Purification, characterization and anticancer efficiency of L-glutaminase from Aspergillus flavus

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The aim of this work was to purify L-glutaminase from Aspergillus flavus. The enzyme was purified 12.47-fold from a cell-free extract with a final specific activity of 613.3 U/mg and the yield was 51.11%. The molecular weight of the enzyme, as estimated by SDS-PAGE, was found to be 69 kDa. The maximal activity of L-glutaminase was recorded at pH 8 and 40°C. The highest activity was reported towards L-glutamine as substrate, with an apparent Km value of 4.5 mmol and Vmax was 20 U/ml. The enzyme was activated by Na+ and Co2+, while it was greatly suppressed by iodoacetate, NEM, Zn2+ and Hg2+ at 10 mM. L-glutaminase activity increased with a gradual increase of sodium chloride concentration up to 15%. In vivo, the median lethal dose (LD50) was approximately 39.4 mg/kg body weight after intraperitoneal injection in Sprague Dawley rats. Also, L-glutaminase showed no observed changes in liver and kidney functions and hematological parameters on rates. Purified A. flavus L-glutaminase had neither a cognizable effect on human platelet aggregation nor hemolytic activity. In addition, MTT assay showed that the purified L-glutaminase has a high toxic impact on Hela and Hep G2 cell lines with an IC50 value 18 and 12 μg/ml, respectively, and a moderate cytotoxic effect on HCT-116 and MCF7 cells, with an IC50 value 44 and 58 μg/ml, respectively.

Key Words: anticancer efficiency; Aspergillus flavus; characterization; L-glutaminase; purification

Introduction

The enzyme L-glutaminase (EC 3.5.1.2) is an amidohydrolase enzyme which synthesizes L-glutamic acid and ammonia from L-glutamine. The genome of human carries the code of two main glutaminase isoforms, the liver isoform (LGA/GLS2) and the kidney isoform (KGA/GLS1) (Aledo et al., 2000).

L-glutamine is used as an obligate nitrogen donor for the synthesizing of pyrimidine and purine nucleotides in a living cell. Tumor cells have no pathway to synthesize L-glutamine and hence take it as an exogenous source. L-glutaminase causes the specific death of glutamine-dependent tumor cells by preventing the cells from accessing L-glutamine, and hence it is used as an effective agent in the remediation of acute lymphocytic leukemia and HIV (Singh and Banik, 2013). Microbial therapeutic enzymes have a great specific treatment as thrombolytic or anticoagulant, and largely as anticancer (Sabu, 2003; Sabu et al., 2005).

Recently, L-glutaminase has acquired awareness as a therapeutic agent against cancer and HIV (Chandrasekaran et al., 1991; Kumar and Chandrasekaran, 2003; Roberts et al., 1970), as a biological factor in controlling glutamine grades in mammalian and hybridoma cell lines (Kashyap et al., 2002; Zhao et al., 2004), for the production of special chemicals like theanine by c-glutamyl transfer reactions, and as a taste booster in the food industry (Sivakumar et al., 2006).

Glutaminase controls the delicious taste of fermented foods, such as soy sauce and in general food products by elevating the glutamic acid (Chou and Hwan, 1994). The use of L-glutaminase as a taste-enhancing factor in Chinese foods has replaced the use of monosodium glutamate which causes an allergic reaction for individuals (Jeon et al., 2009; Masuo et al., 2004; Thongsanit et al., 2008).
l-glutaminase has been purified and characterized from various bacterial species including *Pseudomonas aurantiaca* (Imada et al., 1973), *Streptomyces canariensis* (Reda, 2015), *Streptomyces* sp. (Desai et al., 2016) and *Vibrio Costicola* (Nagendra Prabhu and Chandrasekaran, 1999). Also, the enzyme was purified from various fungi *Aspergillus oryzae* (Koibuchi et al., 2000), *Aspergillus sojae* (Ito et al., 2012) and *Penicillium brevicompactum* (Elshafei et al., 2014).

In this work we focus on the purification and characterization of l-glutaminase from *A. flavus* and also to determine the minimal lethal dose and cytotoxicity of l-glutaminase. The anticancer efficiency of the purified l-glutaminase as an antitumor agent was measured against some types of cancer cells lines.

**Materials and Methods**

**Fungal strain and growth conditions.** *Aspergillus flavus* AUMC 8653 was selected as the most potent fungus for the production of l-glutaminase from our preliminary studies. *A. flavus* was grown on an optimized solid state fermentation medium utilizing wheat bran as carbon and nitrogen sources. The maximum productivity of l-glutaminase 45.0 ± 0.97 U/mg protein was achieved at 35°C, initial pH 7, moisture content 70%, supplementation of the salt basal medium with glucose (1%) and beet molasses (20% v/v).

