Kinetic properties of *Streptomyces canarius* L- Glutaminase and its anticancer efficiency

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Submitted: August 6, 2013; Approved: December 28, 2014.

**Abstract**

L-glutaminase was produced by *Streptomyces canarius* FR (KC460654) with an apparent molecular mass of 44 kDa. It has 17.9 purification fold with a final specific activity 132.2 U/mg proteins and 28% yield recovery. The purified L-glutaminase showed a maximal activity against L-glutamine when incubated at pH 8.0 at 40 °C for 30 min. It maintained its stability at wide range of pH from 5.0-11.0 and thermal stable up to 60 °C with Tm value 57.5 °C. It has high affinity and catalytic activity for L-glutamine (Km 0.129 mM, Vmax 2.02 U/mg/min), followed by L-asparagine and L-aspartic acid. *In vivo*, L-glutaminase showed no observed changes in liver; kidney functions; hematological parameters and slight effect on RBCs and level of platelets after 10 days of rabbit’s injection. The anticancer activity of L-glutaminase was also tested against five types of human cancer cell lines using MTT assay *in vitro*. L-glutaminase has a significant efficiency against Hep-G2 cell (IC50, 6.8 μg/mL) and HeLa cells (IC50, 8.3 μg/mL), while the growth of MCF-7 cells was not affected. L-glutaminase has a moderate cytotoxic effect against HCT-116 cell (IC50, 64.7 μg/mL) and RAW 264.7 cell (IC50, 59.3 μg/mL).

**Key words:** L-glutaminase, anti-cancer, cytotoxicity, MTT assay.

**Introduction**

Microbial sources like actinomycetes are well recognized to produce a variety of chemical structures, several of which are most valuable pharmaceuticals, agrochemicals and industrial products like enzymes (Thadikamala and Reddy, 2011). Actinomycetes are considered to be preferred enzymes sources due to their production of extracellular enzymes. Many enzymes produced by actinomycetes and have been used as drugs like wise L-glutamine amidohydrolase (E.C. 3.5.1.2), commonly referred to as L-glutaminase (Sunil Dutt et al., 2014). Since the discovery of its anti-tumor properties, L-glutaminases have been in prime focus (Lazarus and Panasci, 1986). Nowadays, L-glutaminase is used as enzyme therapy for cancer especially for acute lymphocytic leukemia (Robert et al., 2001; Kyoko et al., 2004).

Where, high rate of glutamine consumption is a characteristic nature of some types of cancerous cells (Lazarus and Panasci, 1986). Based on this characteristic experimental therapies have been developed to deprive L-glutamine to tumor cells (Iyer and Singhal, 2008). 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. This in fact, results in a selective starvation of the tumor cells because unlike normal cells lack properly functioning glutamine biosynthetic machinery (Tanaka et al., 1988; Dang, 2010). Microbial therapeutic enzymes have a broad variety of specific uses as oncolectics, thrombolytics or anticoagulant, and largely as anticancer (Sabu, 2003; Sabu et al., 2005).

The production of enzyme was influenced by a variety of physicochemical and nutritional factors. The factors affecting the production in recent years had received attention as of its great demand in clinical application and also in food industries. It is known that, the factors involved in the process of production, would not only enhance the quantity, but also quality of enzyme because of which it becomes more suitable for a specific application. Optimization of parameters can in turn influence enzyme
synthesis and cell yield (Okami, 1986). The strain of actinomycetes was used for glutamic acid production under optimum growth conditions (Divya Teja et al., 2014). From the compatibility perspective in mass production and as well as beneficial application aspect extracellular enzyme producer as choice of source is always attractive (Pandey, 2004). The objective of this study was to utilize Streptomyces canarius FR with good ability to produce extracellular L-glutaminase and characterize the purification. Biochemical, kinetics and in vitro anticarcenogenic properties of L- glutaminase will be also examined.

Materials and Methods

Collection, isolation and identification of S. canarius FR

Streptomyces canarius FR isolated from Tell Basta, Zagazig, Sharkyia Governorate, Egypt was identified according to identification keys (Kämpfer, 2006). The identification was confirmed by the analysis of 16S rRNA gene sequence (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-glutamic acid 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 L-glutaminase

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 sulfate saturation). The collected precipitate was dissolved in phosphate buffer (0.01 M, pH 8.0) and then dialyzed against the same buffer for 24 h at 4 °C with continuous stirring and occasional changes of the buffers. The dialyze was fractionated by ion-exchange chromatography (DEAE-Cellulose). After column equilibration the enzyme was eluted by gradient NaCl (50-200 mM) dissolved in 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 G100 gel-filtration chromatography) 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 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 and kinetic properties of the purified L-glutaminase

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 described by Amenà et al. (2010) and Sabu et al. (2005).

