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Characterization and Antitumor Activity Study for a Therapeutically Important L-glutaminase Purified from Staphylococcus Aureus

Mustafa Gh. AL-Juhaishy. Asmaa A. Hussein

Abstract--- *L-glutaminase enzyme produced under optimum conditions in a previous study was purified using precipitation with 80% saturation of sulfate ammonium, ion exchange chromatography on column DEAE-cellulose, and filtration gel chromatography during Sephadex G-200 column, the activity specific of the purified enzyme after last step was raised to 1000 U/mg for 38.4 folds of purification and 69% recovery enzyme. MWut. of purified l-glutaminase was estimation through S.D.S-P.A.G.E to be 35 KDa, It was gave highest activity at pH 8.0 and stability at pH 7.5, the enzyme was active at 37 ^oC and stable at a range of temperatures from 20^oC to 37 ^oC. Also l-glutaminase activity was inhibited to (62%) in the presence of $CaCl_2$. L-glutaminase was tested for in-vitro cytotoxic activity using MTT assay against colorectal adenocarcinoma (LS 174T) cell line which was inhibited with an IC50 of 37.19 IU\ml however, it didn't show a important affect on normal cell.*

Keywords--- *L-Glutaminase, MTT Assay, LS 174T Cell Line.*

I. INTRODUCTION

L-glutaminese (L-Glutaminase amidohydrolase EC 3.5.1.2) is one of most important enzymes used to fight the cancer, which considered big health problem in the world. L-glutaminase acts to breakdown l-glutamine and converted it to glutamic acid and ammonia group, the amino acid l-glutamine is considered as very important essential amino acid for cancer cell since these cells lose the mechanism of l-glutamine synthesis, which present in normal cells. natural human cells have mechanism of l-glutamine synthesis, therefore, cells are independent on exogenous supply of glutamine whereas cells cancer insufficiency this mechanism and consist of supply of L-glutamene of blood to the growth, division and survival(1). Depletion of l-glutamine from cancer cells environment can block the proliferation of cancer cells and its division become faster than normal cells (1, 2, 3). Microorganisms like bacteria, fungi and yeast are potential sources for l-glutamines, utilizing the microbes for l-glutaminese making is extra desirable as they has simple growth requirements, cheaper production costs and easy to process (1). A lot of previous studies screened, isolated and purified l-glutaminase from bacterial source from different samples and environment such as *Bacillus subtilis* (5) and *Lactobacillus fermentum* (6). The anticancer activity of this enzyme tested such as l-glutaminase purified from; *Aspergillus niger* (3), *Streptomyces canaries* (7) and *Bacillus cereus* LC13(8). Because no one study in Iraq purified l-glutaminase from *S. aureus* and tested the cytotoxic activity of the enzyme against cancer cells. According to those mentioned above, this study was aimed to characterization of L-glutaminase enzyme purified from clinical locally isolated *staphylococcus aureus* and test its cytotoxic activity against colorectal cancer cells (LS 174Tcell line).

Mustafa Gh. AL-Juhaishy. Asmaa A. Hussein, College of Biotechnology, Al-Nahrain University, Iraq.

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II. MATERIAL AND METHODS

Preparation of crude enzyme

S. aureus was grown in a production medium after optimization from previous study(not shown data), ($MgSo_4.7H_2o$ 0.5g/l,,$Kcl.7H_2o$ 0.5g/l, KH_2Po_4 1.0 g/l $ZnSo_4.7H_2o$ 1.0 g/l and L-glutamine 5.0 g\l) supplemented with 0.1% starch and 0.1% meat extract (pH 8.0) at 37 °C for 48h. The culture was centrefuged at 6,000 rpm, of 30 mint. and cell free supernatant utilizing as crude enzyme.

L-glutaminase activity assay method

L-glutaminese activity was check according to Mannan way (9). In addition, Protein content was assayed according to Bradford method (18).

L-glutaminase purification

Precipitation ammonium sulfate

Added ammonium sulfate in crude enzyme (supernatant) by different of the ratios saturation of (20, 40, 60 and 80 %). The mix was mixed softly in magnatic stirrer to 20 min and using centrifuged at 6 rpm of 20 mint at 4°C.