**Preparation of cell-free extract.** At the end of the incubation period, the content of each flask was thoroughly mixed with 40 ml of 0.1 M citrate-phosphate using a shaker at 200 rpm for 40 min. The entire content of each flask was filtered through muslin then centrifuged at 4000 rpm for 15 min at 4°C. The supernatant was accumulated as the crude enzyme.

**Materials.** Nessler’s reagent, folin reagent, pyridoxine, sephadex G-100 and diethyl amino ethyl cellulose (DEAE-cellulose) were purchased from Sigma-Aldrich Company (St. Louis, Mo.).

**l-glutaminase assay.** Enzyme productivity was calculated by the method of Imada et al. (1973). This method measures the liberated ammonia from l-glutamine in the enzyme reaction by the Nessler’s reaction. The reaction was started by adding 0.5 ml cell-free extract into 0.5 ml 0.04 M L-glutamine and 0.5 ml 0.05 M Tris-HCl buffer, pH 7.0 and incubated at 30°C for 30 min. The reaction was finished by the addition of 0.5 ml of 1.5 M trichloroacetic acid (TCA). The absorbance was measured at 450 nm. One unit of l-glutaminase is the amount of enzyme which induces the synthesizing of 1 μmol of ammonia per min.

**Determination of protein.** The protein amount was evaluated by the method of Lowry et al. (1951). All assessments were achieved in triplicate and the averages were recorded.

**Enzyme purification.** Enzyme purification was started with precipitation of 400 ml of crude enzyme by the gradual addition of (NH₄)₂SO₄ using the range of saturation from 50 to 90%. The precipitate was collected by centrifugation, dissolved in a minimal amount of buffer, applied to a Sephadex G-100 gel filtration column (1.5 × 45 cm) and elution was achieved by citrate-phosphate. The fractions possessing the highest l-glutaminase activity were collected, pooled, concentrated and applied to an ion-exchange chromatography column containing diethyl amino ethyl cellulose (DEAE-cellulose). Elution was achieved by a linear gradient of NaCl up to 0.5 M. The fractions containing the highest activity were collected, desalted by dialysis, lyophilized by Labconco Freeze Dryer at –65°C and 250 μbar and stored at 0°C for further investigations.

**Gel electrophoresis.** The molecular weight of purified l-glutaminase from a culture of *A. flavus* was calculated by SDS-PAGE in accordance with Laemmli (1970) utilizing 10% polyacrylamide gel.

**Determination of kinetic parameters.** The optimum pH of the enzyme activity was investigated by measurements at 30°C in different buffers covering a pH range of 6–11. These buffer solutions were 0.1 M citrate-phosphate, pH 6.0–8.0, and 0.2 M glycine-NaOH, pH 8.5–11.0. The pH stability of the enzyme was calculated by measuring the residual activity of the enzyme after incubation in the buffers in different pH values for different periods at 30°C. The optimum temperature of the enzyme activity was calculated by incubating the enzyme at various temperatures ranging from 20 to 60°C in a buffer (pH 8). The thermostability of the enzyme was investigated after incubating the enzyme at various temperatures for different periods in the optimum buffer. l-glutaminase was incubated with gradual concentrations of l-glutamine and the kinetic parameters were investigated. Michaelis constant (Km) and the maximum velocity (Vmax) of the enzyme activity were calculated by linear regression from a Lineweaver-Burk plot (Lineweaver and Burk, 1943).

**Substrate Specificity of Purified l-glutaminase.** This experiment was designed to evaluate the specificity of the purified l-glutaminase towards various substrates: namely, l-glutamate, L-aspartagine, glutathione, L-albizziiin and L-γ-Glu-p-NA. The assay of l-glutaminase against L-γ-Glu-p-NA (pNA) was measured by calculating the produced paranitroalanine (Penninckx and Jaspers, 1985). The assay mixture contained an enzyme preparation (100 μl, 36.8 U) in 100 mM Tris-HCl at pH 7.2, with 2.5 mM of L-γ-glutamyl-p-nitroanilide in a final volume of 1 ml, and incubated at 37°C for 30 min. The reaction was terminated by the addition of 2 ml of 1.5 M acetic acid to the mixture, and then the release of (pNA) was measured at 410 nm (a yellow color develops).

**Effect of different metal ions and various inhibitors on l-glutaminase activity.** The effect of some metal ions (i.e. K⁺, Na⁺, Ca²⁺, Mg²⁺, Cu²⁺, Mn²⁺, Zn²⁺, Hg²⁺ and Mn²⁺), N-ethylmaleimide (NEM), ethylenediamine-tetraacetat (EDTA), and iodoacetate, on the enzyme activity was tested. The enzyme was pre-incubated with these components (metal ions were added as chlorides) for 15 min at 1 or 10 mM, and then the relative enzyme activity was estimated.