The thermal stability of enzyme was examined after preincubation of the enzyme at 50-80 °C using 0.1 M phosphate buffer (pH 8.0) for different time (10-90 min). The relative activity was determined after the incubation of reaction mixture at 37 °C for 30 min. The thermal inactivation rate (Kt) was calculated by the first-order kinetic model (Whitaker, 1972): ln(A/A$_0$) = k$_T$t, where A$_0$ and A$_t$ are the specific activity zero and t time. T1/2 was calculated from the linear equation for each temperature. The Tm was calculated from the linear equation of different preincubation temperature at 60 min.

Stability of L-glutaminase was examined after preincubation of the enzyme for 2 h at pH 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 (40 mM) 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 (40 mM). 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 (10-100 mM). Michaelis-Menten 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 L-glutaminase was evaluated using 5 New Zealand rabbits as experimental group and one rabbit as control. Experimental group were injected intravenously with 1 mL of L-glutaminase (132.2 U/mg protein/1.5 ± 0.1 kg) every 5 days for two weeks. After the two weeks, blood samples were collected 10; 25; 40 and 50 day of the last injection. Plasma were collected and stored at -20 °C, RBC, WBC, platelets, hemoglobin 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., 1989). The efficiency of L-glutaminase was undergoes fractional precipitation by salt-precipitation (50-80% ammonium sulfate). The enzyme overall activity of the purified L-glutaminase was determined immediately after incubation and stored at -20 °C. RBC, WBC, platelets, hemoglobin 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., 1989).

Histopathological examination:

Histological examination for the experimental group and control was carried out on liver and kidney, 50 days of the last injection, according to Roy and Maity (2007).

Anticancer efficiency

Anticancer efficiency of L-glutaminase as antitumor was measured against: 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 (MCF-7); human colorectal carcinoma cells (HCT-116); HepG2); Human breast adenocarcinoma cell line (MCF-7); human colorectal carcinoma cells (HCT-116); Human cervical carcinoma cell line (HeLa) and Raw (MCF-7); human colorectal carcinoma cells (HCT-116); HepG2); Human breast adenocarcinoma cell line (MCF-7); human colorectal carcinoma cells (HCT-116); Human cervical carcinoma cell line (HeLa) and Raw

Optimization of L-glutaminase production

The submerged fermentation method for producing L-glutaminase was showed that, the L-glutaminase produced, is in its optimum phase (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. The result obtained by Krishnakumar et al. (2011) is in the agreement with present study. They showed that production of L-glutaminase from Streptomyces sp. SBU1 was at 30 °C after 96 h 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 120 rpm. Divya Teja et al. (2014) reported that strain of actinomycetes gave maximum production at pH 7, temperature 30 °C, time 96 h and salinity 3.5%. On the other hands, the optimum conditions (pH and Temperature) for L-glutaminase family is varied from one member to another Usha-Kiranmayi et al. (2013) studied one of glutaminase family he found that, the maximum production of L-asparaginase from Pseudonocardia endophytica VUK-10 was found in the culture medium with pH 8 and temperature 30 °C incubated for four days. Moreover, Suresh Kumer et al. (2013) stated that the response surface methodology predicted that a production containing Temperature 36.31 C, pH 7.34, Time 67.63 h, Galactose 40.20 g/L and L-glutamine 19.09 g/L to be optimum for the production of Serratia marcescens L-Glutaminase. Kiruthika and Saraswathy (2013) revealed that the maximum yield of Vibrio azureus L-glutaminase production (247 U/mL) was achieved in a seawater based medium at pH 8, 37 °C, 1% inoculum concentration and 2% glutamine concentration for 24 h.

Identification of S. canarius FR strain

The present study showed that, the strain is S. canarius FR used (after it has been identified by studying their morphological and biochemical characterization). PCR amplification of 16S rDNA gene confirmed the identity (98%) of 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. The results derived from the sequence analysis of the 16S rDNA 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).
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. The maximum specific activity 132.2 IU/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. These results were in the agreement with that reviewed by Mohana Priya et al.

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. On the other hand, Elshafei et al. (2014) recorded that an intracellular L-glutaminase from *Penicillium brevicompactum* NRCC829 was purified to homogeneity (162.75 fold) with an apparent molecular mass of 71 kDa. So the method used in the present study proved to be a good method in producing and purification of L-glutaminase.