Gel filtration chromatography

L-glutaminase obtained of ion exchange step was used to Sephadex G-200 (1.5x55cm). Elution was achieved at a flow rate of 30 ml/hr part then utilizing the same buffer to equilibration. Absorbance magearment at 280.0 nm. L-glutaminase activity was too estimation in each part. **Determination of the molecular weight and the purity enzyme**

Determined L-glutaminase Purity via electrophorsis on gel SDS-polyacrylamide on the find for the proteins standard (Alpha-lactalbumin, human erythrocyte phosphorylase B soybean carbonic anhydrase, trypsin inhibitor, aldolase, and Myosin). Interval of protein migrated to anode was measured after electrophoresis on polyacrylamide gel to calculate Mwut. of the enzymeL- glutaminese characterization (10).

Determination of pH affects L-glutaminase Activity

Added the enzyme Purified of 0.1 mM L-glutaminese and buffers0.05M of PH about of 5 - 10. The buffers utilized was sodium acetate pH buffer (5 and 6), Tris buffer for pH values (7, 8, 9, and 10). activity Enzymatic for each one was measured. Moreover, the relationship among several pH and activity enzyme was plotted.

Determination of pH affects on L-glutaminase stability

Enzyme was pre-incubated for 30 min. at 37 ^{O}C with buffer of various pH (5, 6, 7, 8, 9, 10). then cooled in ice bath. activity Enzymatic estimation of the continuing activity (%) of L-glutaminase was plotted against value pH.

Determenation of several ions and inhibitors affect on l-glutaminase Activity

Enzyme was incubated with an equal volume of a different metal ions (NaCl, KCl, $CaCl_2$, $MnCl_2$ and $CuCl_2$) prepared at 1 mM concentrations for 30 min. at 37°C. Control was an enzyme solution without any of these compounds. The remaining activity was assayed for each treatment.

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Cytotoxicity assay

Colorectal carcinoma LS-174T cell line was seeded on to 96 well plates for conc. of 1.0 x l05 cells/ml. next incubation to 24 -48 hr at 37°C, when the confluent mono layer of LS-174T cells was complete (80%-100%). Different concentrations (1, 10, 50, 100 and 200 IU/ml) of microtiter composites add to cultured wells of the volume final of 100 µl in each well.

Then 24 hours. incubation in 5% CO_2 at 37°C, the microtiter 96 wells plates was marched out and transferred to biohazard safety cabinet, via sterilized environments to avoid any contamination. Wholly utilized wells media was discarded, The LS-174T cell mono layers washing through 1ml solution PBS (Phosphate Buffer Saline) to eliminate any residual quantity of standard or composites anticancer drugs utilized that might be affect or interacts by reagents MTT,. then dissolving the form azenvia addition di-methyl sulfoxide DMSO (1:1) diluted in isopropanol on each wells containing blank wells, the at 490 nm for reference wavelength at 630 nm via reader ELISA. the Mean blank absorption subscribe of another samples and controls wells absorptions (8, 12, 13).

Cell Capability %= [Absorbance of sample treated / Absorbance of sample non-treated] × 100

III. RESULTS AND DISCUSSION

L-glutaminase purification and molecular weight determination

Purification steps of l-glutaminase enzyme produced from *S. aureus* summarized in table (1), high enzyme activity specific (40.4 U/mg protein) was achieved at 80% ammonium sulfate saturation. Purification by DEAE-cellulose chromatography showed increased in especific activity of the enzyme. However, just one protein peak shown in the wash step, next elution via gradient conc. of sodium chloride two peaks protein were appeared (Figure. 1).

L-glutaminase activity was assayed for all these protein peaks, and most L-glutaminase activity was observed at eluted proteins (fractions 37 to 44). Enzyme activity specific was measured to be (292.5 IU\mg protein), too its purificetion fold reached 11.25 with 76.7 % overall yield. Gel filtration technique chromatography was the last step in the purification of l-glutaminase produced through local isolate *S. aureus* (Figure. 2) with specific activity of 1000U/mg, purification fold of 38.4 and the L-glutaminase produce 69.7%. In study submitted by Elshafei *et al.* (15) l-glutaminase enzyme of *P. brevicompectum* NRC 829 purified via gel filtration with especific activity reached (869.1 U/mg), purification fold (162) and 48% enzyme recovery. Jayabalan *et al.* (16) mentioned, l-glutaminase from marine *Brevundimonas diminuta* MTCC purified used gel filtration with specific activity reached (60.15 U/mg) and 48.12% enzyme recovery. Analysis of protein profile for purified L-glutaminase of *S. aureus* give one band next gel filtration step, which indicates the purity of enzyme (Figure.3). The molecular weight of purified l-glutaminase was estimation via interpolation for linear molecular weight versus the Rm value (mobility relative) to be 35 kDa (Figure.4). Electrophoresis is considered as another step of purification (17). Singh *et al.* (14) mentioned that l-glutaminase with anticancer property has 35 KDa that purified from *B. cereus* MTCC 1305. In other study, molecular weight of l-glutaminase purified from *B. cereus* LC13 was 35 ± 1 kDa (8).

