9

Purification and Characterization of *Streptomyces Canarius* L- Glutaminase and its Anticancer Activity

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L-GLUTAMINASE produced by *Streptomyces canarius* FR (KC460654) was purified to homogeneity with an apparent molecular mass of 44 kDa. The purified enzyme was 17.9 fold with a final specific activity 132.2 U/mg protein and 28% yield recovery. The purified L- glutaminase showed a maximal activity against L- glutamine when incubated at pH 8.0 and 40°C for 30 min. The enzyme maintained its stability at wide pH range for 5.0- 11.0 and is thermally stable up to 60°C with Tm value 57.5°C. The enzyme had high affinity and catalytic activity for L- glutamine (Km 2.4 mM, Vmax 28.1 U/mg/min), followed by L- asparagine and L-aspartic acid. *In vivo*, the purified enzyme showed no obvious changes in liver and kidney functions. Also, it has a normal effect in all hematological parameters except slight effect on RBCs and levels of platelets as shown after 10 days of rabbit's injection with purified enzyme. Interestingly, it showed slight effect on the tested renal and liver tissues comparing with negative control. The anticancer activity of the purified enzyme was determined against five types of human cancer cell lines using MTT assay *in vitro*. The tested enzyme had a high efficiency against Hep-G2 cell (IC_{50}, 6.8 µg/ml) and HeLa cells (IC_{50}, 8.3 µg/ml), while the growth of MCF-7 cells was not affected. On the other hand, the tested enzyme had a moderate cytotoxic effect against HCT-116 cell (IC_{50}, 64.7 µg/ml) and RAW 264.7 cell (IC_{50}, 59.3 µg/ml).

Keywords: L-glutaminase, Anti-cancer, Cytotoxicity, MTT assay.

L-glutaminases (L-glutamine amidohydrolase E.C 3.5.1.2) are ubiquitous in the biological world and organisms ranging from bacteria to human beings have the enzyme (Chantawannakul *et al.,* 2003). The enzyme catalyzes the deamidation of L-glutamine to L-glutamic acid and ammonia (Roberts *et al.,*1970). L-glutaminase has been received significant attention since it was reported extensively as antileukemic agent and use in food industries imparting the flavor and aroma to the foods (Kyoko *et al.,* 2004; Robert *et al.,* 2001).

Since the discovery of its anti-tumor properties, L-glutaminases have been in prime focus and microbial sources of the enzyme are sought. Where, high rate of glutamine consumption is a characteristic nature of some types of cancerous cells

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(Lazarus and Panasci 1986). Based on this character experimental therapies have been developed to deprive L-glutamine to tumor cells (Iyer and Singhal 2008). Tumor growth regulation can be achieved by inhibition of both protein and nucleic biosynthesis in the cancerous cells due to the lack of availability of any component of these acid macromolecules (Tanaka *et al.,*1988). Inhibition of the tumor cell uptake of glutamine is one of the possible ways to stop the growth and this is the best accomplished by the use of L-glutaminase, which breaks down L-glutamine. This in fact, results in a selective starvation of the tumor cells because unlike normal cells they lack properly functioning glutamine biosynthetic machinery (Tanaka *et al.,*1988 and Dang 2010). Warrell *et al.* (1982) used the L-glutaminase against adult leukemia. One of the major problems encountered in the treatment with microbial L-glutaminases is the development of immune responses against the enzyme.

Microbial therapeutic enzymes have a broad variety of specific uses: as oncolytics, thrombolytics or anticoagulant and largely as anticancer (Sabu 2003; Sabu *et al.,* 2005). Although microorganisms are potential sources of therapeutic enzymes, utilization of such enzymes for therapeutic purposes is limited because of their incompatibility with the human body. But there is an increased focus on utilization of microbial enzymes because of economic feasibility (Sabu 2003).

Streptomycetes have attracted much attention because of their ability to make secondary metabolites that have a wide range of bioactivities and thereby may find use as antibiotics, immunosuppressants and anti-canceragents (Kieser *et al.,* 2000). They are soil-inhabiting Gram-positive bacteria with a high G+C content and it belonging to the order of Actinomycetales (Garrity 2002). Wakayama *et al.* (2005) observed that the L-glutaminase produced from actinomycetes has a good salt tolerance; and thermostable (Masayukr *et al.,* 2007). So for only few marine actinomycetes have been explored for their L-glutaminase production (Krishnakumar *et al.,* 2011).

The enzyme is produced throughout the world by both submerged and solid-state cultures. Extracellular glutaminases from actinomycetes are more advantageous than intracellular ones, since they can be produced abundantly in the culture broth under normal conditions, and purified economically. In the light of the previous fact this study aimed to extract and characterize of the purified L-glutaminase from *Streptomyces canarius* FR cultures. Biochemical, kinetics and *in vivo* anti-carcenogenic properties of L- glutaminase will be also studied.

Material and Methods

Identification of L-glutaminase producing actinomycete strain

Streptomyces canarius FR isolated from tombs of New Kingdom, Tell Basta, Zagazig, Sharkyia Governorate, Egypt was identified by various parameters such as colony morphology, spore arrangement, physiologically and biochemically according to identification keys (Kämpfer 2006). The identification was molecularly confirmed by the analysis of 16S rRNA gene sequence. The 16S

rRNA gene sequence, which have been determined in the present study, were deposited at NCBI web server (http://www. ncbi.nlm.nih. gov/ Genbank/ update. html) with the following accession no KC748492. Sequence analysis and comparison to published sequences made using the Basic Local Alignment Search Tool (BLAST) program (http://blast.ncbi.nlm.nih.gov/Blast.cg) (Altschul *et al.,* 1997).

The strain was preliminary tested for L-glutaminase production by streaking on minimal glutamine agar medium (MGA) plates. Components of MGA (g/L) include: KCl 0.5; MgSO$_4$·7H$_2$O 0.5; KH$_2$PO$_4$ 1.0; FeSO$_4$·7H$_2$O 0.1; ZnSO$_4$·7H$_2$O 1.0; glutamine 5 and phenol red 0.012. The plates were incubated at 30°C for 5 days. Formation of pink zones around the microbial growth indicated the positive reaction (Balagurunathan and Subramanian 1993, Balagurunathan *et al.,* 2010).