**Determination of molecular weight for enzyme:**

The molecular homogeneity of the purified L-glutaminase from fermentation condition, as well as their purification steps were evaluated by SDS-PAGE. Single band at 44 kDa was appeared after final purification step (Column Sephadex G100) (Figure 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). And, Singh and Banik (2013) revealed that L-glutaminase extracellularly produced by *Bacillus cereus* MTCC 1305 was purified to apparent homogeneity with a fine band. The molecular weight of native enzyme and its subunit were found to be approximately 140 and 35 kDa, respectively, which indicates its homotetrameric nature.

**Biochemical properties of the purified L-glutaminase**

**Optimal pH and pH stability**

The L-glutaminase maintained its activity over a range of pH 5.0 -9 with optimum at pH 8.0 (Figure 2 A). The activity significantly decreased at both low and high pH values. The pH stability of the L-glutaminase 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; Kumar et al., 2012).

**Optimal temperature and thermal stability**

The optimum temperature for the L-glutaminase activity was determined by incubation of the reaction mixture at various degrees (25-50 °C) using L-glutamine as substrate in 0.1 M potassium phosphate buffer (pH 8.0). The highest enzyme specific activity (53.3 U/mg protein) was obtained at 40 °C. Above and below this temperature the enzyme activity decreased (Figure 2 B). Regarding to thermal stability the enzyme had a catalytically thermal stability below 60 °C, with a slightly decreasing in its activity at 70 °C, while completely lost its activity at 80 °C (Figure 2 C). The enzyme half-life times (T1/2) was 45.7, 38.8, 29.3 and 17.3 min at 50, 60, 70 and 80 °C, 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 (Figure 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. Also, the enzyme retained stability up to 50 and 20% even after treatment at 50 and 55 °C, respectively, for 30 min. Furthermore, Elshafei et al. (2014) recorded that the purified *Penicillium brevicompactum* L-glutaminase showed its maximal activity against L-glutamine when incubated at pH 8.5 at 50 °C for 30 min indicating the thermo-stability nature of this enzyme.

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

| Purification steps | Enzyme activity (U) | Total protein (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        |
| DEAE-Cellulose      | 95                  | 0.77               | 123.4                            | 16.7             | 56        |
| Sephadex G100      | 47.6                | 0.36               | 132.2                            | 17.9             | 28        |
Salt tolerance of *S. canarius* L-glutaminase

The results represented in Figure (2 E) showed that, tolerance in glutaminase activity was observed by increasing the NaCl concentration up to 25%. This is a good tolerance value. Similarly, Wakayama *et al.* (2005) observed that the L-glutaminase produced from actinomycetes has a good salt tolerance.

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

The kinetic parameters for L-glutaminase towards substrates were determined from the Lineweaver-Burk Plot (Figure 2 F and Table 2). The enzyme had relative high affinity and catalytic activity for L-glutamine (Km 0.129 mM, Vmax 2.02 U/mg/min), followed by L-asparagine (Km 0.137 mM, Vmax 1.75 U/mg/min) and low affinity to L-aspartic acid (Km 5.38 mM, Vmax 0.68 U/mg/min). Also, the highest catalytic efficiency (Kcat turnover number) for the enzyme was assessed for L-glutamine (0.75 x 10^{-3} s^{-1}) followed by L-asparagine (0.68 x 10^{-3} s^{-1}) and L-aspartic acid (0.25 x 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, Singh and Banik (2013) revealed that the substrate specificity test of *Bacillus cereus* MTCC 1305 L-glutaminase showed its specificity for L-glutamine. Moreover, the small Km (0.129 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). Also, Elshafei *et al.* (2014) recorded that the highest activity of *Penicillium brevicompactum* L-glutaminase was reported towards its natural substrate, L-glutamine, with an apparent Km value of 1.66 mM.

Influence of metal ions on L-glutaminase activity:

The impact of various inhibitors and activators on catalytic potency of the prepared L-glutaminase was evaluated by pre incubation of the enzyme with each compound for 30 min without substrate. Results in Table 3 showed that, Ca^{2+}, Ba^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+}, Hg^{2+} and Cd^{2+} considered to be inhibitor. On the other hand, Mn^{2+}, Na^{+} and Co^{2+} were shown to be activator. These results were in agreement with Senthil-Kumar and Selvam (2011) for *S. radiopugnans* MS1 L-asparaginase. Also, Singh and Banik (2013) reported that monovalent cations (Na^{+}, K^{+}) and phosphate ion activated the *B. cereus* MTCC 1305 L-glutaminase activity, while divalent cations (Mg^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+}, Ca^{2+}, Co^{2+}, Hg^{2+}, Cd^{2+}, Cu^{2+}) inhibited its activity.