**Effect of different concentrations of NaCl.** The impact of various concentrations of NaCl (0–30%, w/v) on the activity of l-glutaminase was estimated.
at room temperature, the platelet-rich plasma (350 µl) was collected, injected with 200 and 400 µl of enzyme, and incubated for 20 min at room temperature. Platelet aggregation was evaluated with a blood Lumi-Aggregometer.

Hemolytic activity of L-glutaminase. The hemolysis of L-glutaminase was evaluated using a blood agar assay.

Table 1. Purification summary of L-glutaminase from Aspergillus flavus.

| Purification step | Total activity (U) | Total protein (mg) | Specific activity (U/mg protein) | Yield (%) | Purification fold |
|------------------|-------------------|-------------------|---------------------------------|-----------|------------------|
| CEP              | 18000             | 366               | 49.18                           | 100       | 1.0              |
| Ammonium sulphate (70%) | 12989           | 156               | 83.26                           | 72.16     | 1.69             |
| Sephadex G-100   | 10845             | 35                | 309.85                          | 60.25     | 6.3              |
| DEAE-Cellulose   | 9200              | 15                | 613.3                           | 51.11     | 12.47            |

Determination LD50 of L-glutaminase enzyme by intra-peritoneal injection in Sprague Dawley rat. Sprague Dawley rats weighing between (120–150) grams were purchased from the Egyptian Organization for biological products and vaccines, VACSER, Egypt. The rats were housed in plastic airy cages under a controlled environmental condition cycle (12 hours dark, 12 hours light at 25°C) in groups of 5 animals per cage with free access to food and water. The procedure was carried out according to Bass et al. (1982). For the determination of the acute lethal dose (LD100) and the median lethal dose (LD50) of purified L-glutaminase, intraperitoneal injection of doses from 10 to 80 mg/kg body weight with an increasing factor (1.2) were carried out. Mortality was recorded after 24 h, and LD50 was calculated as follows:

\[
\log \text{LD}_{50} = \log \text{LD next below 50%} + (\log \text{increasing factor} \times \text{proportionate distance})
\]

\[
\text{proportionate distance} = \frac{50\% - \text{mortality next below 50\%}}{\% \text{mortality above 50\%} - \% \text{mortality next below 50\%}}.
\]

Cytotoxicity of L-glutaminase. The cytotoxic effect of the L-glutaminase was evaluated using 5 Sprague Dawley rats as an experimental group and one rat as the control. The experimental group was injected intravenously with 1 mL of L-glutaminase (3.49 mg/kg body weight of rats) every 5 days for 15 days. After that, blood samples were collected 10, 20 and 30 days after the last injection. Plasma was collected and stored at −20°C. Various hematological factors, such as hemoglobin, red blood cells (RBC), white blood cells (WBC), platelets (PLT), mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV), were calculated. Also various clinical chemistry parameters such as total protein, bilirubin, direct bilirubin, albumin, alanine aminotransferase (ALT), lactate dehydrogenase (LDH), aspartate amino transferase (AST), blood urea nitrogen (BUN), creatinine, uric acid, glucose, cholesterol and triglyceride, were estimated as described in Ringler and Dabich (1979).

Plate aggregation. A platelet aggregation assay was conducted following the turbidimetric method of Wei et al. (2007). A human blood specimen was collected in 0.4% sodium citrate. After centrifugation at 8,000 rpm for 5 min at room temperature, the platelet-rich plasma (350 µl) was collected, injected with 200 and 400 µl of enzyme, and incubated for 20 min at room temperature. Platelet aggregation was evaluated with a blood Lumi-Aggregometer.

Hemolytic activity of L-glutaminase. The hemolysis of L-glutaminase was evaluated using a blood agar assay. Agarose 1% was prepared in a phosphate buffer saline and human erythrocytes, were added to a final concentration of 2%. Two holes were punched into the plates, and pipetted with 100 and 200 µl purified enzyme (613.3 U/mg proteins). The plates were incubated at 37°C for 48 h, and then the clear zone was observed (Tay et al., 1995).

Antitumor assay. The anticancer efficiency of the purified L-glutaminase as an antitumor was measured against human hepatocellular carcinoma cell line (HepG2); human breast adenocarcinoma cell line (MCF-7), human colorectal carcinoma cells (HCT-116), and human cervical carcinoma cell line (Hela). Carcinoma cell lines were gained from the American Type Culture Collection (ATCC, USA). The antiproliferative effect was measured using MTT assay (Renugadevi and Venus, 2012). Dulbecco’s modified Eagle’s medium (DMEM) supplied with 1% L-glutamine, HEPES buffer, 50 µg/ml gentamycin and 10% heat-inactivated fetal bovine serum was utilized for cultivation of the cells in a 24-well plate for 48 h at 37°C in 5% CO2. The formed monolayer of cells was exposed to various concentrations of purified L-glutaminase (2:20 µg/ml), the incubation was resumed for 48 h and the yield of viable cells was measured by a colorimetric method. Cell viability (%) = (OD of treated cells/OD of control cells × 100).

