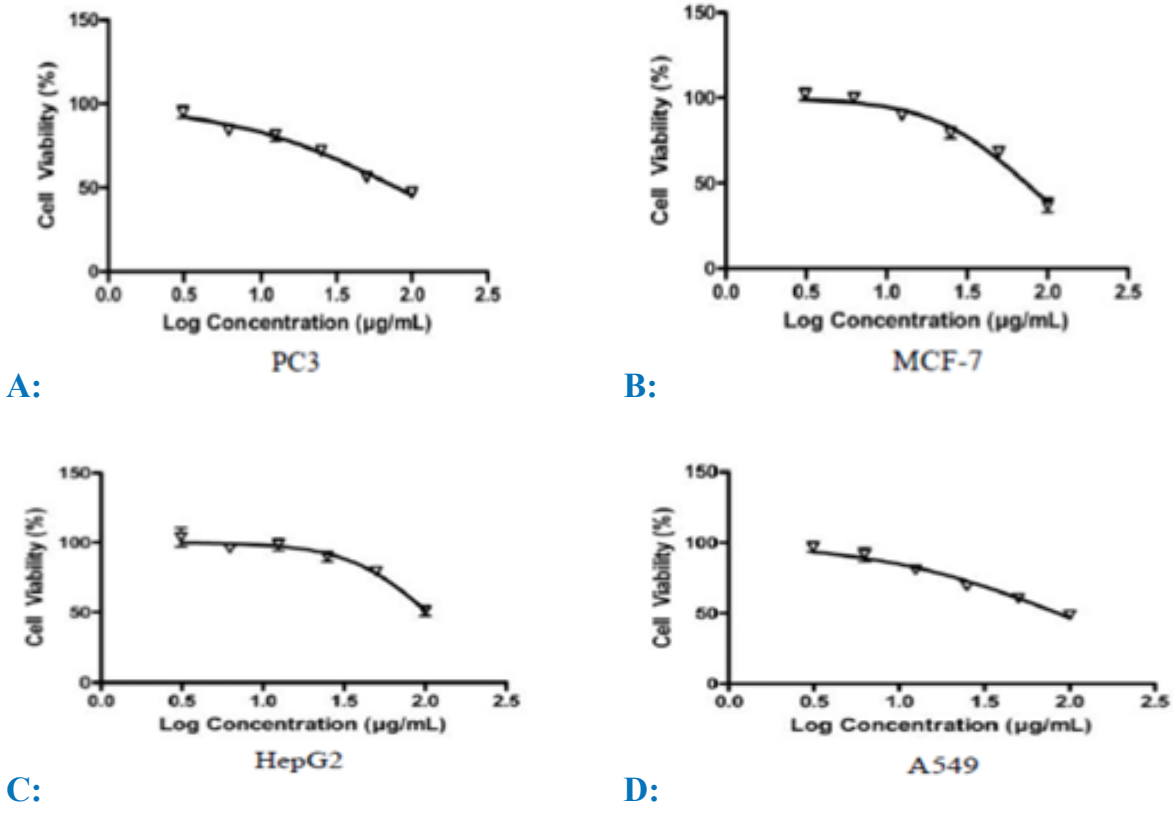

Fig.7. Effects of Intra-cellular L-asparaginase in PC3 (A) and MCF-7 (B) and HepG2 (C) and A549 (D). Effects of Extra-cellular L-asparaginase against the viability of treated cells were evaluated through mitochondrial activity using the MTT assay.

Table 3: Ic50 of Extra and Intracellular Asparaginase in Cancerous cell line by MTT assays after 48h treatment.

L-Asparaginase	HepG2	A594	PC3	MCF-7
Intracellular	**94±8.45**	**82.37±9.04**	**70.12±2.53**	**76.54±4.19**
Extracellular	**45.08±6.32**	**37.93±3.85**	**27.41±2.95**	**34.78±4.01**

Data are mean ± SD

PC3 cell line were more sensitive to extra and intracellular Asparaginase as it gave a lower IC50 value. Therefore, PC3 cell line was selected for further examination in order to examine the feature of apoptosis on this cell. By exposing tumor cells to anticancer drugs, cell death occurs by apoptosis and can be identified by different cellular targets, the combination of these targets is valuable for assessing cell injury as Mitochondrial Membrane Potential (MMP) disruption tends to be an early, reversible event of apoptosis, whereas nuclear shape changes and increase in plasma membrane permeability are indicative of late stage irreversible apoptosis events, correlative analysis of these indicators enables cells in the different stages of apoptosis to be subtly distinguished simultaneously, which is useful for profiling cytotoxicity in the process of drug discovery (18).