Optimization of culture conditions for L-glutaminase production
Streptomyces canarius FR was cultivated in L-glutaminase production modified Czapek's medium contained (g/L): L-glutamine 10; D-glucose 5; MgSO$_4$·7H$_2$O 0.5; KCl 0.05; KH$_2$PO$_4$ 1.0. Enzyme production was tested under different cultured conditions; different incubation periods (0-8 days); different temperatures (25-55°C); different pH-values (pH 4-10) under shaking and static conditions. The culture was harvested and centrifuged at 10,000 rpm for 30 min.; the obtained cell free filtrate was used as crude enzyme according to Dura *et al.* (2002).

Activity assay and Protein determination of enzyme
The activity of glutaminase enzyme is determined by estimating the amount of NH$_3$ liberated from glutamine (Borek *et al.,* 2004). Protein concentration was determined by Lowery *et al.* (1951) using bovine serum albumin (Sigma chemical Co.) as a standard.

L- glutaminase purification
Two liters from the nutritionally optimized submerged 5 days culture of *S. canarius* FR growing in L-glutaminase producing medium was prepared. The precipitated protein was collected by centrifugation at 10.000 rpm at 4°C and dissolved in a minimum volume of phosphate buffer (0.01 M, pH 8.0) (Sabu *et al.,* 2005). L- glutaminase was fractionated by salting out (50-80% ammonium sulphate saturation). The collected precipitate was dissolved in phosphate buffer (0.01 M, pH 8.0) and then dialyzed against the same buffer for 24hr at 4°C with continuous stirring and occasional changes of the buffers. The dialyzate was fractionated by Sephadex G100 gel-filtration chromatography. After column equilibration the enzyme was eluted by phosphate buffer (0.1 M, pH 8.0) with 0.001 M EDTA. The activity, homogeneity (SDS-PAGE) and protein contents of the fractions were determined as above. The most active homogenous fractions were gathered and loaded to pre-equilibrated column of Sephadex G50 using the same buffer for elution. For each fraction activity was assessed as above. The

most active fractions were pooled and concentrated by dialysis against buffer (Nagendra Prabhu, 1997).

SDS-PAGE Analysis

The homogeneity and molecular weight of L- glutaminase from culture of *S. canarius* was carried out using SDS-PAGE according to Lammeli (1970). PageRuler Unstained Protein Ladder, Fermentas marker was used.

Biochemical Properties of the purified L-glutaminase enzyme

The biochemical properties of purified *S. canarius* L- glutaminase as optimum pH, pH stability, reaction temperature, thermal stability salt tolerance, metal ions and substrate specificity enzyme were determined as Amena *et al.* (2010) and Sabu *et al.* (2005).

The thermal stability of enzyme was studied after preincubation of the enzyme at various temperatures (50-80°C) using 0.1M phosphate buffer (pH 8.0) for different time (10-90 min). The enzyme relative activity was determined after the incubation of reaction mixture at 37°C for 30 min. The thermal inactivation rate (Kr min) can be described by the first-order kinetic model (Whitaker, 1972); Ln (A_t/A_o) $k_t T$, where A_o and A_t are the specific activity zero and t time. $T_{1/2}$ (time at which the enzyme loss 50% of its activity) was calculated from the linear equation for each temperature. The temperature at which the enzyme loss 50% of its activity (Tm) was calculated from the linear equation of different preincubation temperature at 60 min.

Stability of L-glutaminase was examined after pre-incubation of the enzyme for 2 hr at different pH values ranging from 4.0-11.0. Acetate (0.2 M), phosphate (0.2 M) and glycine-NaOH buffers were used to covering pH range (4-5), (6-8) and (9-11) respectively. After adding glutamine (40mM) the reaction mixture was incubated at 37°C for 30 min. The activity of the enzyme was determined for each pH.

To assay the metal ions effect, the purified enzyme was pre-incubated with each metal ion separately for 30 min before adding glutamine (40mM). The enzyme relative activity was determined immediately after incubation at 37°C for 30 min.

The kinetic parameters of L-glutaminase as Vmax, Km and Kcat were estimated using different concentrations of glutamine, asparagine and aspartic acid, separately (10-100mM). Michaelis-Mentel constant (Km) and maximum velocity (Vmax) were calculated from Lineweaver-Burk plot. Catalytic efficiency (Kcat) was expressed by the specific activity per mol enzyme.

Cytotoxicity of L- glutaminase

Cytotoxic effect of the purified enzyme was evaluated using five New Zealand rabbits groups each contained five one. Rabbits were intravenous (i.v.)

injected by 1 ml of three successive doses of L- glutaminase (132.2 U/mg protein/1.5±0.1 Kg) during two weeks. Blood samples were collected after 10, 25, 40 and 50 day of the last injection. Control sera (zero time) without enzyme injection were used. Plasma were prepared and stored at -20°C. To evaluate the biological effects of the enzyme on blood chemistry of treated rabbits, various hematological parameters as RBC, WBC, platelets, hemoglobin and biochemical parameters as ALT, AST, GGT, total protein, albumin, cholesterol, glucose and creatinine were determined as described by Birt (1967) and Reitman and Frankel (1957). Hemolytic activity of the purified L-glutaminase was evaluated using a blood agar assay (Tay *et al.,* 1995).

Histopathological characterization
 For histological studies, small portion of liver and kidney of treated and non-treated rabbits after 50 days of the last injection were removed and fixed overnight in 10% formalin according to Roy and Maity (2007).

Anticancer activity of S. canarius L-glutaminase against various cell lines In Vitro
   Human hepatocarcinoma cell line (HepG2), Human breast adenocarcinoma cell line (MCF-7), human colorectal carcinoma cells (HCT-116), Human cervical carcinoma cell line (HeLa) and Raw murine macrophage (RAW 264.7) purchased from ATCC, USA were used.

   Anticancer efficiency of tested enzyme was measured against HepG2, MCF-7, HCT-116, HeLa and RAW 264.7 cells using the MTT Cell Viability Assay (Hansen *et al.,* 1989). % Cell viability = O.D of treated cells / O.D of control cells X 100.

   The IC_{50} value was expressed by the concentration of enzyme required to inhibit 50% of initial growth of tumor cells. IC_{50} for L-glutaminase was determined against five human cell lines, using sigmoidal dose response curve-fitting models (Graphpad Prizm Software, version 3).

Statistical analyses
   All data were the mean of three replicates ± SD (standard deviation). ANOVA test (one way) and t- test were calculated in all cases, significance was measured by LSD at $p < 0.05$ using SPSS program version 10.0 (Salvatore and Reagle, 2001).

