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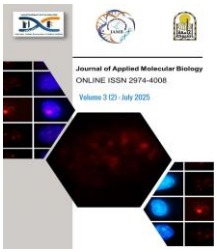

Purification, characterization and anticancer applications of extracellular L-asparaginase produced by the novel endophytic *Aspergillus aflatoxiformans* AUMC 16562

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ABSTRACT

L-asparaginase (L-ASNase) is a multifunctional enzyme utilized for its anticancer properties. The current study delineated the optimization, purification, and application of extracellular L-ASNase produced by the endophytic *Aspergillus aflatoxiformans* AUMC 16562. The generation of L-ASNase was optimized in M9 medium, resulting in L-ASNase high yield with a maximum activity of 62.3 U/mL at pH 8 and 27 ºC. L-ASNase was purified using ethanol precipitation, DEAE-cellulose anion exchange chromatography, and Sephacryl S-200 HR gel filtration. In the present study, the purified L-asparaginase exhibited a molecular weight of approximately 40.5 kDa, as determined by SDS-PAGE analysis. The enzyme reached a maximum specific activity of 3668.9 U/mg under optimal conditions at pH 8.0 and 30ºC. The purification process resulted in a 12.42-fold increase in purity, with a final recovery yield of 39.82%. These findings reflect the outcomes of the current research. The purified enzyme exhibited anticancer efficacy against the human hepatocellular carcinoma cell line (HePG-2) with an IC_{50} of 65.2 µg/mL, the Colon cell line (HCT-116) with an IC_{50} of 48.2 µg/mL, and the Prostate cell line (PC-3) with an IC_{50} of 68.9 µg/mL. Concerning the in-vivo assay of the purified enzyme, the biochemical profiles of pure L-ASNase exhibited no effect on glucose, other electrolytes, the liver, or the kidneys. Conversely, the total bilirubin level and the activities of aspartate aminotransferase (AST) and alanine transaminase (ALT) were marginally raised. The results indicated that L-

ASNase had a little impact on liver function, with liver impairment presumably signified by AST and ALT indicators. All hematological values remained within normal limits during the investigation. The present work identified a robust L-ASNase from *A. aflatoxiformans* AUMC 16562, which demonstrated effective cytotoxic performance against various carcinogenic cell lines under different environmental conditions, highlighting its potential utility in multiple commercial and therapeutic applications.

1. INTRODUCTION

The commercially significant enzyme L-ASNase catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia [1]. Animals, plants, fungi, and bacteria are frequently discovered to have it [2, 3]. The enzyme is mostly utilized to treat hematological and non-hematological diseases, including acute lymphoblastic leukemia (ALL). Additionally, it serves as a biosensor to measure asparagine levels during chemotherapy and is utilized in the food sector to neutralize the carcinogenic acrylamide [4].

By starving cancer cells of asparagine, L-ASNase attacks these cells. Cancer cells are unable to synthesize asparagine de novo because they lack the enzyme asparagine synthetase. As a result, L-ASNase activity depletes asparagine in blood, which inhibits cancer cells' ability to synthesize proteins [5]. The anti-leukemic drug L-ASNase is available in five commercial formulations for the treatment of ALL: three formulations based on *Escherichia coli* L-ASNase (Elspar1, Leukanase, Kidrolase); one formulation is a pegylated form of *E. coli* L-ASNase (Oncaspar1); and the fifth is a recombinant L-ASNase from *Erwinia chrysanthemi* (Erwinase1) [6]. As a result, discovering and developing potent anticancer drugs for these specific illnesses has a significant influence on patient morbidity and death, potentially saving countless lives.

Of the drugs approved for the treatment of cancer, over 60% have a natural origin. The search for other L-ASNase sources, like eukaryotic microorganisms, can lead to an enzyme with less adverse effects. Microbial production of enzymes such as L-ASNase is primarily achieved using bacteria, yeasts, and filamentous fungi, wild or recombinant, through fermentation processes in liquid (submerged) or solid state. Among various microorganisms, filamentous fungi stand out as optimal agents for enzyme production. They are easily manipulable and have the capability to produce enzymes extracellularly. Alternatively, when needed, they can be subjected to straightforward and cost-effective unit operations for intracellular enzyme extraction [7]. In addition, several fungal species are Generally Recognized as Safe (GRAS). So far, the *Aspergillus caespitosus* species has been reported in the literature as a producer of enzymes such as amylase [8], alkaline phosphatase [9], laccase [10], invertase [11], and L-ASNase, a crucial therapeutic enzyme that accounts for 40% of the global demand for enzymes [12] and was first described as a producer recently [13]. There are several sources of L-ASNase isolation and purification but for the commercial purposes, it is currently being isolated from two main bacterial sources first from *E.coli* (mainly from its recombinant and genetically modified versions) and second, from *Erwinia chrysanthemi* [14]. L-ASNase is present in most of the bacteria, fungi, plant tissues, algae and in some animals. Rodents have L-ASNase in their blood serum, however, humans lack this vital enzyme [15, 16]. L-ASNase enzyme is believed to be the most important due to its role in lymphoblastic therapy and for the inhibition of acrylamide production as a side reaction during the industrial processing of