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Characterization of purified l-glutaminase

The maximum activity of l-glutaminese achieved at pH 7and 37°C. However, l-glutaminase was more stable at pH 7.5 and the range of thermal stability 20 °C to 37°C. The enzyme activity increased when $MnCl_2$ was applied, but $CaCl_2$ was found to inhibit enzyme activity to 62% (Table 2). Previous study showed the higher activity of *V. Costicola* l-glutaminase (10) in pH 8.0, 37°C and thermal stability was at 45°C.

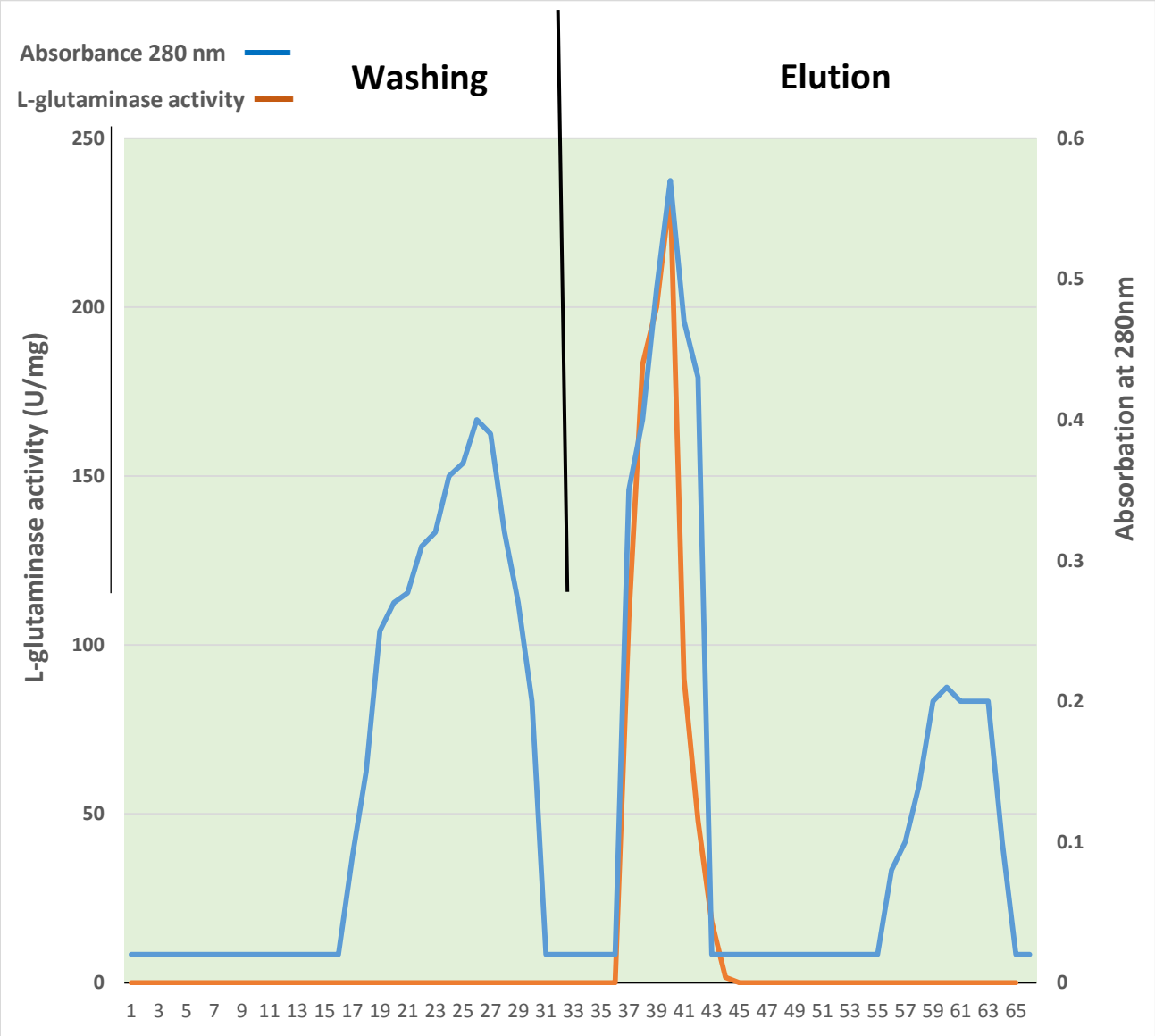

Figure 1: Chromatography Ion exchange of L-glutaminase produced through locally isolated *Staphylococcus aureus* utilizing column DEAE-cellulose (2x35cm) by a flow rate of 30ml/h

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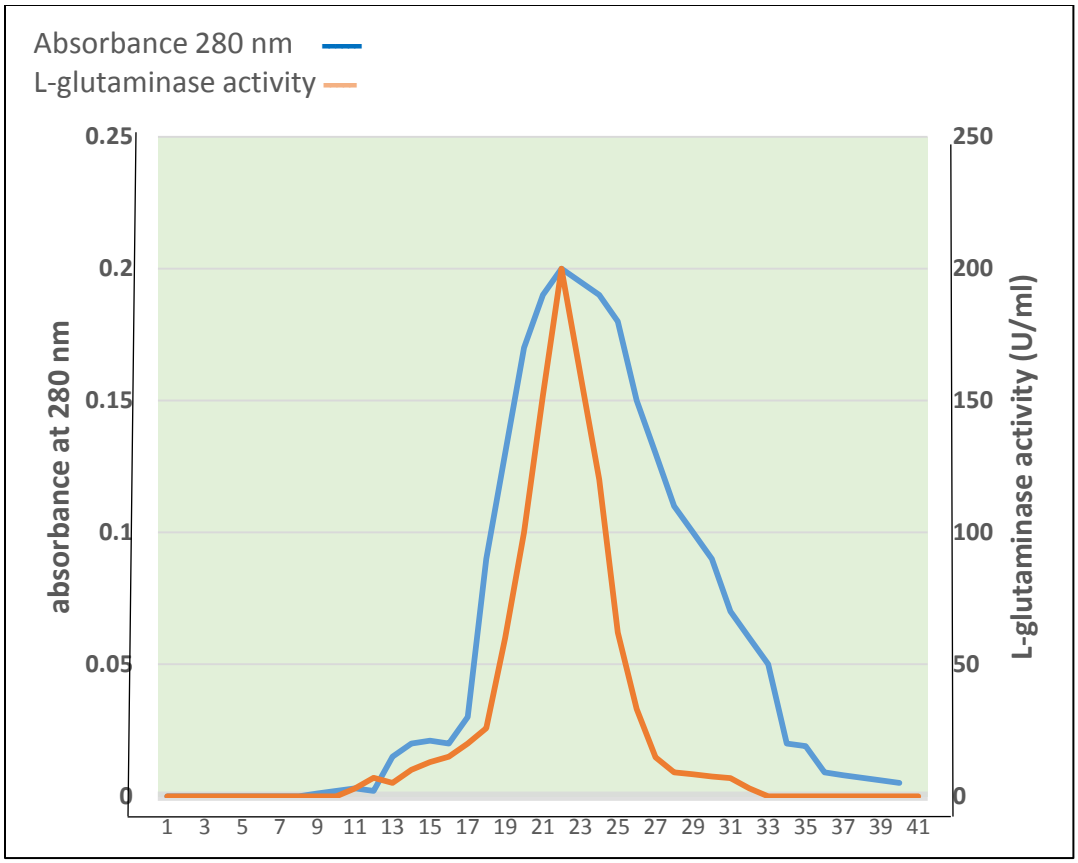

Figure 2: Gel filtration chromatography L-glutaminase produced by locally isolated *Staphylococcus aureus* using SephadexG-200 column (1.5x55cm) equilibrated by 0.10 M Tris-HCL buffer pH8

