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Antitumor Activity of Extra and Intracellular L-Glutaminase Produced From *Burkholderia cepacia* complex.

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ABSTRACT

Tumor cells have an absolute requirement for glutamine as a growth substrate, glutamine is required as a precursor for both DNA synthesis and protein synthesis, the use of glutaminase deprives the tumor cells from L-glutamine and cause selective death of L-glutamine dependent tumor cells.Extra and intra-cellular l-glutaminase were produced from *Burkholderia cepacia* complex, 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 namely 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 glutaminase as it gave a lower IC50 value (27.41±2.95 and 70.12±2.53) for extra and intracellular respectively, extracellular glutaminase treatment increases nuclear condensation, plasma membrane permeability, MMP attenuation and increased cytochrome c in the cystol compared to control.The extra-cellular glutaminase of *Burkholderia cepacia* was a therapeutic agent against human PC3 cell line.

Introduction

L-glutaminase (L-glutamine amidohydrolase E.C. 3.5.1.2) catlyses the hydrolysis of L-glutamine to L-glutamic acid and ammonia (Sajitha *et al*., 2013). This is an essential enzyme for the synthesis of various nitrogenous metabolic intermediates (Teja et al., 2014) and the enzyme plays a major role in the nitrogen metabolism of both prokaryotes and eukaryotes (Katikala et al.,2009). It is widely distributed in plants, animal tissues and microorganism including bacteria, fungi and yeast (Pallem et al.,2010). Interest on amidohydrolases started with the discovery of their antitumor properties (Broome, 1961; El-Asmar and Greenberg, 1966; Santana et al., 1968; Roberts et al., 1970) and since then, a lot of efforts have gone into extensive studies on microbial L-glutaminases with the intention of developing them as antitumor agents (Athira et al.,2014).

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Cancer cells are often referred to as being "glutamine addicted", as they typically are extremely sensitive to glutamine deprivation and hence cannot proliferate in cell culture without it (Erickson and Cerione, 2010). Glutamine 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 (Suba, 1993). Un like normal cells, tumor cell do not demonstrate the L-glutamine synthetase, thus it is dependent on the exogenous supply of L-glutamine for their growth and survival (Rohde et al.,1969). Cancer cells require a robust supply of reduced nitrogen to produce nucleotides, non-essential amino acids and a high cellular redox activity, glutamine provides a major substrate for respiration as well as nitrogen for the production of proteins, hexosamines, and macromolecules therefore, glutamine is one of key molecules in cancer metabolism during cell proliferation (Unissa et al.,2014). The L-glutaminase causes selective death

of glutamine dependent tumor cells by blocking these cells of glutamine. The present study deals with the purification of intra and extracellular L-glutaminase from *Burkholderia cepacia* complex, 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.

Materials and Methods

Bacterial strain

The strain *Burkholderia cepacia* complex used in this study was isolated from soil during a screening study for l-glutaminase producing bacteria, the culture was maintained on nutrient agar slant at 4° C.

Molecular identification

DNA was prepared for PCR amplification from overnight cultures using promega kit for chromosomal DNA extraction, PCR was performed as described by Mahenthiralingam et al., (2000) using PreMix reagents (Bioneer Trade(shanghai) Co.,Ltd. PCR OF *Burkholderia cepacia* complex recA genes was carried out using primer BCR1, TGACCGCCGAGAAGAGGCAA and BCR2, CTCTTCTTCGTCCATCGCCTC, amplifying a 1043-bp fragment. Thermal cycling was carried out in a thermal cycler (Esco Micro Pte Ltd) for 30 cycles of 30s at 94°C, annealing for 45s at 58°C and extension at 72°C for 60s, with a final 10min extension at 72°C. Approximately 2µl of each PCR product was visualized by 2% agarose gel electrophoresis.

Growth conditions for bacterial culture

Burkholderia cepacia complex was grown on modified glutamine broth containing: glucose 20 g, Ammonium sulphate 10 g , Sodium sulphate 10 g, KCl 0.5 g , $MgSO_4.7H_2O$ 0.5 g , $FeSO_4.7H_2O$ 0.1 g , $ZnSO_4.7H_2O$ 1.0 g , KH_2PO_4 1.0 g, Distill water 1000 ml. The pH of the medium was adjusted to 7.0 and incubated at 28°C for 24 h in a shaking incubator at 150 rpm.

Glutaminase 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 .