food [17]. The importance of this enzyme is also evident from its high global demand which was 380 million USD in 2017 and is estimated to rise up to 420 million USD by 2025 [18]. Due to such vital importance of the enzyme in health and industry, L-ASNase is currently being investigated for its efficient role against different types of cancers including breast cancer [19]. The stunning properties of the enzyme in health, pharmaceuticals and food industry have a demand that this enzyme must be studied thoroughly. This study has discussed thoroughly its microbial sources most importantly the fungal one with special emphasize on its purification methods and characterization.

2. MATERIALS and METHODS

2.1. Fungal strain

The endophyte *Aspergillus* isolate utilized in this study has been isolated from *Chenpodium ambrosioides* collected from El-Kharga city, New Valley Governorate, Egypt [20]. The pure culture of the fungal strain has been preserved in the culture collection of the Assiut University Mycological Centre under the designation AUMC 16562. It was chosen for the optimization of L-ASNase production due to its elevated activity in producing L-ASNase on M9 agar medium (Figure 1).

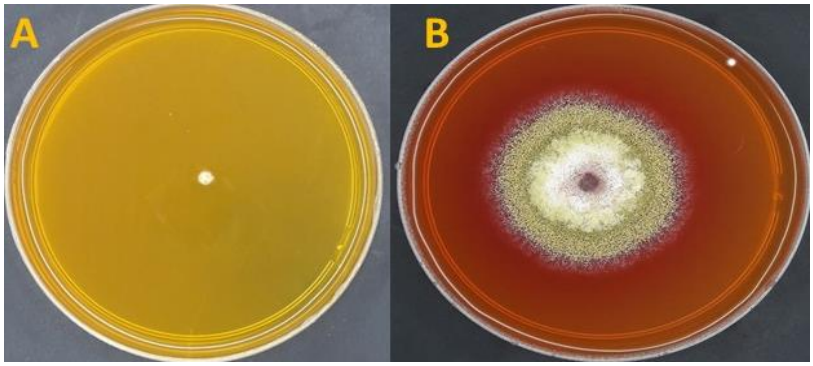


Figure 1. Qualitative assessment of L-ASNase activity demonstrated by red coloration on modified M9 agar plates (**A**) Control M9 medium (**B**) M9 media inoculated with the fungal strain exhibiting a color shift.

2.2. Fermentation medium

Modified M9 medium [21] was used as the fermentation medium in the optimization process of the L-ASNase production. The medium contained (g/L): L-asparagine, 10; glucose, 2.0; $Na_2HPO_4.2H_2O$, 6.0; KH_2PO_4, 3.0; NaCl, 0.5; 1.0 M $MgSO_4.7H_2O$, 2.0 mL; and 0.1 M $CaCl_2.2H_2O$, 1.0 mL.

2.3. Molecular identification of the *Aspergillus* isolate

Following the procedure outlined by [22], DNA isolation of the *Aspergillus* isolate AUMC 16562 was carried out. The internal transcribed spacer (ITS) region was amplified using the universal primers ITS1 and ITS4 [23]. PCR procedures were carried out at SolGent Company (Yuseong-Gu, Daejeon, South Korea) according to Al-Bedak O and Moubasher A [24]. ITS sequence of the *Aspergillus* isolate AUMC 16562 in this study along with the most similar ITS sequences of *Aspergillus* species in GenBank were

aligned by MAFFT (version 6.861b) [25]. *Aspergillus niger* ATCC 16888 served as an outgroup. BMGE (Block Mapping and Gathering with Entropy) [26] optimized alignment gaps and parsimony uninformative characters. MEGA X (version 10.2.6) conducted the Maximum-likelihood (ML) and Maximum parsimony (MP) analyses [27], using a heuristic search of 1000 replications [28]. Akaike information criterion (AIC) as implemented in Modeltest 3.7 selected the best model of nucleotide substitution [29].