Results and Discussion

Identification of L-glutaminase producing strain
   Streptomyces canarius FR strain was identified by studying their morphological and biochemical characterization according to identification keys. PCR amplification of 16S rDNA gene confirmed the identity (98%) of selected potent strain as *S. canarius* FR. The partial nucleotide sequence of amplified gene was submitted in GenBank (http://www. ncbi. nlm. nih. gov/

GenBank/update.htm) under accession number KC748492. Our results derived from the sequence analysis of the 16S rRNA gene show a G + C content of 58.9 mol % which is in good agreement with the data published for *Streptomyces* by Kannan and Vincent (2011). The tested strain was characterized by formation of a pink zone around colonies using MGA medium due to breakdown of amide bond in L-glutamine and ammonia liberation (Ranjekar and Sridhar, 2002).

Optimization of L-glutaminase production by submerged fermentation

The enzyme was produced from the submerged fermented culture of *S. canarius* under optimum conditions at 30°C for 5 days and pH 7.5 under shaking condition at 120 rpm (Data was not shown). Dura *et al.* (2002) and Sabu (2003) stated that submerged fermentation was the routinely used methods for L-glutaminase production from various microorganisms. Krishnakumar *et al.* (2011) showed that production of L-glutaminase from *Streptomyces* sp. SBU1 was at 30°C after 96 hr of incubation and initial pH 9.0. Balagurunathan *et al.* (2010) stated that, the optimum conditions for marine *Streptomyces olivochromogenes* L-glutaminase production were determined at pH 7, temperature 30°C and 3.5% salinity for 5 days under shaking condition at 120rpm.

Purification of L-glutaminase

The enzyme was purified from 5 days *S. canarius* culture to assess their yield and purity. The crude enzyme was undergoes fractional precipitation by salting out (50-80% ammonium sulfate). The enzyme overall purification profile from S. *canarius* culture was summarized in (Table 1). The crude extract contained 23.0 mg of protein and showed a total glutaminase activity 170 units with specific activity of 7.4 U/mg protein. At all purification steps, the specific activity increased compared to crude enzyme. The maximum specific activity 132.2 U/mg protein with a yield of 28 % was attained after Sephadex G100 purification. The partial purified was increased more than 17 fold compared with crude enzyme. These results were in similarity with that reviewed by Mohana Priya *et al.* (2011). Also, Kumar *et al.* (2012) stated that *Bacillus* sp. LKG-01 (MTCC 10401) L-glutaminase activity was purified 49-fold from cell-free extract with 25% recovery with specific activity 584.2 U/mg protein after gel filtration.

TABLE 1. Summary of purification steps of *Streptomyces canarius* L- glutaminase.

Purification steps	Enzyme activity (U)	Total sprotein (mg)	Specific activity (U/mg protein)	Purification fold	Yield (%)
Crude extract	170	23	7.4	1	100
70 % Amm.Sulph.ppt	145	17	8.5	1.2	85
Sephadex G100	95	0.77	123.4	16.7	56
Sephadex G50	47.6	0.36	132.2	17.9	28

Determination of molecular weight for enzyme

The molecular homogeneity of the purified enzyme from fermentation condition, as well as their purification steps were evaluated by SDS-PAGE. Single band of 44 kDa was appeared after final purification step (Column

Sephadex G_{100}) for the enzyme from *S. canarius* (Fig. 1). Similarly, the molecular mass of the native enzyme from *Stenotrophomonas maltophilia* NYW-81 was estimated to be 41 kDa by gel filtration (Wakayama *et al.,* 2005).
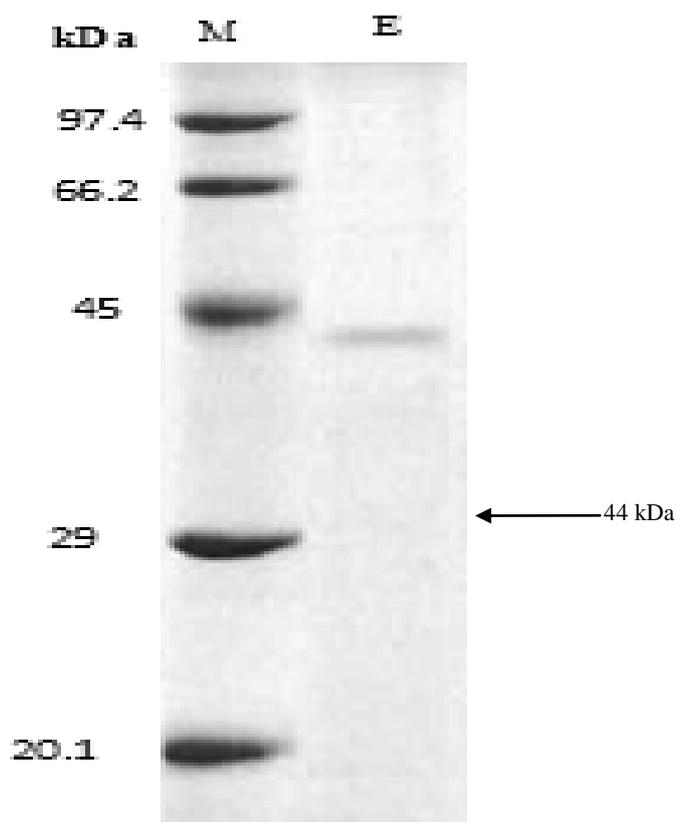

Fig. 1. SDS- PAGE of purified L-glutaminase from *S. canarius*. Lane M (marker protein), Lane E, the purified enzyme.

Biochemical properties of the purified L-glutaminase
   Optimal pH and pH stability
   The enzyme maintained its activity over a range of pH 5.0 -9 with optimum at pH 8.0 (Fig. 2 A). The activity significantly decreased at both low and high pH values. The pH stability of the enzyme also showed a similar trend, the enzyme was stable at wide range pH 5.0-11.0 with being most stable at pH 7.0-9.0. Practically, at acidic pH values a higher rate of enzyme inactivation was appeared, comparing to alkaline side, assuming the enzyme basic identity. The negative effect on enzyme activity at lower and higher pH values, suggesting the effect on ionization state of enzyme, modifying the enzyme surface charge,

dissociation of subunits/ coenzyme, consequently disrupt its binding with substrate. Consistently, the activity of L-glutaminase from *S. canarius* showed alkaline optimum pH 7- 8, this neutral pH stability of enzyme, being a favored criterion for enzyme action *in vivo*. Similarly, *Streptomyces gulbargensis* L-asparaginase was more stable at the alkaline pH than at the acidic one (Amena *et al.,* 2010 and Kumar *et al.,* 2012).