Assay of L-glutaminase Activity

Assay of L-glutaminase activity was carried out as per Imada et al. (1973) . 0.5 ml of 0.2 M glutamine 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-glutaminase is the amount of enzyme which liberates 1µmole of ammonia under optimum condition . The enzyme yield was expressed as unit/ml (U/ml).

Determination of protein concentration

Protein concentration was determined (Lowry et al., 1951) using bovine serum albumin as the standard.

Glutaminase purification

The intra and extracellular glutaminase 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

MTT cytotoxicity assay

PC3 cells were plated at 1 x 10^4 cells/well by adding 200 µL of a 5 x 10^4 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 glutaminase 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% CO_2, 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% CO_2, 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 (Al-Qubaisi et al,2011)

Multiple cytotoxicity assay

Cellomics Multiparameter Cytotoxicity 3 Kit (thermo Scientific) was used. Cells were plated at 1x10^4 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 DyLight™ 649. Cells were

Results and Discussion

The bacterial isolate was confirmed as Bcc by PCR amplification of the *recA* gene with specific primers for Bcc, BCR1 and BCR2, an amplicon of about 1,043 bp was obtained (fig.1). The *recA* gene has been proven very useful in discriminating Bcc from closely related species (Mahenthiralingam et al., 2000; Leite et al., 2011; Medina-Pascual et al.,2012), recA (RecA is a protein essential for repair and recombination of DNA) has been shown to be diploid, with a single copy of the gene residing on each of the two large chromosomes present in Bcc strains (Li et al.,2010).

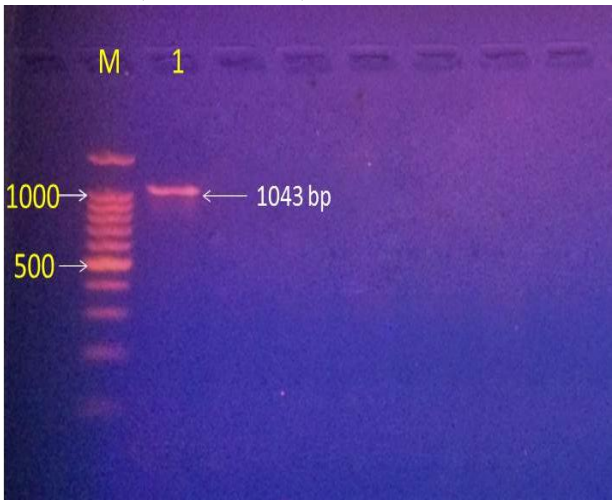


Fig.1: Gel electrophoresis demonstrates the PCR product of recA bacterial gene using (BCR-1/BCR-2) primers. (M) lane shows the 100bp DNA leader, lane 2 show the *Burkholderia cepacia* complex amplified DNA product.

The production of L-glutaminase by the bacterial cells may be endo- or exo-enzymes, which

was based on the metabolic activity of bacterial cells (Prakash *et al*,2009). The results showed that *Burkholderia cepacia* have the ability to produce intra- and extra-cellular glutaminase and the extra-cellular glutaminase was higher than the intra-cellular. Renu & Chandrasekaran, (1992) reported the presence of intra- and extra cellular glutaminase 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-glutaminase is secreted extracellulary also by strains of *Bacillus subtilis* and *B. licheniformis* (Cook *et al*,1981), and *Debaryomyces sp*.(Dura *et al*,2002).

Enzymes purification

Extracellular glutaminase:

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-glutaminase from *Burkholderia capacia* complex

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 glutaminase 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.

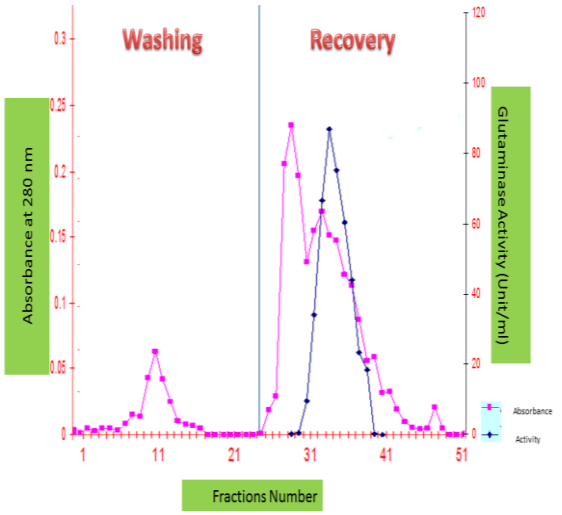


Fig.2. Elution diagram of Extra-cellular L-glutaminase of *Burkholderia capacia* complex from DEAE-cellulose