IC50; enzyme concentration resulted in 50% inhibition was determined from the graph plotting percentage of cell viability against enzyme concentration. The lower the IC50 value indicates a higher antitumor capacity.

Statistical analysis. The recorded data were analyzed with SPSS version 20, in which the equations of the hypothesis tests, including the mean, standard deviation, and T-statistics value, were used. Results were considered highly significant, significant, or non-significant, corresponding to p ≤ 0.01, p ≤ 0.05 and p > 0.05, and represented by ***, ** and *, respectively.

Results

Purification of L-glutaminase from Aspergillus flavus

The results of the purification procedure for L-glutaminase from the crude enzyme preparation (CEP) of A. flavus grown on the optimized solid state fermentation medium utilizing wheat bran as substrate are summarized in Table 1. The results showed that precipitation of proteins from the CFP with 70% saturation of ammonium sulfate provided a fraction containing the highest enzyme preparation. The precipitation process was followed by the steps of gel filtration through Sephadex G-100 which resulted in an overall recovery of 60.25% with a 6.3 fold purification and ion-exchange chromatography with DEAE-cellulose by which the overall recovery of the apparently
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The purified L-glutaminase was calculated to be 51.11% with 12.47 fold purification.

**SDS-PAGE**

Results showed the electrophoretogram of the purified L-glutaminase from *A. flavus* as determined using SDS-PAGE. The molecular weight of the purified enzyme was estimated after finishing purification to be 69 kDa (Fig. 1).

**Influence of pH on enzyme activity and stability**

The results reveal that the enzyme showed a great activity at pH 6–8 with a maximum at pH 8. A decline in the enzyme activity was recorded on either side of this pH range (Fig. 2A). The influence of pH on the enzyme stability showed that it retained full activity after one hour of incubation at pH 8 and about 77% of the original activity after one hour of incubation at pH 8.5 (Fig. 2B).

**Effect of temperature on enzyme activity and stability**

The optimum activity of purified glutaminase was re-

![Fig. 1. SDS-PAGE of purified L-glutaminase from *A. Flavus*. Lane 1, L-glutaminase sample, Lane M, molecular weight standards (Marker).](image)

![Fig. 2. Characterization of L-glutaminase of *A. flavus*. A. Effect of pH value on activity of the purified L-glutaminase of *A. flavus*; B. Effect of pH value on stability of the purified L-glutaminase of *A. flavus*; C. Effect of temperature on activity of the purified L-glutaminase of *A. flavus*; D. Effect of temperature on stability of the purified L-glutaminase of *A. flavus*.](image)

| Substrate           | Relative enzyme activity (%) |
|---------------------|------------------------------|
| L-glutamine         | 100                          |
| L-asparagine        | 44                           |
| glutathione         | 0                            |
| l-albizzin          | 11                           |
| L-γ-Glu-p-NA        | 0                            |

**Table 2. Activity of the purified L-glutaminase from *A. flavus* on different substrates.**

| Components | Conc. (mM) | Relative enzyme activity (%) |
|------------|------------|------------------------------|
| Control    | 0.0        | 100                          |
| Metal ions*|            |                              |
| K⁺         | 1          | 100                          |
| 10         | 107        |
| Na⁺        | 1          | 100                          |
| 10         | 129        |
| Ca²⁺       | 1          | 100                          |
| 10         | 80         |
| Co²⁺       | 1          | 105                          |
| 10         | 115        |
| Cu²⁺       | 10         | 2                            |
| Mg²⁺       | 1          | 88                           |
| 10         | 55         |
| Zn²⁺       | 1          | 45                           |
| 10         | 30         |
| Hg²⁺       | 1          | 70                           |
| 10         | 35         |
| Mn²⁺       | 1          | 100                          |
| 10         | 105        |

**Table 3. Effect of some chemical components on activity of the purified L-glutaminase.**

| Components | Conc. (mM) | Relative enzyme activity (%) |
|------------|------------|------------------------------|
| NEM        | 1          | 84                           |
| 10         | 60         |
| EDTA       | 1          | 100                          |
| 10         | 98         |
| Iodoacetate| 1          | 44                           |
| 10         | 23         |

*Metal ions were added as chlorides.

![Fig. 3. Effect of different concentration of NaCl on the purified L-glutaminase of *A. flavus*.](image)
corded at 40°C, and at higher than 40°C, the enzyme activity decreased gradually (Fig. 2C). The results of thermal stability showed that the purified enzyme retained full activity after one hour of incubation at 40°C and could tolerate up to 45°C for 30 min without loss of more than 12% of its initial activity and also retained about 74% of this activity at 50°C (Fig. 2D).