2.4. L-ASNase assay

Enzyme assay was done by Nesslerization method [30]. Absorbance was measured at 480 nm, and the amount of released ammonia was determined using ammonium sulfate as standard. One unit of L-ASNase is defined as the amount of enzyme that liberates 1 µmol of ammonia at the standard assay conditions (Equation 1).

$$\textbf{L-ASNase activity} = \frac{\text{Absorbance} \times 1000 \times \text{Enzyme DF}}{\text{Slope} \times \text{M.Wt.} \times \text{Time}} \text{ U/mL} \qquad \textbf{(1)}$$

Where: DF = dilution factor for the enzyme. M.wt. = molecular weight of asparagine. The concentration of proteins was determined according to the Lowry's method [31, 32]. Bovine serum albumin standard curve was used to estimate the protein concentrations.

2.5. Optimization of fermentation parameters

Using one factor at time (OFAT), the maximization of L-ASNase production was studied at various pH values (4-10), nitrogen sources (ammonium chloride, ammonium sulphate, sodium nitrate, sodium nitrite, urea, peptone, and yeast extract; each at 0.2%), incubation temperatures (20, 25, 30, 35, 40, 45, and 50 °C), and fermentation period (1-8 days). The enzyme assay was carried out as previously described, and the optimum parameters were chosen for enzyme production.

2.6. Purification of L-ASNase

2.6.1. Ethanol precipitation

After the incubation period, cell-free supernatant was recovered by centrifuging at 10,000 rpm for 10 min at 4 ºC. Cold absolute ethyl alcohol (-25 ºC) was employed in triple volume to isolate the enzyme at 4 °C. The isolated protein was dissolved in Tris buffer (pH 8.0), dialyzed twice for two hours at room temperature (cutoffs: 12-14 kDa), and then cooled overnight at 4 °C to remove salts and other small molecules.

2.6.2. DEAE-cellulose ion exchange column

Diethylaminoethyl cellulose (DEAE-cellulose) anion exchanger column (60 cm × 2.2 cm; bed volume 100 cm^3) was activated by 0.5 M NaOH for 60 min. A 5.0 mL sample was placed onto the column after phosphate buffer (100 mM, pH 8.0) had been used to equilibrate it. The column flow rate was adjusted at 0.25 mL/min and the fractions had a 5.0 mL volume. The previously mentioned method was used to measure the L-ASNase

activity. The most active fractions were combined, concentrated, and stored for further purification.

2.6.3. Sephacryl S 200 HR gel filtration column

In a glass column (65 cm × 2.5 cm; bed volume 120 cm^3), Sephacryl S 200 HR gel was packed. The protein was eluted using phosphate buffer (100 mM, pH 8.0) after this column was loaded with the concentrated enzyme sample. The L-ASNase activity was evaluated using the mentioned method in fractions of 5.0 mL. The most active fractions were pooled, concentrated, and lyophilized.

2.6.4. Formulas for determining purification profile parameters

The following equations were used to calculate the purification fold and the yield (%) in purification profile of L-ASNase:

$$\text{Purification Fold} = \frac{\text{Specific Activity of crude extract (U/mg)}}{\text{Specific Activity at a given step (U/mg)}} \quad \textbf{(2)}$$

$$\text{Yield (\%)} = \frac{\text{Total Activity at a given step (U)}}{\text{Total Activity of crude extract (U)}} \times 100 \quad \textbf{(3)}$$

These formulas were adapted according to the standard methods described by Scopes 2013 [33].

2.7. Determination of L-asparaginase molecular weight using SDS-PAGE

Whole cell lysates were produced by suspending the cells in 100 µL of lysis buffer (20 mM Tris/HCl, pH 7.4) (Invitrogen, USA), which included 4.0 % sodium dodecyl sulfate (SDS), 20 % glycerol, 10 % 2-mercaptoethanol, and 0.0025% bromophenol blue (tracking dye). A 12% SDS polyacrylamide gel was loaded with the entire cell lysates, which were then cooked for five minutes at 100 °C and run at 100 mA and 150 V for 45 min. Coomassie brilliant blue dye R 250 was used to stain the proteins bands. Once the unbound dye was removed from the gel and the stained proteins were visible as blue bands, the gel was placed into a de-staining solution. Quantity One Software (Version 4.6.2) was then used to analyze the gel.2.8. Impact of pH, temperature and metal ions on the activity of pure L-asparaginase