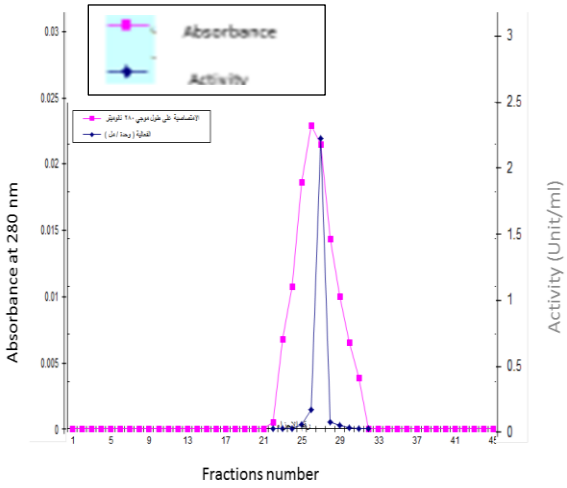


Fig.3. Elution diagram of Extra-cellular L-glutaminase of *Burkholderia capacia* complex from Sephadex G-300

Intracellular glutaminase:

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. Sequential multi-steps process for purification of Intra-cellular L-glutaminase from *Burkholderia capacia* complex

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 glutaminase activity having 20.7% yield. The most active fractions (F22-F32) were pooled together, concentrated with lyophilizer and stored at -20°C.

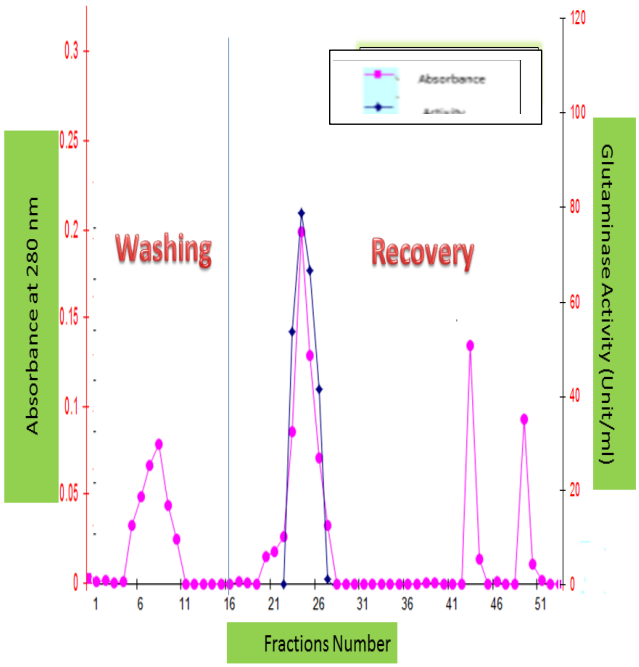


Fig.4. Elution diagram of Intra-cellular L-glutaminase of *Burkholderia capacia* complex from DEAE-cellulose

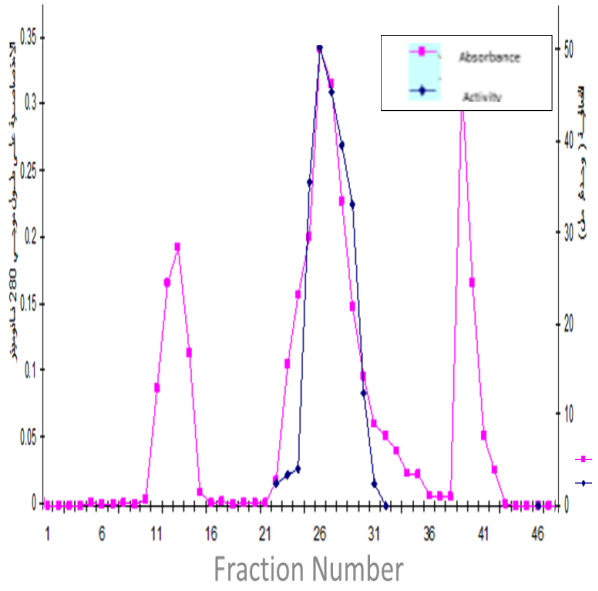


Fig.5. Elution diagram of Intra-cellular L-glutaminase of *Burkholderia capacia* complex from Sephadex G-300