**Substrate specificity of L-glutaminase**

The results shown in Table 2 reveal that the enzyme had a relative activity towards various substrates. The enzyme showed the highest affinity towards L-albizziin and did not show any activity against glutathione and L-γ-Glu-p-NA. V_max of the purified enzyme was calculated to be 20 Uml⁻¹. Km value was calculated from the Lineweaver-Burk plot of reciprocals of initial velocities and substrate concentrations. It is of interest to note that the enzyme inhibited by iodoacetate and NEM retaining only about 23 and 60% of its initial activity, respectively. While the activity of enzyme was apparently not affected by EDTA.

| Dose (mg/Kg b.wt.) | Number of animals | Survivals | Deaths | % Mortality |
|---------------------|-------------------|-----------|--------|------------|
| 70                  | 10                | 10        | 0      | 100%       |
| 60                  | 10                | 9         | 1      | 90%        |
| 50                  | 10                | 7         | 3      | 70%        |
| 43                  | 10                | 5         | 5      | 50%        |
| 36                  | 10                | 6         | 4      | 40%        |
| 30                  | 10                | 8         | 2      | 20%        |
| 25                  | 10                | 9         | 1      | 10%        |
| 20                  | 10                | 10        | 0      | 0%         |

**Effect of different metal ions and inhibitors on L-glutaminase activity**

Metal ions may act as activators or inhibitors in numerous enzymatically catalyzed reactions. Therefore, the effect of some metal ions on L-glutaminase activity was tested. The results (Table 3) show that Na⁺ and Co²⁺ act as potent activators, where the relative enzyme activity significantly increased to 129 and 115%, respectively, at a final concentration of 10 mM. Moreover, K⁺ and Mn²⁺ had a slightly stimulatory effect on enzyme activity. However, the enzyme activity was suppressed in the presence of Cu²⁺, Zn²⁺, Hg²⁺ and Ca²⁺. The effects of various enzyme inhibitors on the activity of A. flavus L-glutaminase are also evaluated. L-glutaminase was significantly inhibited by iodoacetate and NEM retaining only about 23 and 60% of its initial activity, respectively. While the activity of enzyme was apparently not affected by EDTA.

| Parameters                                | Control (zero time) | After 10 days | After 20 days | After 30 days |
|-------------------------------------------|---------------------|---------------|---------------|---------------|
| **Control**                               | After 10 days       | After 20 days | After 30 days |
| Total protein (g/dl)                      | 60.3 ± 0.3          | 60.1 ± 1.36   | 62.45 ± 1.09  | 60.22 ± 1.08  | 59.23 ± 0.99  | 59.78 ± 0.15  | 60.44 ± 0.84  |
| Bilirubin (µmol/L)                        | 1.62 ± 0.08         | 1.6 ± 0.04    | 1.51 ± 0.10  | 1.58 ± 0.11   | 1.51 ± 0.25   | 1.61 ± 0.05   | 1.56 ± 0.05   |
| Direct Bilirubin (µmol/L)                 | 0.44 ± 0.02         | 0.41 ± 0.03   | 0.55 ± 0.05  | 0.42 ± 0.11   | 0.47 ± 0.07   | 0.40 ± 0.04   | 0.37 ± 0.03   |
| Albumin (g/L)                             | 28.44 ± 0.22        | 28 ± 0.13     | 25.56 ± 0.14 | 28.02 ± 0.09  | 26.44 ± 0.14**| 28 ± 0.21     | 26.78 ± 0.14**|
| ALT (U/L)                                 | 25.45 ± 0.22        | 27.22 ± 0.13  | 33.67 ± 0.14 | 28.02 ± 0.13  | 26.66 ± 0.49**| 30.62 ± 0.25  | 26.33 ± 0.10***|
| Lactate dehydrogenase (mg/Kg b.wt.)       | 259 ± 0.10          | 259 ± 0.13    | 270.2 ± 1.27**| 258.3 ± 0.13  | 261.4 ± 0.19**| 259 ± 0.08**  | 260.3 ± 0.10**|
| LDH (U/L)                                 | 45.23 ± 1.01        | 41.81 ± 0.13  | 44.74 ± 0.13 | 42.56 ± 0.13  | 40.09 ± 0.13  | 43 ± 0.07     | 40.13 ± 0.09   |
| Aspartate amino transferase (mg/Kg b.wt.)  | 70.23 ± 0.94        | 73.75 ± 0.94  | 82.78 ± 1.57**| 71.03 ± 1.22  | 76.09 ± 1.21**| 70.2 ± 1.59   | 72.67 ± 1.12**|
| AST (U/L)                                 | 8.85 ± 0.14         | 8.27 ± 0.14   | 10.67 ± 0.27**| 7.89 ± 0.23   | 9.56 ± 0.13   | 9.02 ± 0.25   | 8.38 ± 0.12***|
| Blood urea nitrogen (mg/Kg b.wt.)          | 50.12 ± 0.14        | 50.13 ± 0.13  | 50.27 ± 0.14 | 50.13 ± 0.13  | 50.49 ± 0.25**| 50.25 ± 0.12**| 50.13 ± 0.12**|
| Creatinine (µmol/L)                       | 45.23 ± 1.01        | 41.81 ± 0.13  | 44.74 ± 0.13 | 42.56 ± 0.13  | 40.09 ± 0.13  | 43 ± 0.07     | 40.13 ± 0.09   |
| Uric acid (µmol/L)                        | 63.89 ± 0.94        | 60.12 ± 0.83  | 57.19 ± 1.57**| 62.12 ± 1.22  | 60.00 ± 1.21**| 61.76 ± 1.15**| 60.31 ± 1.22**|
| Glucose (mmol/L)                          | 10.22 ± 0.14        | 10.01 ± 0.09  | 13.56 ± 0.17**| 11.23 ± 0.23  | 12.13 ± 0.19**| 10.78 ± 0.12  | 11.41 ± 0.24**|
| Cholesterol (mmol/L)                      | 0.97 ± 0.08         | 0.91 ± 0.05   | 1.23 ± 0.08**| 0.91 ± 0.11   | 0.83 ± 0.09** | 0.93 ± 0.08   | 0.91 ± 0.14** |
| Triglyceride (mmol/L)                     | 2.05 ± 0.09         | 1.94 ± 0.24   | 2.25 ± 0.10**| 1.97 ± 0.31   | 1.82 ± 0.25** | 1.98 ± 0.19   | 1.94 ± 0.25** |