The effect of pH (3.0–11.0) on the activity of pure L-ASNase was determined at temperatures of 21, 24, 27, 30, 33, 36, 39, 42, and 45 °C. Citrate buffer (pH 3–6), Phosphate buffer (pH 7–8) and Glycine-NaOH buffer (pH 9–11) were utilized, each buffer was prepared at a concentration of 0.1 M to ensure consistent ionic strength across the pH range. Enzyme activity was measured after allowing the enzyme to equilibrate in the respective buffers, and all experiments were conducted at the optimal temperature to

minimize external variability. The reaction was started by adding 0.01 g of enzyme powder with 0.01 g of L-asparagine (each was separately dissolved in 1.0 mL of buffer solution at the desired pH values). After 30 min, the reaction was terminated by the addition of 2.0 mL of Trichloroacetic Acid (TCA). Furthermore, metal ions, such as NaCl, KCl, $CaCl_2$, $MgSO_4$, $MnSO_4$, $FeSO_4$, $CuSO_4$, $ZnSO_4$, $CoCl_2$, $CdCl_2$, and $BaCl_2$, were examined by adding them to a solution at a concentration of 5 mM. These metal ions included Na^+, K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+}, and Ba^{2+}. Moreover, enzyme chelation was examined via the use of 5 mM ethylenediaminetetraacetic acid (EDTA). To determine 100% activity, the activity of L-ASNase was measured under conventional reaction conditions without the presence of metal ions or EDTA. There were three runs of the experiment.

2.9. Determination of K_m and V_{max}

The Michaelis–Menten constant (k_m) and maximum reaction velocity (V_{max}) values of the pure L-ASNase were determined by measuring the enzyme activity at different concentrations of L-asparagine (2-20 mM) via a Line weaver–Burk plot [34] according to Equation (4).

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \times \frac{1}{S} \quad (4)$$

2.10. Amino acid analysis

Amino acid analysis was performed on the ethanol-precipitated fraction to confirm the presence of the target protein and guide subsequent purification steps. A validated standard was included, and the analysis confirmed that the amino acid composition of the sample matched that of the target protein, with no evidence of interference from unwanted proteins. This preliminary analysis provided valuable compositional information for optimizing the purification process. The final purified protein was subjected to additional validation to ensure data accuracy. The amino acid analysis and determination were carried out by the Chromatography Laboratory at the National Research Centre, Giza, Egypt, via the techniques outlined by [35], [36], and [37]. After 0.1 g of pure L-ASNase was mixed with 5.0 mL of water and 5.0 mL of 6 M HCl, the mixture was heated to 120 °C for 24 hours before being filtered. In the end, 1.0 mL of the filtrate was added to an Agilent 1260 series HPLC after being dried and suspended in 0.1 M HCl. An Eclipse Plus C18 column (4.6 mm × 250 mm; 5 µm diameter) was used for separation. The mobile phase was created by mixing a solvent mixture of acetone, methanol, and water (45:45:10) at a flow rate of 1.5 mL/min with sodium phosphate dibasic/sodium borate buffer (pH 8.2). A linear gradient was used to program the mobile phase in sequential order.

2.11. Cytotoxic effect of the pure L-asparaginase on human cell lines

2.11.1. MTT assay

The National Research Centre in Giza, Egypt's Bioassay-Cell Culture Laboratory carried out and analyzed an in vitro bioassay on human tumor cell lines. RPMI 1640 medium containing 1.0 % antibiotic-antimycotic combination (10,000 U/mL Penicillin G potassium, 10,000 µg/mL Streptomycin Sulfate, and 25 µg/mL Amphotericin B) and 1.0 % L-glutamine, was used to suspend HePG-2, PC-3, HCT-116 cell lines at 37 ºC under 5.0 % CO_2 conditions [38].Using a water jacketed carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA), cells were batch grown for 10 days before being seeded at a concentration of 10×10^3 cells/well in new complete growth medium using a 96-well microtitre plate at 37 ºC for 24 hours under 5% CO_2. The medium was aspirated after 48 hours of incubation, and 40 µL of MTT salt (2.5µg/mL) were added to each well. The wells were then incubated for an additional four hours at 37 ºC with 5% CO_2. A 100 µg/mL of Doxorubicin was employed as a positive control [39, 40]. Afterwards, using a microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA), the absorbance was measured at 595 nm to determine the IC_{50}. The % change in viability was calculated using Equation (5).

$$\% \text{ Cell viability} = \left[\frac{\text{Reading of sample}}{\text{Reading of negative control}}\right] - 1 \times 100 \quad \textbf{(5)}$$

2.11.2. Apoptosis assay

The apoptotic effect of the Aspergillus L-ASNase was examined on HCT-116, HepG-2 and PC-3 cells using Annexin V-FITC/PI-PE double-staining assay. Determination of the apoptotic effect of the enzyme was determined by obeying to the manufacturer's instructions [41, 42]. Accordingly, the cells (3×10^5 cells/well) were planted into a 6-well culture plate and exposed to the 25, 50 and 100 µg/mL of the enzyme for 48 h. After incubation, the collected cells in 1 mL of complete medium including fetal bovine serum (FBS, 1%). Afterward, the cell suspension (100 µL) was mixed with FITC AnnexinV/ propidium iodide and vortexed for 3-5 seconds. After incubation for 20 min at room temperature and monitoring by flow cytometry Analyzer. The flow cytometry results were represented with dot blot graphs. Untreated cells were used as control group cells.