Antitumor activity

To evaluate the cytotoxic activity, extra and intracellular glutaminase 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 glutaminase (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 (IC_{50}), however, the highest antitumor was recorded towards PC3 cells with IC_{50} 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 IC_{50} 45.08±6.32 and 94±8.45 for extra and intracellular respectively (Table3). In this connection, the cytotoxicity of L-glutaminase from *Aspergillus flavus* KUGF009 towards MCF-7 cell line by the MTT assay (IC_{50} 250 µg/ml) was reported by Nathiya et al.(2012) and El shafei et al.(2014) reported purified L-glutaminase IC_{50} 63.3 µg/ml and partially purified L-glutaminase IC_{50} 109.9 µg/ml from *Penicillium brevicompactum* NRC 829 towards Hep-G2 cell line. While the cytotoxicity of L-glutaminase from *Aspergillus oryzae* towards MCF-7 cell line by the MTT assay (IC_{50} 283.288µg/ml) was reported by Sunil Dutt et al.(2014).

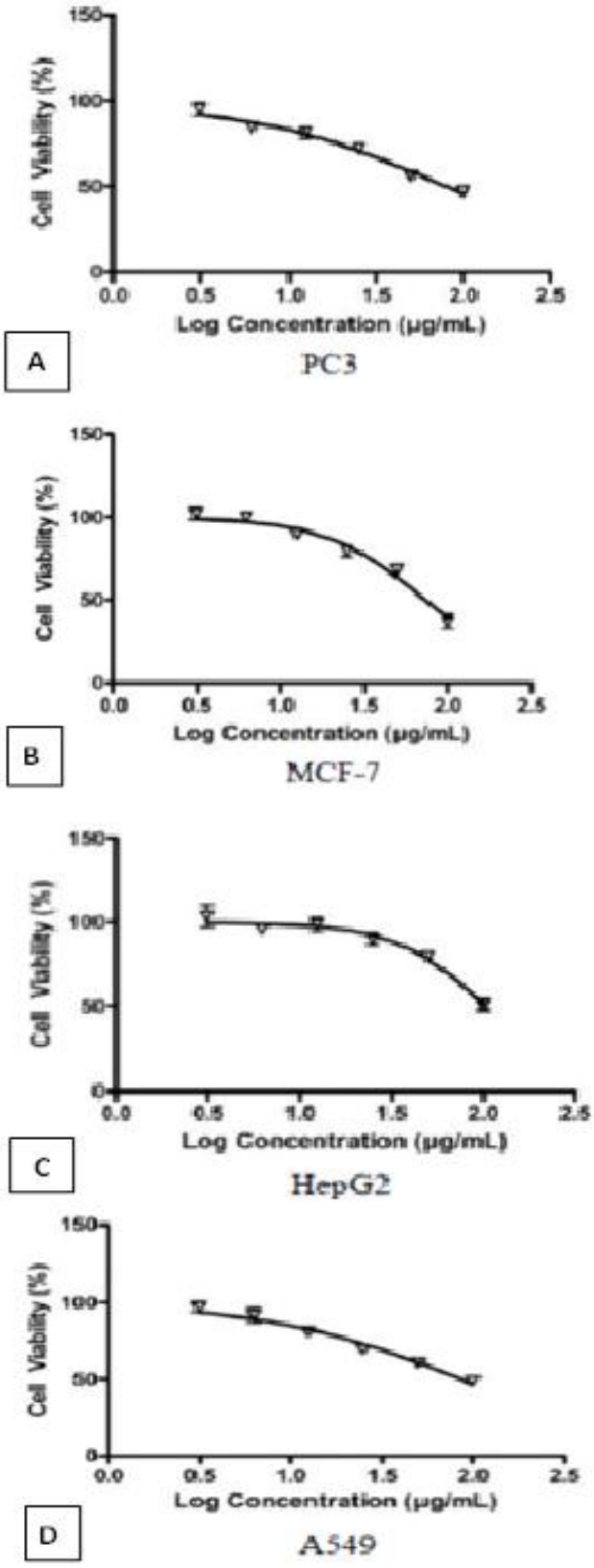


Fig.6. Effects of Extra-cellular L-glutaminase in PC3 (A) and MCF-7 (B) and HepG2 (C) and A549 (D). Effects of Extra-cellular L-glutaminase against the viability of treated cells were evaluated through mitochondrial activity using the MTT assay.