**Effect of different concentrations of NaCl**

Figure 3 shows that L-glutaminase activity increased gradually with the increase of NaCl concentrations up to 15%, and then it decreased with gradual increase of NaCl concentrations. It is of interest to note that the enzyme retained about 87% of its original activity at 25% NaCl.

**Medium lethal dose (LD50) of L-glutaminase**

The injection of doses ranged from the concentration 10 to 80 mg/kg body weight. The results presented in Table 4 reveal that the median lethal dose (LD50) was approximately 39.4 mg/kg body weight or 24152.2 units/kg body weight for L-glutaminase after intraperitoneal injection in rats.

| Dose (mg/Kg b.wt.) | Number of animals | Survivals | Deaths | % Mortality |
|---------------------|-------------------|-----------|--------|------------|
| 70                  | 10                | 10        | 0      | 100%       |
| 60                  | 10                | 9         | 1      | 90%        |
| 50                  | 10                | 7         | 3      | 70%        |
| 43                  | 10                | 5         | 5      | 50%        |
| 36                  | 10                | 6         | 4      | 40%        |
| 30                  | 10                | 8         | 2      | 20%        |
| 25                  | 10                | 9         | 1      | 10%        |
| 20                  | 10                | 10        | 0      | 0%         |
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Cytotoxicity of L-glutaminase
The cytotoxicity effect of purified L-glutaminase was based on the biochemical parameters and hematological parameters for Sprague Dawley rats. Commonly available tests include aminotransferase (ALT), lactate dehydrogenase (LDH), aspartate amino transferase (AST), and serum bilirubin, are used to reflect different functions of the liver (Limdi and Hyde, 2003). Table 5 showed a slight increase in the level of ALT, LDH, AST and glucose, especially after 10 days from the last injection but this inducing effect decreased after 20 or 30 days from injection. Also results showed a slight increase in the levels of urea, creatinine, cholesterol and triglyceride after 10 days of the last injection, but returned to normal values by 30 days after the injections.

Table 6 shows the effect of L-glutaminase on the hematological parameters of rats. Various hematological parameters, such as hemoglobin, RBC, WBC, PLT, MCH and MCV showed a mild decrease within the normal range after 10 days from the last injection, while many parameters subsequently increased to reach their normal range after 30 days.

Platelet aggregation and hemolytic activity of L-glutaminase
Platelet aggregation was not monitored with a blood Lumi-Aggregometer. Also, the results illustrated that L-glutaminase displayed no hemolytic activity to human blood (Fig. 4).

Antitumor assay
MTT assay showed that the purified L-glutaminase has a high toxic effect on Hela and Hep G2 cell lines with an IC50 values of 18 and 12 µg/ml, respectively (Figs. 5A and B). Moreover, the growth-inhibitory effect of L-glutaminase on HCT-116 and MCF7 cells (Figs. 5C and D) indicates that there was a moderate cytotoxic effect with an IC50 values of 44 and 58 µg/ml, respectively.