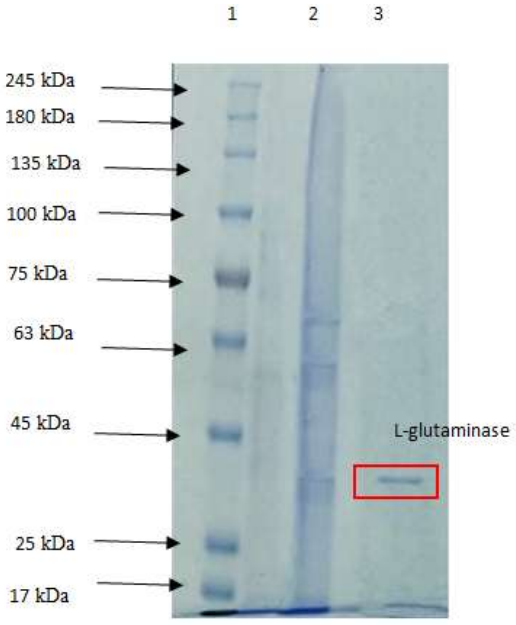

Figure 3: SDS Polyacrylamide gel electrophoresis of crude and purified l-glutaminase produced by *Staphylococcus aureus*

(1): Proteins marker (2): Proteins bands in crude filtrate

(3): Protein band after gel filtration step

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Cytotoxic activity of l-glutaminase utilizing MTT assay

The sensitivity of LS174T cell line to purified L-glutaminase appeared to be dose dependent, resulting in a significant decrease in viable cells viability reached to maximum inhibition with 200 IU/ml concentration of purified L-glutaminase (Table 3). The IC_{50} that the half-maximal concentration inhibitory of growth cell constructing dose-response curve was measured. Purified L-glutaminase appeared IC_{50} value of 37.19IU/ml against LS174T cell line (figure 5). While doxorubicin IC_{50} value was 8.89 IU/ml (figure 6). 36.7% and 21.4% cell capability was observed next handling with 1 IU/ml and 200 IU/ml concentration of purified l-glutaminase respectively (figure 4). While, 43.3% and 29.1% cell capability was observed next handling by 1 IU/ml and 200 IU/ml concentration of doxorubicin respectively (figure 5). However, purified *S. aureus* l-glutaminase did not appear important affect on the viability of normal cell tested (WRL) cell line.

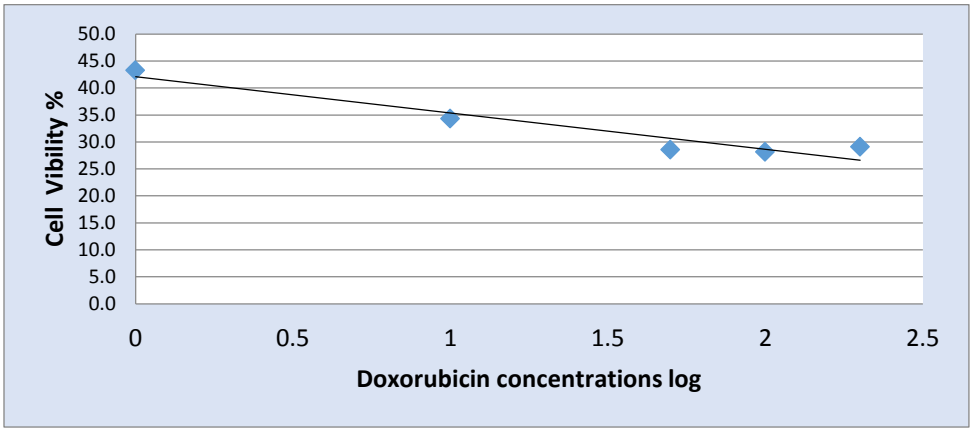

Figure 4: Cytotoxic activity of Doxorubicin on colorectal adenocarcinoma LS174T utilizing MTT test then 24 hs. at 37^{o} C