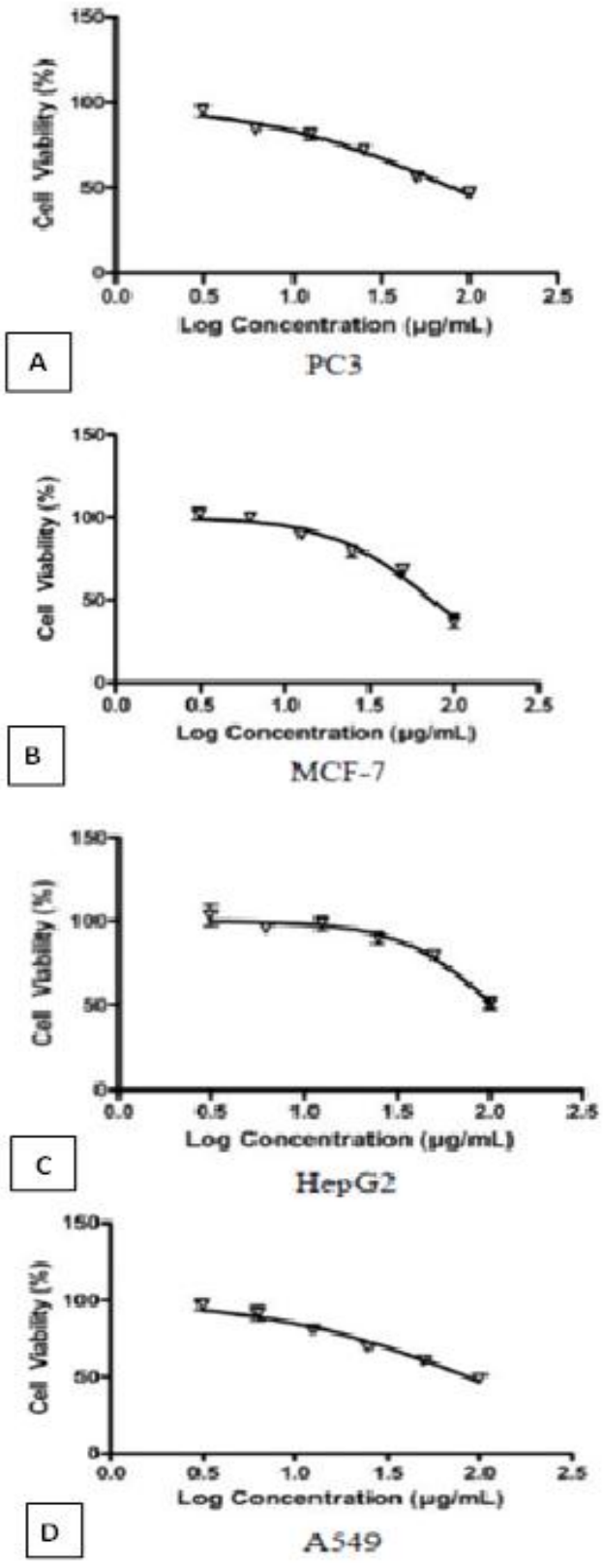


Fig.7. Effects of Intra-cellular L-glutaminase in PC3 (A) and MCF-7 (B) and HepG2 (C) and A549 (D). Effects of Extra-cellular L-glutaminase against the viability of treated cells were evaluated through mitochondrial activity using the MTT assay.

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

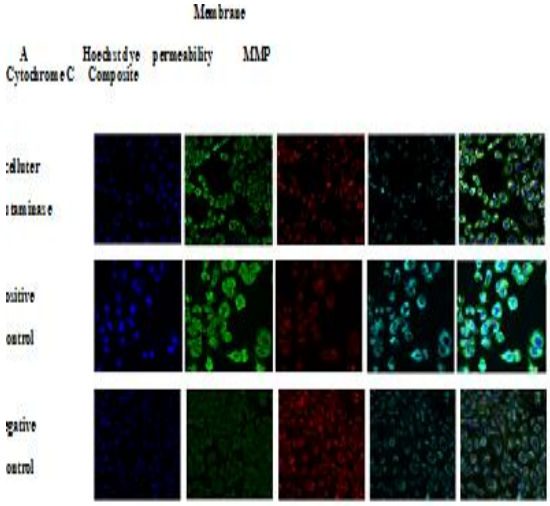
L-glutaminase	HepG2	A594	PC3	MCF-7
Intracellular	94±8.45	82.37±9.04	70.12±2.53	76.54±4.19
Extracellular	45.08±6.32	37.93±3.85	27.41±2.95	34.78±4.01

Data are mean ± SD

PC3 cell line were more sensitive to extra and intracellular glutaminase as it gave a lower IC_{50} 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 (Ye et al, 2007).