Optimal temperature and thermal stability

The optimum temperature for the enzyme activity was determined by incubation of the reaction mixture at various degrees (25-50ºC) using L-glutamine as substrate in 0.1M potassium phosphate buffer (pH 8.0). The highest enzyme specific activity (53.3 U/mg) was obtained at 40ºC. Above and below this temperature the enzyme activity decreased (Fig. 2 B). Regarding to thermal stability the enzyme had a catalytically thermal stability below 60 ºC, with a slightly decreasing in its activity at 70ºC for 20 min and completely lost its activity at 80ºC (Fig. 2C). The enzyme half-life times ($T_{1/2}$) was 45.7, 38.8, 29.3 and 17.3 min at 50, 60, 70 and 80 ºC, respectively. Also, the Tm of enzyme was 57.5°C, assuming the retaining of about 50% of its initial activity by heating for 60 min (Fig. 2 D). Theoretically, thermal inactivation rates (Kr) are 0.0184, 0.0227, 0.0544 and 0.0449 S-1 at 50, 60, 70 and 80°C respectively, suggesting the dissociation of co-enzyme or denaturation of subunits by heating per unit time. These results were in consistent with those reported for Amena *et al.* (2010) and Kumar *et al.* (2012).

Salt tolerance of S. canarius L-glutaminase

The results represented in (Fig. 2 E) showed that, tolerance in glutaminase activity was observed by increasing the NaCl concentration upto 25%. Marine actinomycete isolates were tolerated high salt conc. (Krishnakumar *et al.,* 2011 and Kumar *et al.,* 2012).

Substrate specificity and kinetic properties of S. canarius L-glutaminase

The kinetic parameters for L-glutaminase towards substrates were determined from Michaelis-Mentel constant and the Lineweaver-Burk Plot (Fig. 2 F and Table 2). The enzyme had relative high affinity and catalytic activity for L-glutamine (Km 2.4 mM, Vmax 28.1 U/mg/min), followed by L- asparagine (Km 2.6 mM, Vmax 25.5 U/mg/min) and low affinity to L-aspartic acid (Km 6.2 mM, Vmax 9.5 U/mg/min). Also, the highest catalytic efficiency (Kcat turnover number) for the enzyme was assessed for L-glutamine (0.64×10^{-3} s-1) followed by L-asparagine (0.58×10^{-3} s-1) and L-aspartic acid (0.22×10^{-3} s-1). The high affinity of *S. canarius* L-glutaminase to L-glutamine as substrate was detected previously by Senthil-Kumar and Selvam (2011) and Kumar *et al.* (2012) for *Streptomyces radiopugnans* MS1 and *Pseudomonas* sp.BTMS-51, respectively. Also, the small Km (2.4 mmol) of the tested *S. canarius* L-glutaminase indicated high affinity of the enzyme to glutamine meaning that the rate will approach Vmax more quickly (Lehninger *et al.,* 2005).
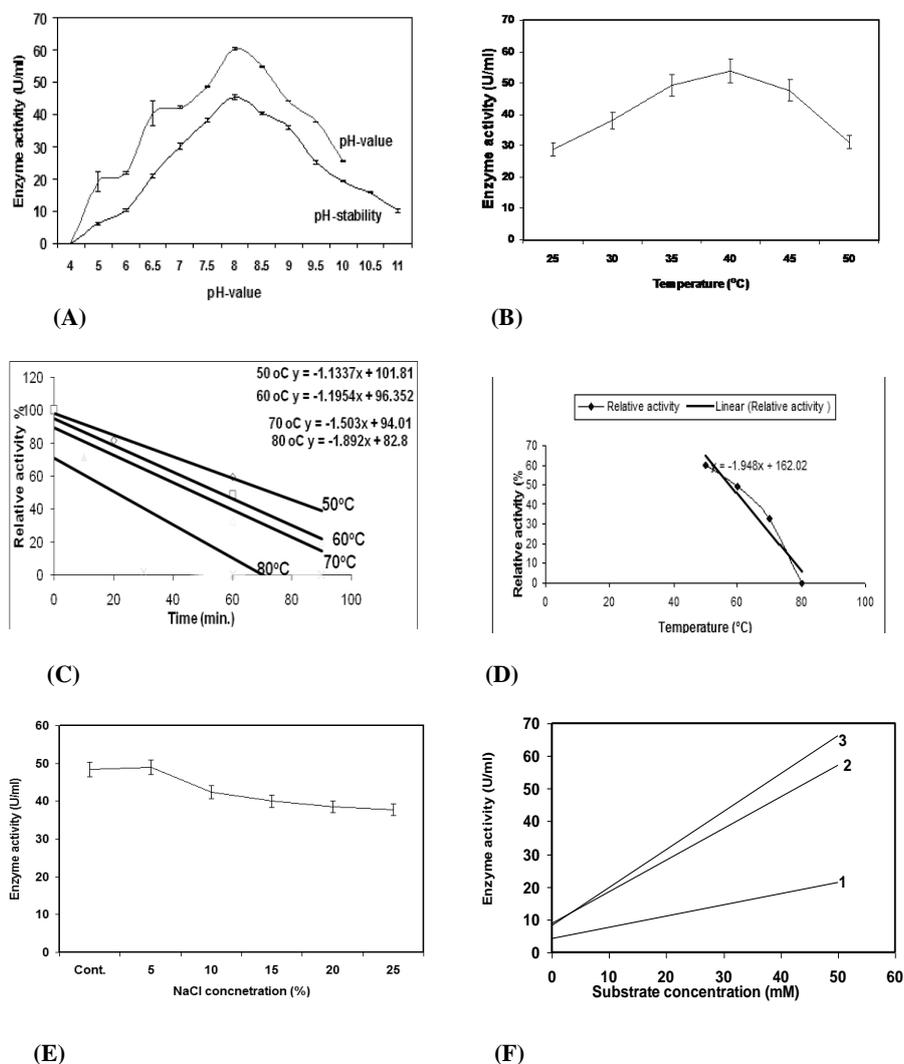
Fig. 2. Characterization of *S. canarius* L-glutaminase.
(A) pH value and pH stability profile. The enzyme was preincubated for 2 hr at various pHs (4.0 -11.0), then measuring the residual deaminating activity; (B) The optimal temperature for activity was assessed by measuring the enzyme activity at different temperatures between 25 and 50°C; (C) Thermal stability profile. After incubation of enzyme in different temperature (50-80°C) at various periods (10-90 min), the residual activity was determined by the standard assay method; (D) Thermal inactivation profile. Tm is temperature degree at which the enzyme retains half of its initial activity at 60 min. (E) Different NaCl concentrations from 0-25%; (F) Enzyme specificity towards substrates, (1) L- aspartic acid y = 0.344x + 6.894; (2) L-asparagine y = 0.975x + 15.29; (3) L-glutamine y = 1.081x + 16.66.