2.12. *In vivo* cytotoxicity of the pure L-ASNase in mice

Six- to eight-week-old Swiss albino mice weighing approximately 50 g were used in the experiment. The mice were graciously donated by the Veterinary Teaching Hospital at Assiut University, Assiut Governorate, Egypt, where the experiment was conducted. Among the mice, there were two groups, each with ten mice. The first group was given 1X phosphate-buffered saline as a control. Two times a week, 500 IU/kg *Aspergillus* isolate AUMC 16562' L-ASNase was injected into the second group. Seven weeks were dedicated to the experiment. Blood samples were obtained 15, 30, and 45 days after the final injection. After extraction, the serum and plasma were stored at –40 °C. The

complete blood count and the levels of transaminases, albumin, total protein, urea, creatinine, and alkaline phosphatase were determined [43, 44]. Using universal biochemical keys, the measurements were carried out in the Clinical Chemistry and Hematology laboratories of the Clinical Pathology Department, South Egypt Cancer Institute, Assiut University, Assiut, Egypt, and served as toxicity markers.

2.13. Statistical analysis

The mean and standard deviation (SD) of the tentative study performed in triplicate were used to express all data. Analysis of the statistical significance was conducted according to Stahle and Wold [45].It was deemed significant at $p \leq 0.05$.

3. RESULTS

3.1. Molecular identification of the *Aspergillus* isolate AUMC 16562

The complete ITS dataset included 10 species. The maximum parsimony dataset comprised 622 characters, of which 527 were constant (without gaps or N), 57 were classified as variable, and 12 were deemed informative. The phylogenetic tree depicted the evolutionary relationship of *Aspergillus* isolate AUMC 16562 with other *Aspergillus* species in section Flavi, emphasizing its genetic similarities and distinctions. The strain in this study was situated alongside the type species of *Aspergillus aflatoxiformans* on the same branch and clustered with *A. flavus* ATCC 16883 and *A. oryzae* NRRL 447 within the same clade, demonstrating robust support values for ML (92%) and MP (93%). As a result, the strain in this study was identified as *A. aflatoxiformans* (Figure 2).