The multiparameter cytotoxicity kit 3 from Cellomics was used to examine the effect of extra and intracellular glutaminase 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 glutaminase-treated cells, cytochrome c stained in the cytosol, indicating glutaminase-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 (Gogvadze *et at.*,2008), change in the MMP increase the release of apoptogenic factors such as cytochrome c from the outer mitochondrial membrane space into the cytosol (Looi *et al.*, 2013). 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 (Chalan *et al.*,2008). 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 (Kwong *et al.*, 2007) 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 glutaminase was less toxic to PC3 cell line compared to extracellular glutaminase (Fig.9 A-E).



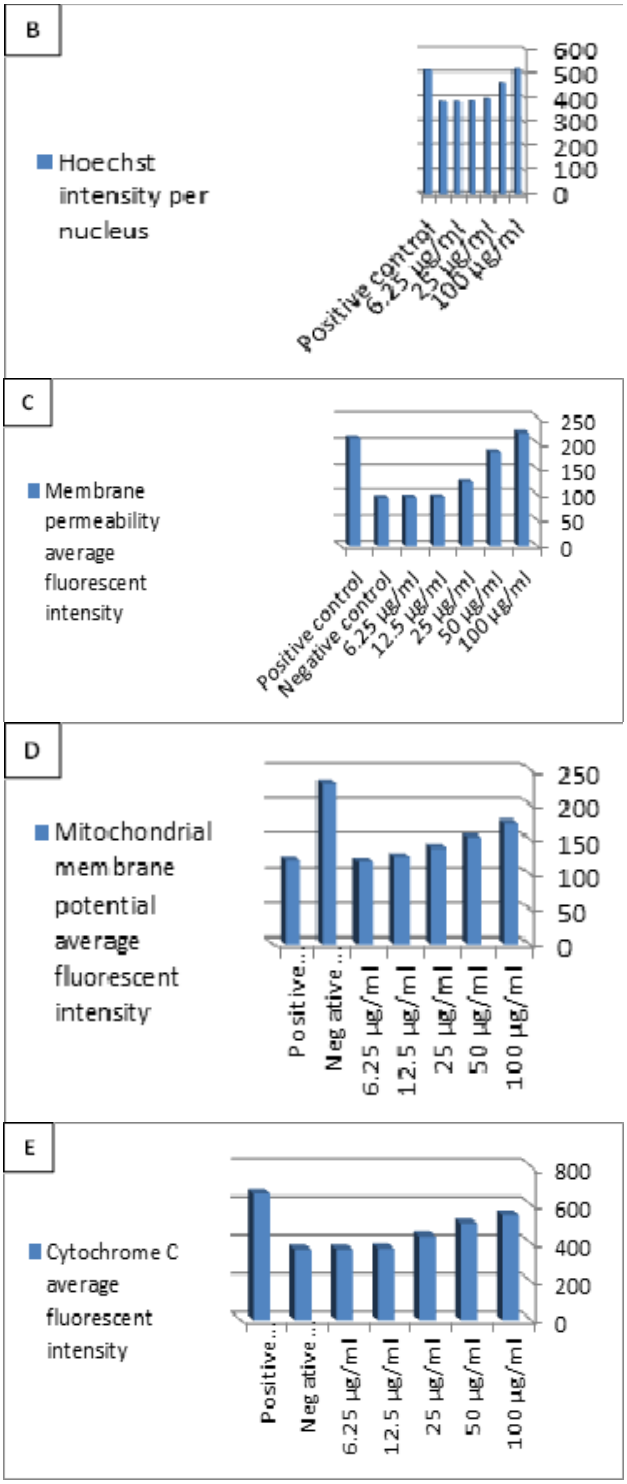
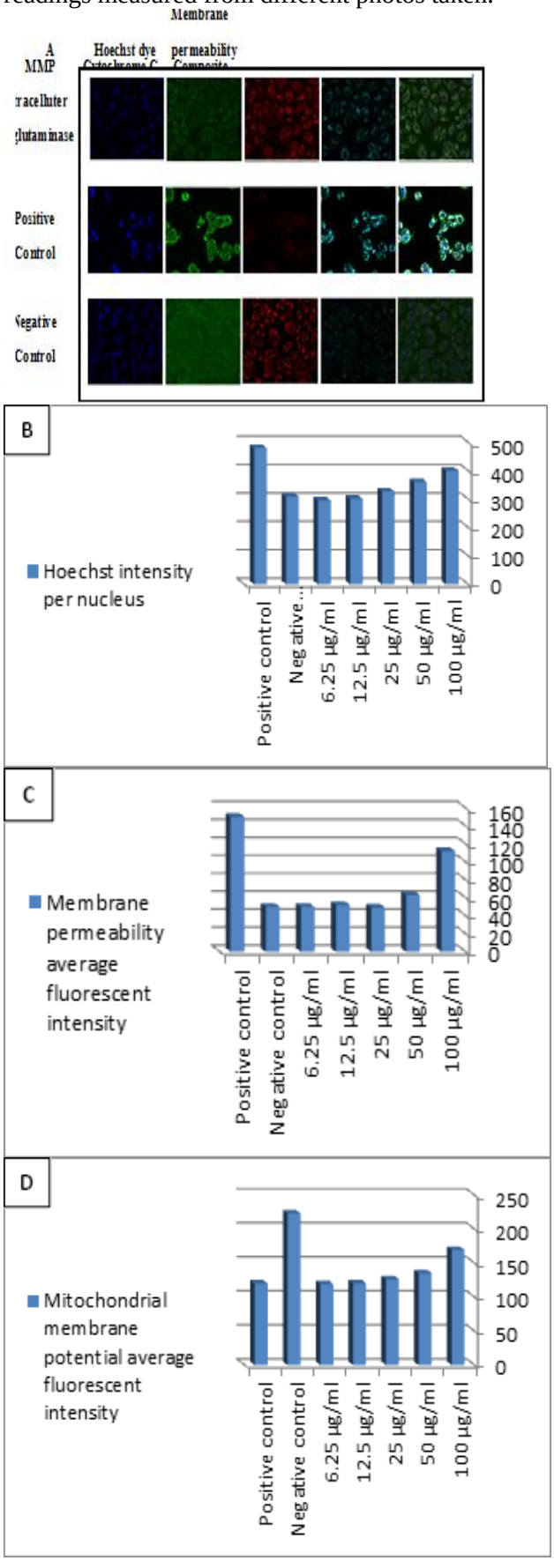