TABLE 2. Kinetics of *S. canarius* L- glutaminase.

Substrates (mM)	Km (mmol $^{-1}$)	Vmax (U mg^{-1} protein min^{-1})	Kcat (s^{-1})
L- glutamine	2.4	28.1	0.64×10^{-3}
L- asparagines	2.6	25.5	0.58×10^{-3}
Aspartic acid	6.2	9.5	0.22×10^{-3}

The kinetic parameters were determined by incubation of the enzyme (132.2 U/mg protein) in potassium phosphate buffer (pH 8) with various concentrations of substrate (10-100 mM) under the standard assay conditions, then measuring the deaminating activity of the enzyme. Maximum velocity (Vmax) was expressed by activity of enzyme in μmol of NH3 compounds formed per minute per mg protein enzyme. Km is the substrate concentration (mM) at half of maximum velocity. Kcat is the maximum velocity of the enzyme per mol per sec.

Influence of metal ions on L-glutaminase activity

The impact of various inhibitors and activators on catalytic potency of the enzyme was evaluated by pre incubation of the enzyme with each compound (10mM final conc.) for 30 min without substrate. Results in (Table 3) showed that among the tested ions, Ca^{+2}, Ba^{+2}, Fe^{+3}, Zn^{+2}, Cu^+, Hg^{+2} and Cd^{+2} decreased significantly the enzyme residual. On the other hand, Mn^{+2}, Na^+ and Co^{+2} were enzyme inducer. These results were in agreement with Senthil-Kumar and Selvam (2011) for *Streptomyces radiopugnans* MS1 L-asparaginase.

TABLE 3. Effect of inhibitors and activators on L- glutaminase activity.

Metal ions (5mM)	Relative activity (%)
None (control)	100.67 abc ± 0.15
Ca^{+2}	75.33 cd ± 0.76
Na^{+2}	105.00 ab ± 0.50
Ba^{+2}	56.70 d ± 5.29
Mg^{+2}	81.33 bc ± 60.48
Hg^{+2}	20.00 e ± 0.50
Co^{+2}	122.00 a ± 0.50
Fe^{+3}	53.00 d ± 0.87
Mn^{+2}	125.00 a ± 0.50
Cu^{+2}	50.00 d ± 0.50
Zn^{+2}	87.00 bc ±0.92
Cd^{+2}	17.00 e ± 0.50
l.s.d	27.49
F	12.669

The enzyme activity was determined in the absence and presence of different ions at concentration 10 mM after a 30 min exposure to each ion. The relative activity was expressed as the percentage ratio of the activity of the enzyme incubated with the effectors to that of the untreated enzyme.

Cytotoxicity effect of L-glutaminase enzyme

The cytotoxicity effect of purified *S. canarius* L-glutaminase was explored based on the hematological and blood chemistry pictures for the New Zealand rabbits (Table 4). The rabbits were injected by 1 ml of three successive doses of enzyme (132.2 U/mg protein) during two weeks. From the biochemical profiles, there is no obvious negative effect on liver, renal functions, glucose, lipids and other electrolytes, with slight inducing effect on the activity of ALT, AST and level of Random Sugar Glucose. As well as, rabbits were alive along the experimented periods. Depending on these results, the enzyme had relatively no negative effect on liver functions, where AST and ALT was the most potential indicator for liver dysfunction (Pratt and Kaplan 2000).

Also, the cellular toxicity of the purified *S. canarius* L-glutaminase was evaluated by estimation of the degree of platelets aggregations and hemolytic activity as described by Wei *et al.* (2007). All the hematological parameters (Table 4) were in normal range along the experimented period, however, the red blood cells, hemoglobin, white blood cells and the platelets slightly decreased after 10 days of L-glutaminase injection, comparing to control (before injection). The enzyme displayed no hemolytic activity to human blood (Plate 1). Platelet aggregation and hemolytic activity are the most relevant biochemical assays (Pratt and Kaplan 2000). The lack of ability to aggregate human platelets and lyses of human RBCs are unique supportive criteria from therapeutic point of view. Similar results approved the non cytotoxicity of microbial glutaminase (Baskerville *et al.,*1980).
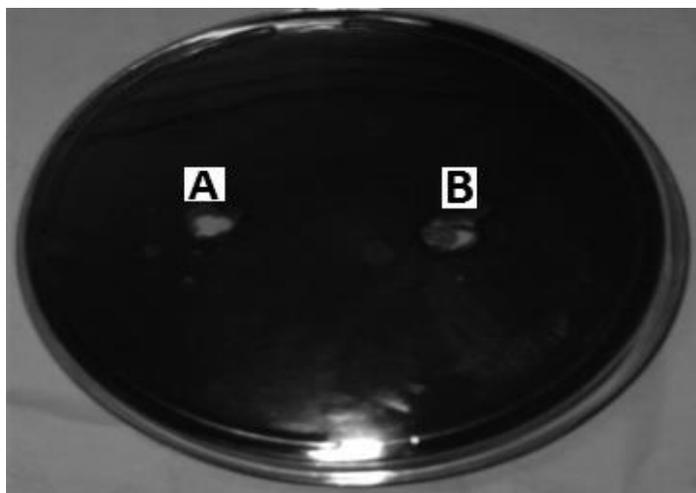

Plate 1. Hemolytic activity of *Strepromyces canarius* L- glutaminase, 100 µl of purified enzyme (A) and 200 µl purified enzyme (B).

TABLE 4. Hematological and biochemical parameters of rabbits in respons e to *S. canarius* L-glutaminase.