Discussion
L-glutaminase is a powerful anti-leukemic factor. Leukemic cells do not demonstrate L-glutamine synthetase, and thus, it depends on the external outfit of L-glutamine to produce α-ketoglutarate, to provide their Krebs cycle fuels, and also produce intermediates for the formation of lipids, nucleosides and other biomolecules, needed for their proliferation and survival (Altman et al., 2016; Rohde et al., 1996; Wise and Thompson, 2010). Therefore, searching for a new enzyme with unique therapeutic properties is a challenge for many biotechnology laboratories. This study was conducted to perform the purification and char-
acterization of L-glutaminase from *A. flavus* solid cultures. The produced enzyme was best precipitated with 70% saturation of ammonium sulfate. This finding is in agreement with that reported by Desai et al. (2016) who recorded the same concentration of ammonium sulphate for L-glutaminase purification from *Streptomyces* sp. To remove the other mixed enzymes in the partially purified L-glutaminase solution of *A. flavus*, it was further purified by two steps of purification with overall recovery of 60.25% and 6.3 fold purification was obtained. This finding was in accord with results obtained by Elshafei et al. (2014), who recorded a 48.21% yield for purified L-glutaminase from *Penicillium brevicompactum*.

The purified L-glutaminase appeared as a single band on the denaturing gel. The molecular weight of the purified enzyme under denaturing conditions was 69 kDa. The appearance of L-glutaminase as a single band confirms the homogeneity and purity of the enzyme. These results are in agreement with previous findings on other L-glutaminases from *Aspergillus sojae* (Ito et al., 2012) and *Penicillium brevicompactum* (Elshafei et al., 2014).

The purified L-glutaminase of *A. flavus* was found to have a good activity at an alkaline value (pH 8) and this optimum pH is equal to that reported for purified L-glutaminase from *Debaryomyces* sp. (Dura et al., 2002), *Streptomyces canariensis* (Reda, 2015) and *Penicillium politans* NRC (Ali et al., 2009). Maximum L-glutaminase acquired was acquired at 40°C, and the decrease of the activity above this temperature may be due to denaturation of the enzyme subunits by the effect of heat (Dias and Weimer, 1998). Our results were in complete agreement with that recorded for the enzyme of *Debaryomyces* sp. (Dura et al., 2002), *Streptomyces canariensis* (Reda, 2015) and *Aspergillus oryzae* (Koibuchi et al., 2000). Meanwhile, a higher optimum temperature was reported for purified L-glutaminase from *Aspergillus sojae* (Ito et al., 2012).

The high affinity of *A. flavus* glutaminase to L-glutamine as a substrate was recorded previously by Elshafei et al. (2014) and Reda (2015) for *Penicillium brevicompactum* and *Streptomyces canariensis*, respectively. While the low activity of L-glutaminase towards L-albizziin, and the absence of activity towards glutathione and 1-γ-Glu-p-NA are in agreement with that recorded for L-glutaminase from *Saccharomyces cerevisiae* and *Debaryomyces* spp. (Dura et al., 2002; Penninckx and Jaspers, 1985; respectively), the Michaelis constant (Km value) of the purified enzyme was calculated to be 4.5 mmol and this value is very near that recorded for the same enzyme from *Debaryomyces* spp. (Dura et al., 2002).

Activity of the enzyme was promoted by the presence of Na⁺ and Ca²⁺. On the other hand, Cu²⁺, Zn²⁺, Hg²⁺ and Ca²⁺ produced the main inhibitory effect and this finding was in agreement with that reported for fungal L-glutaminase by (Reda, 2015; Senthil-Kumar and Selvam, 2011). Generally, the stimulatory impact of metal ions may be ascribed to their stabilizing effect on the conformational structure, and the protection of the enzyme from autoproteolysis and thermal denaturation (Secades and Guijarro, 2001). The enzyme was completely inactivated by a thiol reducing agent such as iodoacetate and NEM, which react with sulfur amino acids. The complete inhibition of L-glutaminase with this agent provides evidence for the presence of -SH group in the active sites of enzyme. These results are in complete agreement with that recorded by (Dura et al., 2002; Sayre and Roberts, 1958). L-glutaminase activity was not affected by EDTA which suggests that L-glutaminase might not be a metalloenzyme. Similar results for the effect of EDTA on L-glutaminase were reported (Dura et al., 2002; Elshafei et al., 2014; Hartman, 1985; Holcenberg, 1985).

The gradual increase of L-glutaminase activity with increasing NaCl concentration up to 15% indicates that our enzyme is characterized by a high salt tolerance, so it may play an important role in those industries that require a high salt culture, such as the soy sauce industry. Similar results were recorded for L-glutaminase from *Bacillus subtilis* (Iwasa et al., 1987) and *Penicillium brevicompactum* (Elshafei et al., 2014). On the contrary, L-glutaminases from *Aspergillus oryzae* and *Aspergillus sojae* are remarkably inhibited by high salt concentrations as demonstrated by (Shimizu et al., 1991; Yano et al., 1988; respectively).