Figure 8 Effect of Extra-cellular l-glutaminase on nuclear morphology, membrane permeabilization, MMP and cytochrome c release.

(A) Representative images of PC3 cells treated with medium alone (Negative control), intracellular l-glutaminase 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.



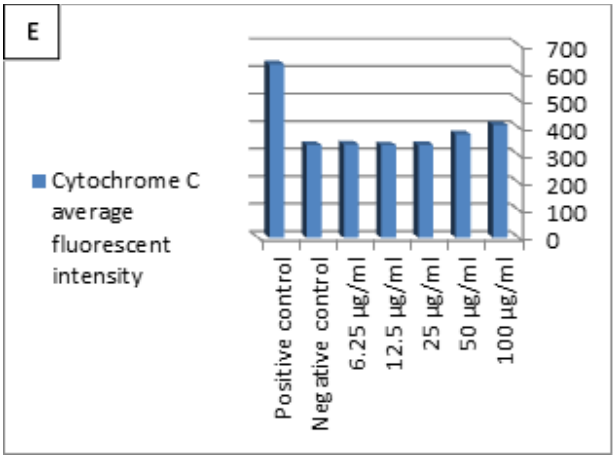


Figure 9 Effect of Intra-cellular l-glutaminase on nuclear morphology, membrane permeabilization, MMP and cytochrome c release.

(A) Representative images of PC3 cells treated with medium alone (Negative control), extracellular l-glutaminase 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.

Conclusion:

The extracellular glutaminase 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 glutaminase as it has a lower IC_{50} value 27.41±2.95 and 70.12±2.53 for extra and intracellular respectively. The cytotoxicity of extracellular glutaminase 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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