The multiparameter cytotoxicity kit 3 from Cellomics was used to examine the effect of extra and intracellular Asparaginase on the subcellular structures. For this purpose, the PC3 cells were stained with Hoechst 33342, a membrane permeability dye, MMP and cytochrome C antibody. The stained samples were visualized with the HCS system. As a positive control, we treated the tumor cells with Paclitaxel 5µM , a cancer chemotherapy drug. As shown in Fig.8, cytochrome c in negative control cells was distributed evenly and localized with MMP dye (red) indicating that cytochrome C was not release from the mitochondria while in extracellular Aspara-

ginase-treated cells, cytochrome c stained in the cytosol, indicating Asparaginase-induced cytochrome C release from the mitochondria (fig.8A). The results also showed attenuation in MMP in treated cell as compared to negative control cells. The mitochondria played a key role in the apoptotic process (19), change in the MMP increase the release of apoptogenic factors such as cytochrome c from the outer mitochondrial membrane space into the cytosol (20). Cytochrome c is considered a key regulator of apoptosis because once it is released from the mitochondrial intermembrane space, the cell is irreversibly committed to death (21). Once in the cytosol it bind to Apoptotic protease activating factor-1 (Apaf-1) helping in its oligomerization and the recruitment of procaspase-9 to form a functional apoptosome, consequently caspase-9 dissociates from the complex and goes on to activate effector caspases (3,6, and 7) which cleave specific cellular substrates and collectively orchestrate the execution of apoptosis (22) In addition, we observed an increase in nuclear condensation, increased membrane permeability in treated cells while in negative control samples, nucleus remained normal and plasma membrane was intact as shown by the weak staining of permeability dye (green). (Fig 8 A-E). The intracellular Asparaginase was less toxic to PC3 cell line compared to extracellular Asparaginase (Fig.9 A-E).