The median lethal dose of *A. flavus* L-glutaminase was evaluated by intraperitoneal injection in Sprague Dawley rats. It was about 39.4 mg/kg body weight, so L-glutaminase enzyme is a hopeful antitumor factor owing to a moderate (LD₅₀) compared with the median lethal dose of the famous anticancer drug paclitaxel which recorded 19.5 mg/kg in rats (Hureaux et al., 2010).

The cytotoxic effect of the purified L-glutaminase was tested in vivo using Sprague Dawley rats. From the biochemical analysis, the enzyme had no stimulatory effect on the levels of ALT and LDH, AST and glucose in the blood especially 30 days after injection, revealing an absence of a direct effect on the liver. ALT and AST are the most relevant indicators of potential liver dysfunction (Pratt and Kaplan, 2000). Our results are in agreement with that recorded for the rhesus monkeys serum LDH (Hambleton et al., 1980). Thus, purified L-glutaminase had relatively no negative effect on liver functions, in agreement with that reported by Reda (2015) and Schoch and Whiteman (2007).

The concentration of creatinine and urea illustrates the filtration capability of the glomerulus, and it can be used as an index of renal function (Salazar, 2014). Our enzyme has no effect on the level of urea, creatinine, cholesterol and triglyceride in the blood of Sprague Dawley rats, and these results were in complete agreement with that recorded for the effect of glutaminase on rhesus monkeys and rabbits by Hambleton et al. (1980). L-glutaminase may have no effect on the kidneys, and there were no detectable biochemical changes in rats so it can be used as a beneficial therapeutic drug, but more research should be carried out to explore its mode of action in vivo.

Injection of purified L-glutaminase caused a mild decrease in the level of hemoglobin, RBC, WBC, PLT, MCH and MCV, in rats which returned to a normal level within 30 days of injection. Our results are in complete agreement with that recorded for the purified L-glutaminase from *Streptomyces canariensis* by Reda (2015).

Purified *A. flavus* L-glutaminase had neither a cogniza-
ble impact on platelet aggregation, nor hemolytic activity of human. These results were also reported by Reda (2015) who reported that L-glutaminase of *Streptomyces canarius* has no hemolytic activity on human blood. The lack of ability to lyse of human RBCs is unique supportive evidence from a therapeutic point of view. Similar results concluded the absence of cytotoxicity of microbial glutaminase Baskerville et al. (1980).

The antitumor studies on cell line showed that L-glutaminase has a significant efficiency against Hep G2 and Hela cell lines and a moderate impact against MCF7 and HCT-116 cells. These results are in agreement with previous findings (Reda, 2015) who reported that the IC<sub>50</sub> value for L-glutaminase on Hela and Hep G2 cell lines were 8.3 and 6.8 μg/ml respectively. Also, Pandian et al. (2014) concluded that purified L-glutaminase by *Alcaligenes faecalis* KLU102 inhibited the growth of Hela cells with an IC<sub>50</sub> value of 12.5 μg/ml. Similar results were obtained by Singh and Banik (2013) who recorded that an IC<sub>50</sub> value for L-glutaminase produced by *Zygosaccharomyces rouxii* which inhibited the growth of the MCF7 cell line, was 82.27 μg/ml, and Elshafei et al. (2014) reported that the purified enzyme derived from *Penicillium brevicompactum* inhibited the growth of MCF7 cell, with an IC<sub>50</sub> values of 63.3 μM. While a less toxic effect of L-glutaminase on MCF7 was reported by Nathiya et al. (2012) and Sunil Dutt et al. (2014) with IC<sub>50</sub> values of 250 and 283.5 μg/ml, respectively. Meanwhile, Reda (2015) recorded that the growth of MCF-7 cells was not affected by the *Streptomyces canarius* L-glutaminase and that the HCT-116 cell was inhibited with an IC<sub>50</sub> value of 64.7 μg/ml.

**Conclusion**

This study has demonstrated the ability of *Aspergillus flavus* to produce L-glutaminase, which has different practical applications, especially in cancer therapy. The enzyme was purified to homogeneity with an overall yield of 51.11%. by simple procedures of purification. The purified enzyme was stable over a wide range of pH and temperature. It has a high affinity to L-glutamine and a high thermal stability. Also, the purified enzyme has a moderate (LD<sub>50</sub>). Moreover, the enzyme had neither an effect on human platelet aggregation, nor hemolytic activity. Moreover, the enzyme has been found to possess significant antitumor activity against different cell lines in *vitro*. Therefore, we can say that L-glutaminase is a promising candidate for cancer therapy. To the best of our knowledge, this is the first report to determine the median lethal dose (LD<sub>50</sub>) of glutaminase.

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