Parameters	Control (at zero time)	After 10 days			After 25 days			After 40 days			After 50 days		
		Control	Sample	t-test	Control	Sample	t-test	Control	Sample	t-test	Control	Sample	t-test
Serum Bilirubin: Total	0.81	0.77	0.65	6.29*	0.74	0.63	12.85**	0.75	0.66	9.0*	0.72	0.64	2.28 n.s
Direct	0.22	0.19	0.14	4.330	0.20	0.64	-.936	0.21	0.17	11.000	0.20	0.17	2.598 n.s
Serum Proteins: Total	4.50	4.64	4.81	11.930-**	4.58	4.35	39.837**	4.94	5.09	-8.660*	4.90	4.81	3.118 n.s
Albumin	3.55	3.58	2.88	2.177 n.s	3.50	3.18	19.4**	3.52	3.48	1.857 n.s	3.45	3.38	2.291 n.s
Globulins	3.12	3.17	4.10	-46.50**	3.32	3.45	-8.51*	3.34	3.37	-2.219 n.s	3.38	3.34291	1.571 n.s
S.Alk.Phosphatase	239	243.00	254.67	-4.27*	245.33	274.00	43.00-**	247.33	251.67	3.606- n.s	245.00	249.00	-3.464 n.s
GGT	11	11.67	15.00	-4.0n.s	13.17	14.50	-8.0*	12.63	13.00	-.78 n.s	12.10	12.50	-2.62 n.s
S.GOT (AST)	22	29.00	43.00	**23.89-	32.33	41.00	-8.549*	31.00	39.00	**10.474-	30.00	34.00	-2.619n.s
S.GPT (ALT)	66	68.00	80.67	-38.0**	66.00	69.00	-1.44n.s	64.00	67.00	-1.29n.s	63.00	65.00	-.756n.s
Serum cholesterol: Total	45	53.00	56.00	-2.59n.s	49.00	46.00	1.964n.s	47.00	47.00	.000n.s	52.00	51.00	.577n.s
Blood Urea	0.75	0.80	1.05	-21.65*	0.88	1.03	0.78 n.s	0.93	0.99	2.268-n.s	0.90	0.99	-7.794*
S.Creatine	135	138.00	125.33	4.865*	140.00	134.00	2.598n.s	145.00	138.00	2.782 n.s	142.00	140.00	.866n.s
Random S.Glucose	93	100.33	115.00	-15.81**	107.00	118.00	-19.053**	100.00	106.00	10.392**	104.00	102.00	1.0n.s
Red Blood cells (RBC)	6.12	6.29	5.00	4.338**	6.37	5.93	44.00**	6.53	6.17	23.568**	6.50	6.47	1.299n.s
Hemoglobin)	11.7	11.33	10.00	3.941 n.s	11.40	11.20	1.23 n.s	11.70	11.00	2.646 n.s	11.90	11.20	6.062*
White blood cells (WBC)	9.8	9.50	8.90	4.32*	9.90	7.60	9.959*	9.80	9.80	.000 n.s	9.70	9.00	1.732n.s
Platelets	382	379.00	360.00	11.332**	378.00	373.33	1.257n.s	376.33	374.67	1.7814n.s	383.00	372.67	2.23n.s

*, ** and NS indicate $p < 0.05$, $p < 0.01$ and not significant, respectively.
Five New Zealand rabbits were i.v. injected by 1 ml of three successive doses of *S. canarius* L- glutaminase (132.2U/mg protein/1.5±0.1 Kg) during two weeks. Blood samples were collected after 10, 25, 40 and 50 day of the last injection. Control sera (zero time) without enzyme injection were used.

Histological studies on the effect of enzyme induced in rabbits
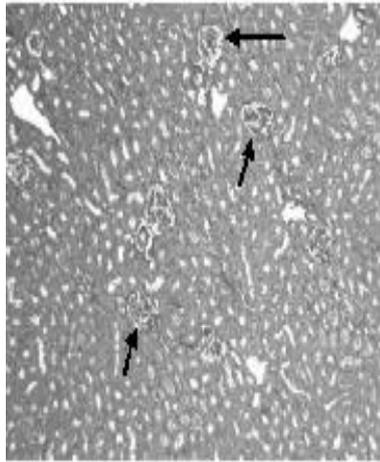
For histological studies, small portion of liver and kidney of treated and non treated rabbits after 50 days of the last injection were investigated. From photomicrograph of renal and liver tissues (Fig. 3 and 4), there is obvious slight effect on the tested tissues, comparing with the negative controls. Photomicrograph of renal tissue of negative control showing normal glomeruli surrounded by normal renal tubules lined cubical epithelial cells, whereas in treated tissue showed round distal convoluted tubules lined by cubical epithelium and longitudinal collecting duct lined by columnar epithelium (Fig. 3). Also, photomicrograph of tested liver tissue showing variable sized central veins surrounded by cords and rows of hepatocytes, comparing with negative control (Fig. 4). These results were in similarity with that obtained by Baskerville *et al.* (1980) and Roy and Maity (2007).
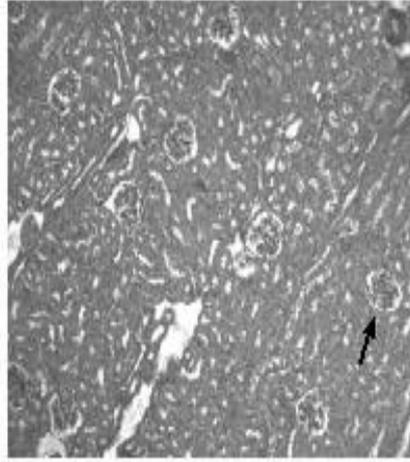
Anticancer efficiency In Vitro

Using MTT assay in the present investigation, the effect of the purified enzyme on the growth of Hep-G2, MCF7, HCT-116, HeLa and RAW 264.7 cells were studied after 48 hr of incubation. As shown in (Fig. 5), the tested enzyme had a high efficiency against Hep-G2 cell (IC_{50}, 6.8 µg/ml) and HeLa cells (IC_{50}, 8.3 µg/ml), while the growth of MCF-7 cells was not affected by the treatment. On the other hand, the treatment of HCT-116 and RAW 264.7 cells with the tested enzyme indicated that there was a moderate cytotoxic effect as concluded from their high IC_{50} calculated values: 64.7 µg/ml and 59.3 µg/ml, respectively, compared with the known anticancer drug paclitaxel, which its IC_{50} values for these cell lines ranged from 0.5-1.2 µg/ml. Similarly, Devi and Azmi (2012) showed that the purified L-asparaginase from *Erwinia carotovora* MTCC 1428 used for killing of Hep-2C cell line. Also, *E. carotovora* MTCC 1428 asparaginase was showed better *in vitro* toxicity on Hep-2C cell lines (84% survival) in comparison to commercial L-asparaginase preparation (90% survival) obtained from *E. coli*. Meanwhile, Nathiya *et al.* (2012) indicated a crucial role of *Aspergillus flavus* KUGF009 L-glutaminase in breast cancer (MCF7). Moreover, Roberts *et al.* (1970) found that L-glutaminase from *Pseudomonas* sp. 7A is administered to inhibit HIV replication in infected cells. The enzyme brings about inhibition of tumor (melanoma) and DNA biosynthesis in affected cells. Glutaminase and asparaginase enzymes have produced prolonged remissions of certain experiment tumors (Wriston and Yellin, 1973). During treatment, plasma glutamine and asparagines are depleted. The degree of amino acid depletion depends on the kinetic properties of the enzymes, its biological half life in the animal and the rate of input of the amino acid into circulation (Wriston and Yellin, 1973).