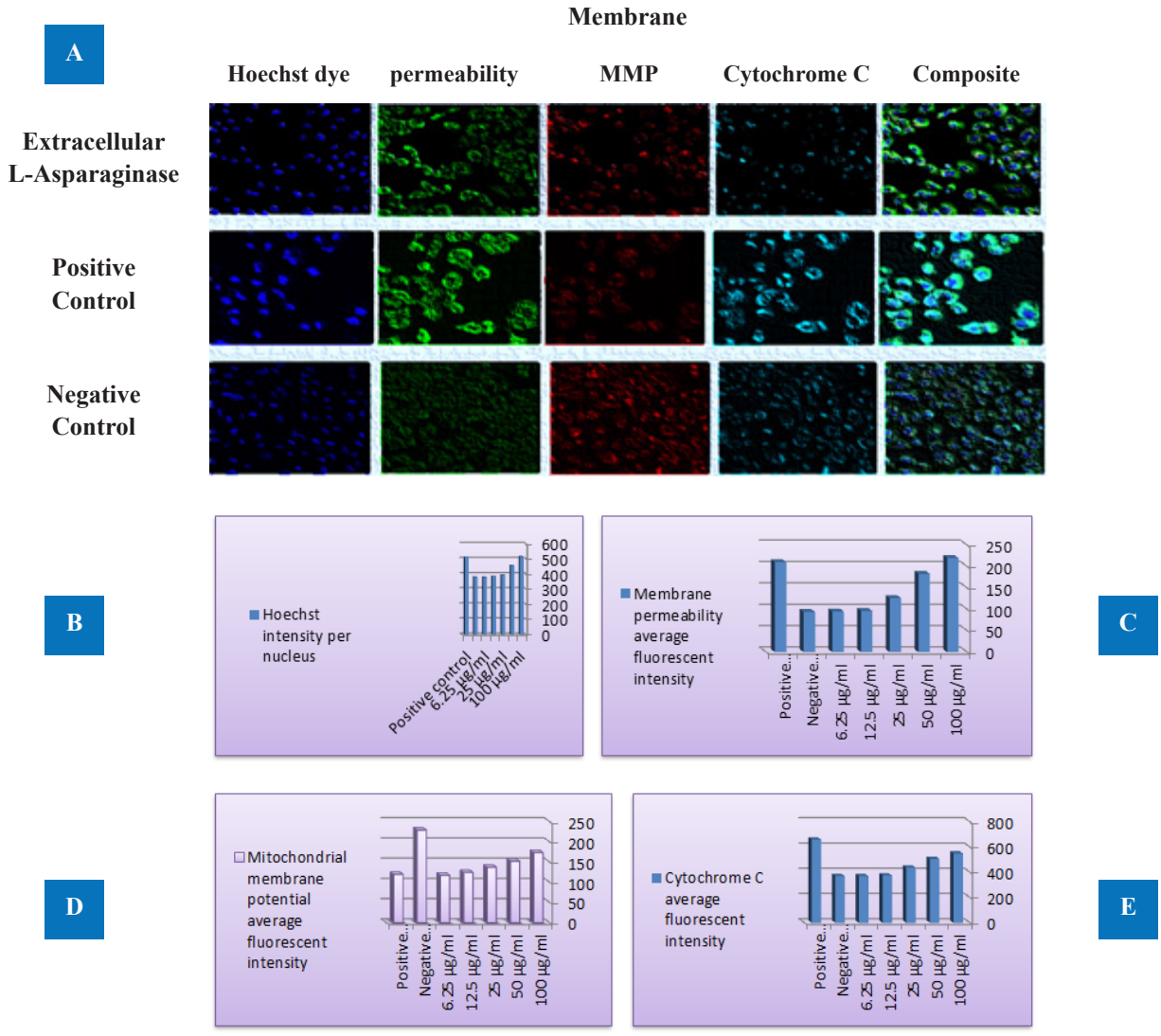

Figure 8: Effect of Extra-cellular L-asparaginase on nuclear morphology, membrane permeabilization, MMP and cytochrome c release.
(A) Representative images of PC3 cells treated with medium alone (Negative control), intracellular L-asparaginase and Paclitaxel 5µM (Positive control). The cells stained with Hoechst for nuclear, cell permeability dye, MMP and Cytochrome c. The images from each row were obtained from the same field of each sample (20X). (B-E) Average fluorescence intensities of Hoechst dye, cell permeability dye, MMP and cytochrome c in PC3 cells. Data were mean± SD of fluorescence intensity readings measured from different photos taken.