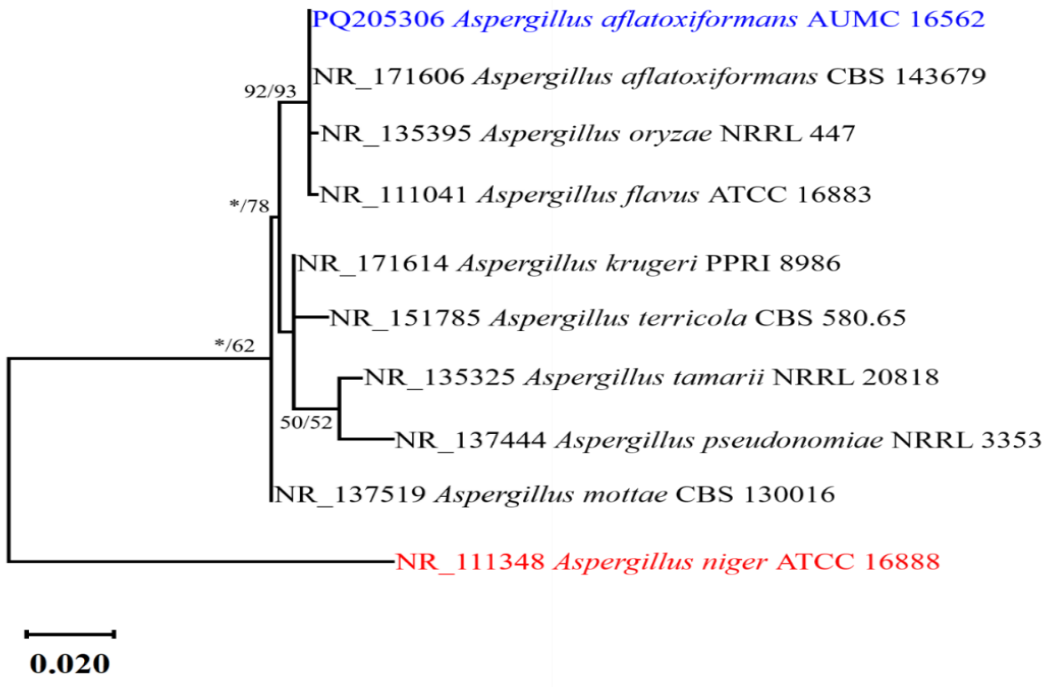


Figure 2. Maximum likelihood phylogenetic tree generated from ML/MP analysis via a heuristic search (1000 replications) of the ITS sequence of *Aspergillus aflatoxiformans* AUMC 16562 (in blue) compared with other closely related species to the genus *Aspergillus* in GenBank. Bootstrap support values for ML/MP ≥50% are indicated near the respective nodes. The tree is rooted to *A. niger* ATCC 16888 as an out group (in red).

3.2. Optimization of L-asparaginase production parameters

The current results revealed that *A. aflatoxiformans* AUMC 16562 presented the highest level of L-ASNase activity 69.29±3.6 U/mL at pH 8.0 when all nitrogen sources were removed from the fermentation medium, with the exception of asparagine. L-ASNase activity decreased when a nitrogen source was added to the fermentation medium; yeast extract was the most significantly effective source (p<0.05), resulting in 61.72±4.2 U/mL L-ASNase activity. L-ASNase activity significantly (p<0.05) increased to 79.11±8 U/mL after 7 days of incubation at 30 °C, and asparagine was the only nitrogen supply needed (Figure 3).

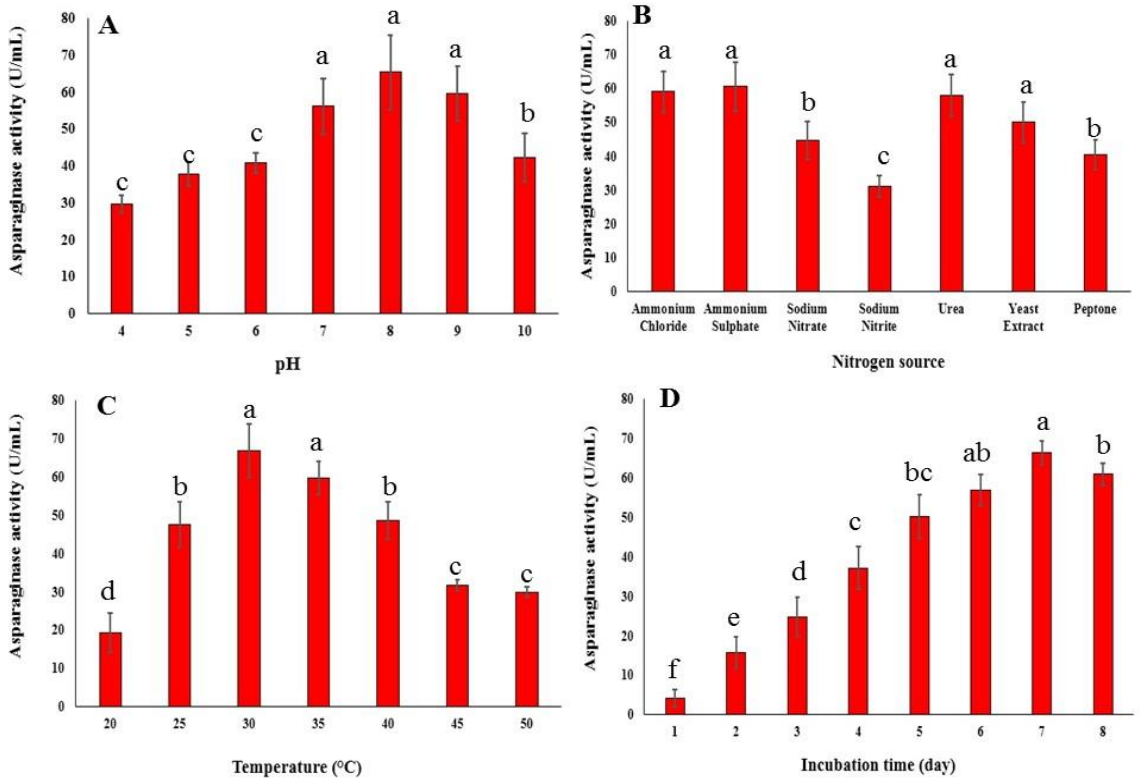


Figure 3. Effect of fermentation parameters on the activity of L-ASNase produced by *A. aflatoxiformans* AUMC 16562 in SmF: (**A**) pH of the medium, (**B**) nitrogen supply, (**C**) incubation temperature, and (**D**) incubation period (Means with different values between treatments are significantly different; p≤0.05).