In conclusion, *S. canarius* (KC460654) had remarkable capacity to produce L- glutaminase. The purified enzyme showed a unique specificity to glutamine, broad pH stability, and high thermal stability. Also, the purified enzyme is being promising candidate for application as antitumor agent in the future work.
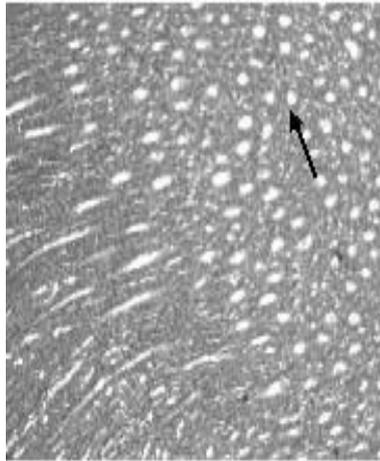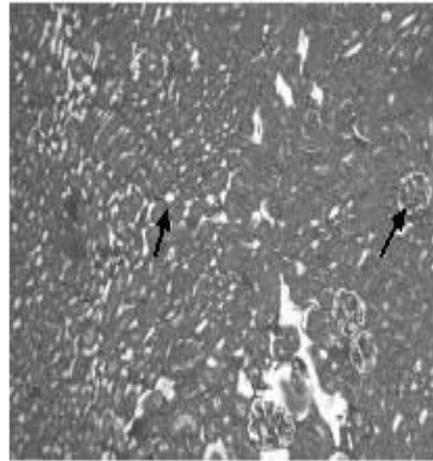
A. Hematoxylin and Eosin (H & E) X 150   A. H & E X 200

B. H & E X 150   B. H & E X 200

Fig. 3. Photomicrograph (at Hematoxylin and Eosin X 150 and X200) of renal tissue of (A) negative control showing normal glomeruli (↑) surrounded by normal renal tubules lined cubical epithelial cells. (B) treated with *S. canarius* L-glutaminase showing round distal convoluted tubules (↑) lined by cubical epithelium and longitudinal collecting duct lined by columnar epithelium.
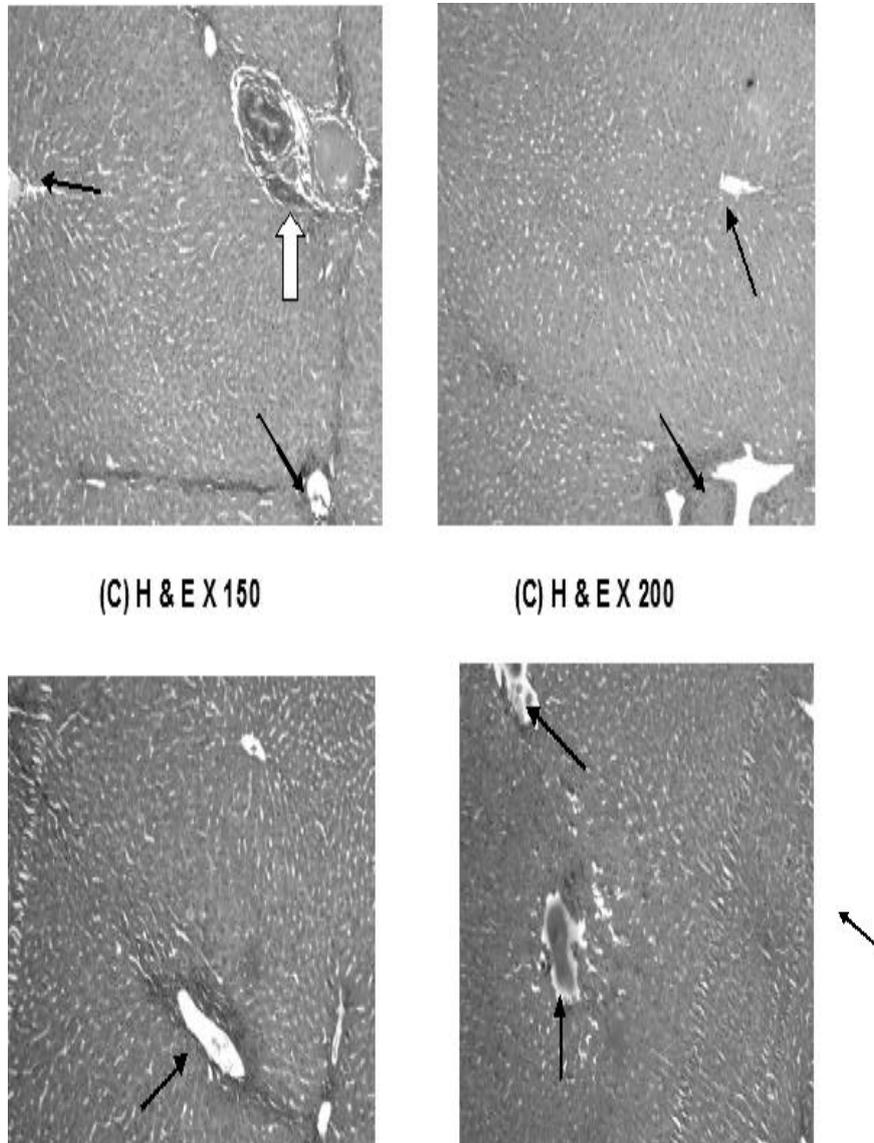
(C) H & E X 150

(C) H & E X 200

Fig. 4. Photomicrograph (at H & E X 150 and X200) of liver tissue of (C) negative control showing normal architecture formed of central vein ( ) and portal tract ( ) surrounded by cords and rows of normal hepatocytes. (D) Treated with *S. canarius* L-glutaminase showing variable sized central veins ( ) surrounded by cords and rows of hepatocytes.