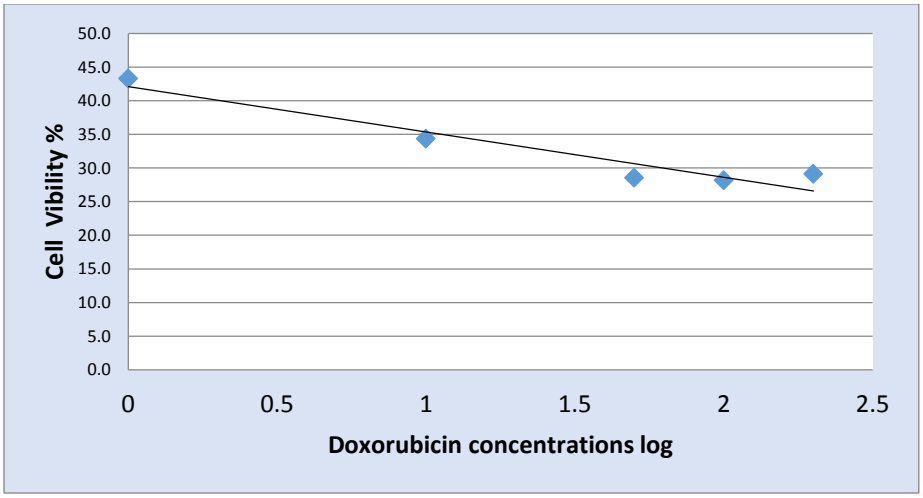

Figure 5: Cytotoxic activity of Doxorubicin on colorectal adenocarcinoma LS174T utilizing MTT test then 24 h. at 37^{o} C

The specially effected of l-glutaminase on cancer cells due to absent l-glutamine amino acid synthesis mechanism. Which present in nonmalignant cells (4). L-glutaminase is a potential antitumor drug for the handling of

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l-glutamine-auxotrophic tumors like hepatocellular carcinomas, breast cancer and specially on cervical carcinoma (HeLa) which had addiction to l-glutamine (7). In other experiment, l-glutaminase purified from *A. oryzae* exhibited cytotoxic activity with an IC50 283.288 µg/ml against the human cell line MCF-7 (breast cancer cell line) (3). The cytotoxicity of l-glutaminase purified purified from *S. canaries* against five types of cancer cell lines MCF-7 (Not effected), Hep-G2(IC50, 6.8 µg/ml), HCT-116(IC50, 64.7 µg/ml), RAW264.7(IC50, 59.3 µg/ml) and HeLa (IC50, 8.3 µg/ml)(7).

Table 1: Purification steps of l-glutaminase produced by Staphylococcus aureus

Purification steps	Volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (folds)	Yield (%)
Crude enzyme	100	43	1.65	26	4300	1	100
Ammonium sulfate precipitation (80%)	25	141	3.49	40.4	3525	1.5	81.9
DEAE-cellulose	15	220	0.8	292.5	3300	11.25	76.7
Sephadex G-200	15	200	0.2	1000	3000	38.4	69.7

Table 2: Characterization of purified l-glutaminase

Characterizations factors		
Effect of pH on activity of L glutaminase	**pH**	**l-glutaminase activity (U/ml)**
	5	**26**
	6	**112.3**
	7	**188**
	8	**203**
	9	**178**
	10	**123.6**
Effect of pH on stability of L glutaminase	**pH**	**Remaining activity %**
	5	**40**
	6	**56**
	7	**83.2**
	7.5	**100**
	8	**86.4**
	9	**80.45**
	10	**59**
Effect of temperature on activity of L glutaminase	**Temperature ºC**	**l-glutaminase activity (U/ml)**
	20	**28**
	25	**50**
	30	**146**
	37	**200**
	40	**113**
	45	**49**
	50	**41**
	60	**14**
Effect of temperature on stability of l- glutaminase	**Temperature ºC**	**Remaining activity %**
	20	**100**
	25	**100**
	30	**100**
	37	**100**
	40	**85**
	45	**45**
	50	**30**
	60	**0**
Effect of ions and inhibitors on l-glutaminase activity	**Metal ions at (1mM)**	**Remaining activity (%)**
	NaCl	98
	KCl	96
	$CuCl_2$	97.5
	$MnCl_2$	107
	$CaCl_2$	62

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Table 3: Inhibitor ratio of LS174T cell line by different purified l-glutaminase and doxorubicin concentrations

Dose IU/ml	Cell inhibition (%)by l-glutaminase	Cell inhibition (%)by doxorubicin
1	21.51 ± 0.15423	15 ± 0.32146
10	^{24}.49 ± 0.21879	23.9 ± 0.25725
50	25.16 ± 0.17586	29.6 ± 0.18230
100	32.51 ± 0.22866	30.0 ± 0.31991
200	36.74 ±0.22866	29.1 ± 0.26153

IV. CONCLUSION

L-glutaminase was tested for *in-vitro* cytotoxic activity using MTT assay against colorectal adenocarcinoma (LS 174T) cell line which was inhibited with an IC50 of 37.19 IU\ml however, it didn't show a important affect on normal cell.

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