3.3. Purification of L-asparaginase

A. aflatoxiformans produced L-ASNase after seven days of culture at pH 8.0 and 30 °C without a nitrogen source other than asparagine. The DEAE-cellulose column produced 48 pooled fractions with the greatest active L-ASNase and protein peaks. After the highest-activity fractions (25-73) from the DEAE-cellulose column were collected, they were further purified via a Sephacryl S-200 HR column. The most active L-ASNase components isolated from *A. aflatoxiformans* were purified via Sephacryl S-200 HR (Fractions no. 27-67) to yield two prominent broad peaks of L-ASNase and protein

activity. After two processing cycles, there was a 12.42-fold increase in the specific activity of the purified L-ASNase, resulting in a protein yield of 39.82% and a specific activity of 3668.9 U/mg (Table 1).

Table 1. Purification profile of L-ASNase produced by *A. aflatoxiformans* AUMC 16562.

Purification steps	Volume (mL)	Activity (U/mL)	Total activity (U)	Protein (mg/mL)	Total protein(mg)	Specific activity (U/mg)	Yield %	Fold
Fermentation media	1150	62.3	71645	0.211	242.65	295.26	100	1
Ethyl alcohol	215	158.5	34077.5	0.312	56.16	606.8	47.56	2.0
DEAE-cellulose	40	793.2	31728	0.283	11.32	2802.8	44.28	9.49
Sephacryl S 200 HR	25	1127.1	28177.5	0.256	7.68	3668.9	39.32	12.42

Table 1 presents the purification steps of an enzyme, showing changes in volume, activity, protein concentration, specific activity, yield, and purification fold. The initial fermentation media contained a total activity of 71,645 U and a specific activity of 295.26 U/mg. The purification process involved three major steps: ethanol precipitation, DEAE-cellulose ion exchange chromatography, and Sephacryl S-200 HR gel filtration. Ethanol precipitation reduced the total protein significantly while increasing the specific activity to 606.8 U/mg, achieving a 2.0-fold purification with 47.19% yield. The DEAE-cellulose step further enhanced purification, increasing specific activity to 2802.8 U/mg (9.49-fold) while maintaining 44.28% of the enzyme activity. Finally, Sephacryl S-200 HR gel filtration resulted in the highest specific activity of 3668.9 U/mg, achieving a 12.42-fold purification with a final yield of 39.82%. The data indicate effective purification with increasing enzyme purity but decreasing total yield, which is expected in multi-step purification processes.

3.4. SDS-PAGE

The SDS-PAGE examination demonstrated that the L-ASNase generated by *A. aflatoxiformans* AUMC 16562 was homogenous and totally purified. The calculated molecular weight was 40.5 kDa (Figure 4).

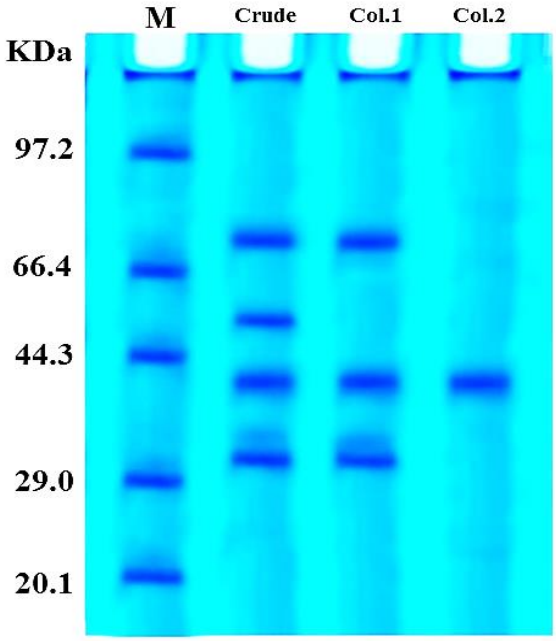


Figure 4. SDS-PAGE of L-ASNase produced by *A. aflatoxiformans* AUMC 16562. A: pre-stained marker. B: crude enzyme. C: pure L-ASNase.

3.5. Effects of metal ions on *A. aflatoxiformans* AUMC 16562' L-asparaginase

Among all the ions tested, K^+ had the greatest stimulatory effect, increasing to the top position, followed by Ca^{2+}, which exhibited substantial increases of 141.53±5.82% and 125.03±4.11%, respectively. The activity of L-ASNase was reduced by the presence of all the other tested ions, with residual activity levels ranging from 10.43±0.4% for Cd^{2+} to 123.93±6.39% for Na^+**,** indicating significant variation in the enzyme's tolerance to different metal ions (Table 2).