(A)

(B)

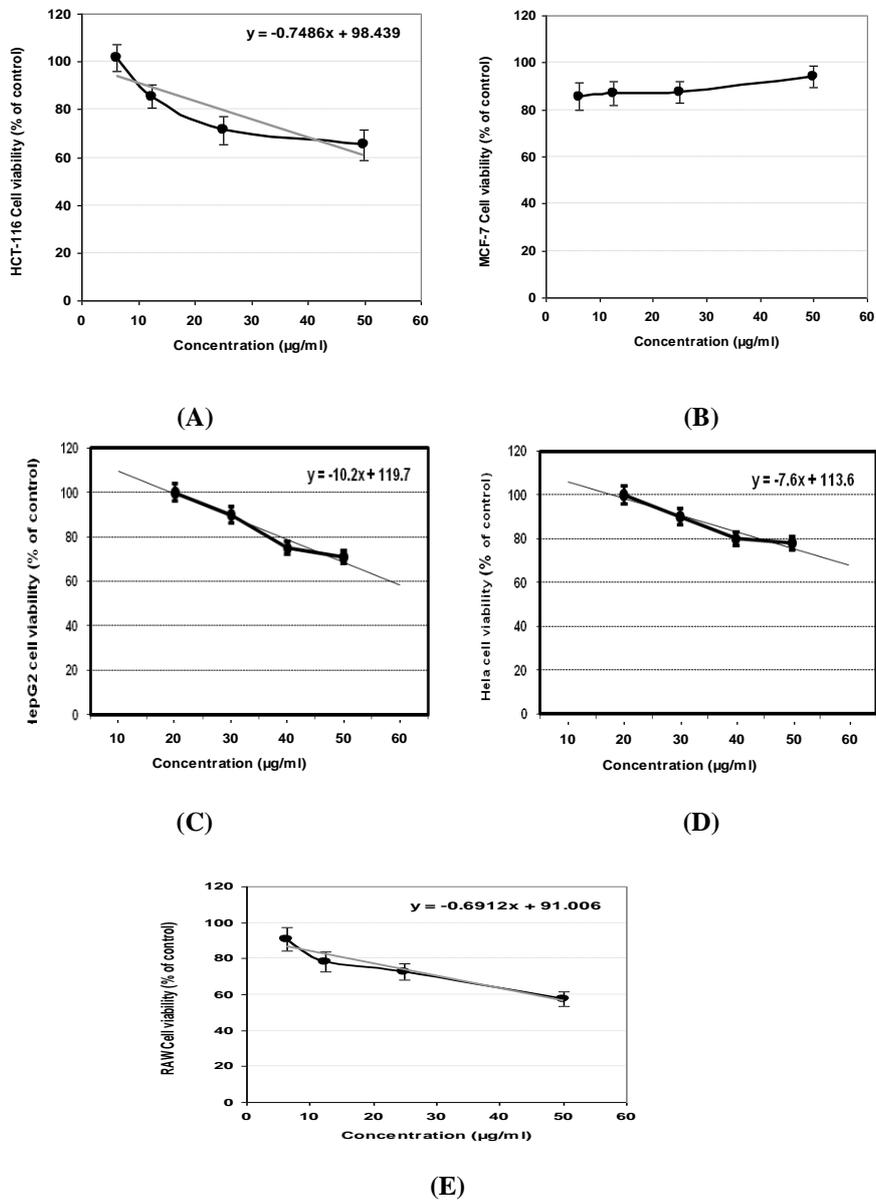
(C)

(D)

(E)

Fig. 5. Cytotoxic effect of *S. canarius* L-glutaminase on tumor cell lines using MTT assay (n=4), HCT-116 (A), MCF-7 (B), HepG2 (C), HeLa (D) and RAW 264.7 (E). Cells exposed to different concentrations of the drug for 48 hours. All data are expressed as the mean value of cell viability (% of control) ± S.E.S.

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تنقية وتوصيف انزيم ل الجلوتاميناز المنتج من استربتوميس كناريس ونشاطه كمضاد للسرطان

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تم تنقية انزيم ل جلوتاميناز المنتج بواسطة استربتوميس كناريس وكان الوزن الجزيئى له ٤٤ كيلو دالتون . كان الانزيم المنقى ١٧,٩ أضعاف مع نشاط محدد النهائي ١٣٢,٢ وعائد ٢٨٪. انزيم ل جلوتامين النقى أظهر درجة نشاطه القصوى ضد L- الجلوتامين عندما حضنت في درجة الحموضة ٨,٠ عند ٤٠ درجة مئوية لمدة ٣٠ دقيقة . حافظ الانزيم على ثباته في نطاق واسع عند درجة الحموضة من ٥,٠ حتي ١١,٠ ودرجة ثباته الحرارية تصل إلى ٦٠ درجة مئوية مع قيمة Tm ٥٧,٥ درجة مئوية. ويتفاعل الإنزيم النقي بدرجة عاليه مع مادة التفاعل ل الجلوتامين وكانت قيمة الـ V_{max} =٢٨,١وحدة / مليجرام/ دقيقة ، km=٢,٤ ملى مول، يليه -L الأسباراجين و حمض -L الأسبارتيك. وأوضحت التجارب على الأرانب أن الأنزيم النقي لم يحدث أي تغيير واضح في وظائف الكبد و الكلى لهم . أيضا ، كان لديه تأثير طبيعي في جميع مكونات الدم ما عدا تأثير طفيف على كرات الدم الحمراء و الصفائح الدموية بعد عشرة أيام من حقن الأرانب. ومن المثير للاهتمام ، أنه أظهر تأثير طفيف على خلايا الكلى و الكبد المختبرة مقارنة مع الارانب التى يتم حقنها. تم تحديد نشاط الانزيم المنقى المضاد للسرطان ضد خمسة أنواع من الخلايا السرطانية باستخدام مقايسة الإنتقالي العسكري في المختبر. كان اختبار كفاءة الانزيم عالية ضد خلايا الكبد (٦,٨ميكروجرام / مل)، و خلايا هيلا (٨,٣ميكروجرام / مل) ، في حين أن نمو الخلايا السرطانية للثدى لم تتأثر بواسطة العلاج. في المقابل ، كان للانزيم تأثير معتدل ضد خلايا سرطان القولون والمستقيم الإنسان (٧ميكروجرام/ مل)، والبلاعم الفئران الخام ٢٦٤,٧ (٥٩,٨ ميكروجرام / مل).