Cytotoxicity effect of L-glutaminase

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). From the biochemical profiles, generally, there is no observed effect on liver, renal functions, glucose, lipids and other electrolytes, with slight inducing effect on the activity of ALT, AST and level of Random S. Glucose. Depending on these results, the L-glutaminase had relatively no negative effect on liver functions, where AST and ALT was the most potential indicator for liver dysfunction, this in the agreement with that obtained by Pratt and Kaplan (2000).

Also, the cellular toxicity of the purified *S. canarius* L-glutaminase was evaluated by estimation of the degree of
Figure 2 - Characterization of *S. canarius* L-glutaminase. (A) pH value and pH stability profile. The enzyme was preincubated for 2 h 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) Km values (Lineweaver-Burk plot) were calculated by fitting the 1/S of substrate vs. the 1/activity of the enzyme. (1) L- aspartic acid $y = 7.4538x + 1.3854$; (2) L-asparagine $y = 3.4933x + 0.4514$; (3) L-glutamine $y = 3.7046x + 0.5076$. 
platelet 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 within normal range after 10 days of L-glutaminase injection. The L-glutaminase displayed no hemolytic activity to human blood (Figure 3). 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).

### 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    | 0.129       | 2.02                        | 0.75 x 10⁻³|
| L-asparagines  | 0.137       | 1.83                        | 0.68 x 10⁻³|
| Aspartic acid  | 5.38        | 0.68                        | 0.25 x 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 second.

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 h of incubation. As shown in Figure (6), the tested enzyme had a high efficiency against Hep-G2 cell (IC₅₀, 6.8 μg/mL) and HeLa cells (IC₅₀, 8.3 μg/mL), while the growth of MCF-7 cells was not affect by the treatment. In 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 IC₅₀ calculated values: 64.7 μg/mL and 59.3 μg/mL, respectively, compared with the known anticancer drug paclitaxel, which its IC₅₀ 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...
### Table 4 - Hematological and biochemical parameters of rabbits in response 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 | 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   | -0.936  | 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  | -4.0 n.s| 13.17  | 14.50  | -8.0*   | 12.63  | 13.00  | -7.8 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  | -2.619 n.s |
| S. GPT (ALT)               | 66      | 68.00  | 80.67  | -38.0** | 66.00  | 69.00  | -1.44 n.s| 64.00  | 67.00  | -1.29 n.s | 63.00  | 65.00  | -0.756 n.s |
| Serum cholesterol: Total    | 45      | 53.00  | 56.00  | -2.59 n.s| 49.00  | 46.00  | 1.96 n.s | 47.00  | 47.00  | .00 n.s | 52.00  | 51.00  | .577 n.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 | 2.598 n.s| 145.00 | 138.00 | 2.782 n.s | 142.00 | 140.00 | .866 n.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 | 1.0 n.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   | 1.299 n.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   | .00 n.s | 9.70   | 9.00   | 1.732 n.s |
| Platelets                  | 382     | 379.00 | 360.00 | 11.332**| 378.00 | 373.33 | 1.257 n.s| 376.33 | 374.67 | 1.7814 n.s| 383.00 | 372.67 | 2.23 n.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 (132.2 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.
Figure 4 - Photomicrograph (at Hematoxylin and Eosin X 150 and X200) of renal section 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.

Figure 5 - Photomicrograph (at H & E X 150 and X200) of liver section of (C) negative control showing normal architecture formed of central vein (†) and portal tract (ﬁ) 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.
**vitro** toxicity on Hep-2C cell lines (84% survival) in comparison to commercial L-asparaginase preparation (90% survival) obtained from *E. coli*. Also, Singh and Banik (2013) reported that the gradual inhibition in growth of hepatocellular carcinoma (Hep-G2) cell lines was found with IC50 value of 82.27 µg/mL in the presence of different doses of L-glutaminase (10-100 µg/mL). Moreover, Elshafei et al. (2014) recorded that the purified enzyme produced by *Penicillium brevicompactum* was inhibited the growth of human cell line hepatocellular carcinoma (Hep-G2), with IC50 value of 63.3 µg/mL. Meanwhile, Nathiya et al. (2012) indicated a crucial role of *Aspergillus flavus* KUGF009 L-glutaminase in breast cancer (MCF7). Furthermore, 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 en-

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**Figure 6** - 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
zyme is being promising candidate for application as anti-tumor agent in the future work.

Acknowledgments

The author would like to thank Dr. Akmal Sakr for collecting of samples, and staff members of Pathology Department, Faculty of Medicine, Zagazig University, Zagazig for their guidance for Histological studies.

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