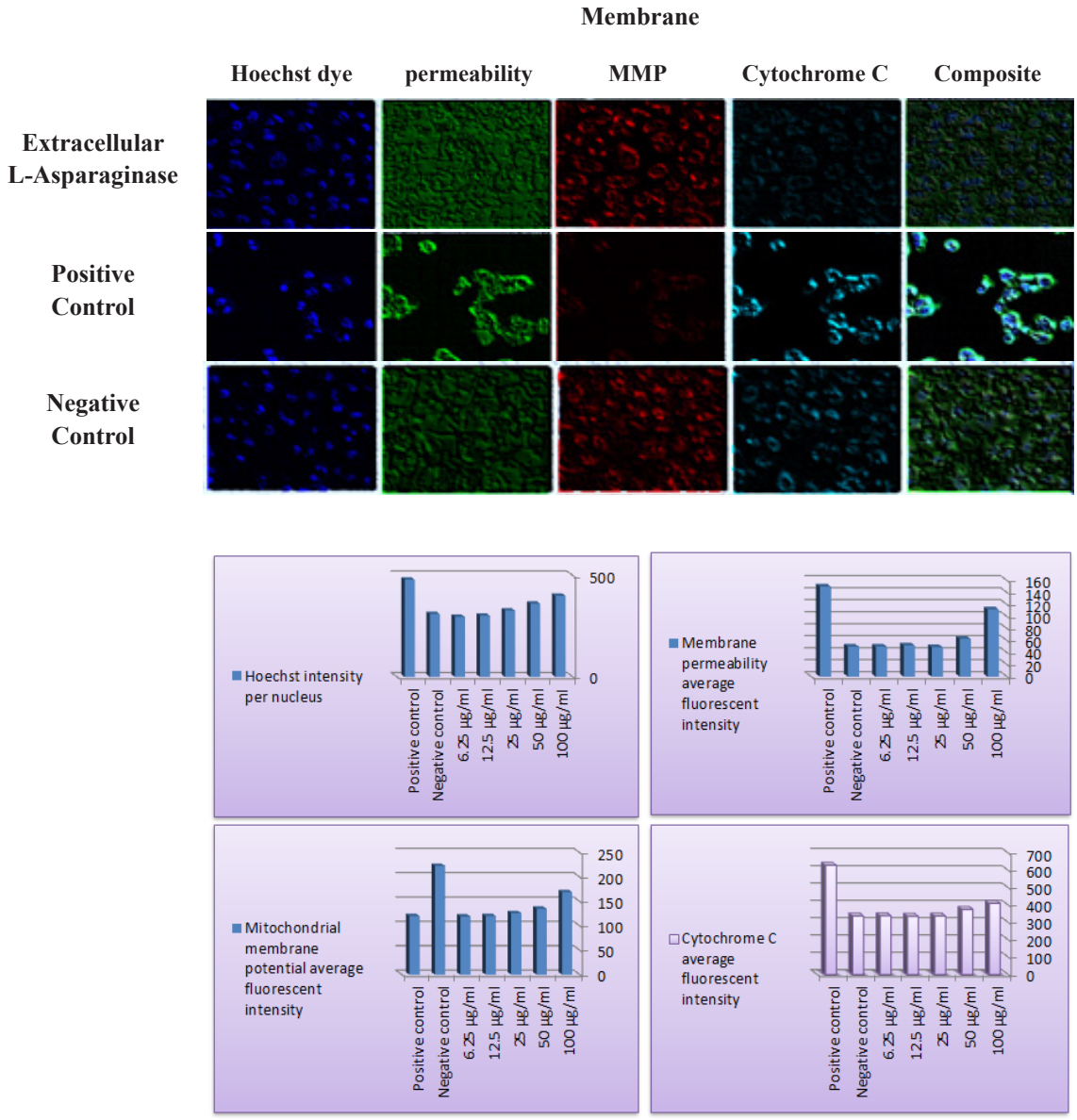

Figure 9 Effect of Intra-cellular L-asparaginase on nuclear morphology, membrane permeabilization, MMP and cytochrome c release. (A) Representative images of PC3 cells treated with medium alone (Negative control), extracellular L-asparaginase and Paclitaxel 5µM (Positive control). The cells stained with Hoechst for nuclear, cell permeability dye, MMP and Cytochrome c. The images from each row were obtained from the same field of each sample (20X). (B-E) Average fluorescence intensities of Hoechst dye, cell permeability dye, MMP and cytochrome c in PC3 cells. Data were mean± SD of fluorescence intensity readings measured from different photos taken.

L-asparaginase is used in treatment of acute lymphoblastic leukemia and non-Hodgkin's lymphoma [23]. The use of L-asparaginase in anticancer therapy is based on its ability to cleave Lasparagine,an amino acid essential for lymphoblasts' growth,to ammonia and L-aspartic acid in serum and cerebrospinal fluid, since lymphoblasts are unable to produce endogenous L-asparagine which leads to death of these cells [24]. Most of the cancer cells are dependent on an exogenous source of this amino acid for survival. However, normal cells are able to synthesize L-asparagine and thus are less affected by its rapid depletion due to treatment with this enzyme. L-asparaginase can also be used to reduce the formation of acrylamide in fried and oven-cooked foods especially in potato chips [25].The formation of acrylamide was attributed to the reaction of free asparagine and reducing sugars . The depletion of asparagine by asparaginase prevented acrylamide

formation[26].

Conclusion: The extracellular Asparaginase showed a cytotoxicity effects against Human prostate cancer cells (PC3) as it reduced the cell viability after 48 hours. PC3 cells were more sensitive to extracellular Asparaginase as it has a lower IC50 value 27.41±2.95 and 70.12±2.53 for extra and intracellular respectively. The cytotoxicity of extracellular Asparaginase was related to the treatment increases nuclear condensation, increment of membrane permeability, MMP attenuation and increased cytochrome c in the cystol.

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الفعالية المضادة للاورام لانزيم لـ ـ اسباراجينيز المنتج من بكتيريا القولون

خالد فاروق الراوي

كلية العلوم/ جامعة الأنبار

الخلاصه:

تم في هذه الدراسة انتاج انزيم لـ-اسباراجينيز من بكتريا القولون بنوعيه داخل خلوي وخارج خلوي وتم تنقيته باتباع خطوات الترسيب بكبريتات الامونيوم وكروماتوغرافيا التبادل الايوني وكروماتوغرافيا الترشيح الهلامي وتم اختبار فعاليتهما المضادة للاورام ضد اربع أنواع من خلايا السرطان البشري (خارج الجسم الحي) وشملت MCF-7 و PC3 و A549 و Hep-G2 باستخدام تقنيات MTT و HCS. بينت النتائج ان الانزيم امتلك فعالية مثبطة للخلايا السرطانية المدروسة ، كانت الخلايا السرطانية من نوع PC3 هي الأكثر حساسية للانزيمين داخل وخارج خلوي اذ بلغت قيمة LC50 لها 27.41 ±2.95 و 70.12 ±2.53 للانزيميني خارج وداخل خلوي على الترتيب.ادى الانزيم خارج خلوي الى زيادة في كثافة المادة النووية للخلايا وزيادة نفاذية الاغشية الخلوية وتقليل القدرة الكامنة لاغشية المايتوكوندريا وازدياد فعالية سايتوكروم سي في سايتوبلازم الخلايا السرطانية مقارنة بخلايا السيطرة.امتلك الانزيم لـ-اسباراجينيز الخارجي فعالية علاجية ضد خلايا السرطان من نوع PC3 خارج الجسم الحي.