3.6. Effects of pH and temperature on the activity of pure L-asparaginase

The present study revealed that pure L-ASNase exhibited increased activity with the pH increase from 3 to 8, then it declined. pH 8.0 was identified as the optimal condition, achieving a peak activity of 4402.7±267 U/mg at 39 °C. (Figure 5).

3.7. Determination of kinetic parameters (K_m and V_{max}) and substrate specificity

K_m and V_{max} were determined using L-asparagine and L-glutamine as substrates at various concentrations (2-20 mM). The Line Weaver Burk plots indicated that the most appropriate substrate for *A. aflatoxiformans* AUMC 16562' L-ASNase was L-asparagine, which presented the highest affinity for the enzyme. The K_m and V_{max} values for L-asparagine and L-glutamine were determined to be 8.11×10^{-2} mM and 111.98 µmol/min , 10.23 mM and 52.32 µmol/min respectively (Table 3).

Table 2. Effects of metal ions and EDTA (5 mM) on the activity of pure L-ASNase produced by *A. aflatoxiformans* AUMC 16562 (mean±SD, n = 3). The residual activity (%) results are expressed as the proportion of the activity under the tested inhibitory conditions relative to the L-ASNase activity in the control without inhibitors.

Variables	Residual activity (%) (Mean±SD)	Specific activity (U/mg) (Mean±SD)	P value
Control	100.03±5.2^{d}	3620.23±218.5^{d}	**<0.001****
Na^{+}	123.93±6.39^{b}	4486.55±224.33^{b}	
K^{+}	141.53±5.82^{a}	5123.71±308.11^{a}	
Ca^{2+}	125.03±4.11^{b}	4526.37±223.3^{b}	
Mg^{2+}	61.57±3.82^{e}	2228.97±133.02^{e}	
Mn^{2+}	52.53±3.1^{ef}	1901.7±68.6^{f}	
Cu^{2+}	18.66±1.08^{i}	675.53±33.11^{i}	
Fe^{2+}	112.82±2.25^{c}	4084.34±268.23^{c}	
Zn^{2+}	34.27±2.41^{g}	1240.65±41.22^{g}	
Co^{2+}	48.4±2.33^{fg}	1752.19±58.32^{g}	
Cd^{2+}	10.43±0.4^{i}	377.58±12.035^{i}	
Ba^{2+}	22.67±1.33^{h}	820.7±36.72^{h}	
EDTA	99.81±5.1^{d}	3613.35±212.26^{d}	

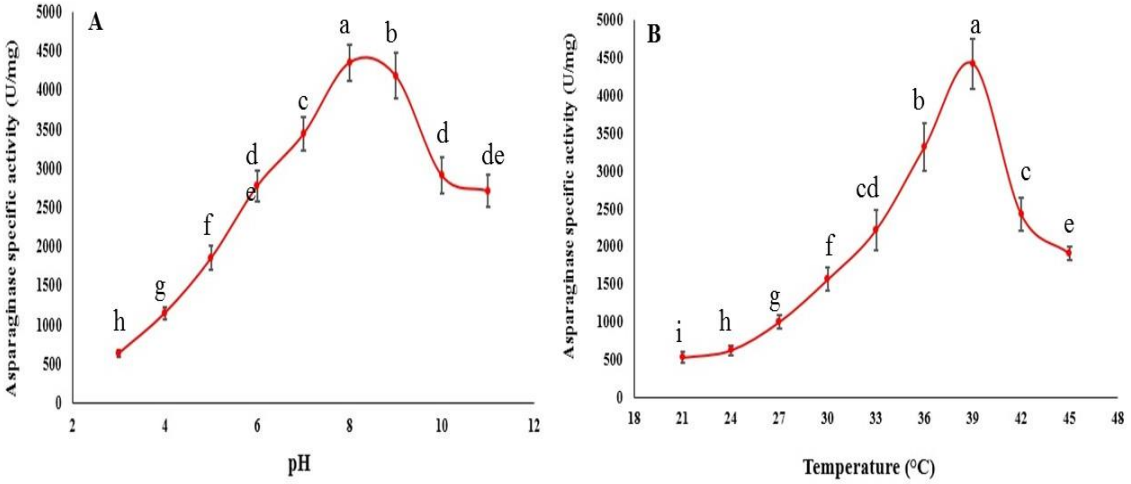


Figure 5. Effects of (**A**) pH and (**B**) temperature on the activity of pure L-ASNase produced by *A. aflatoxiformans* AUMC 16562 (Means with different values between treatments are significantly different; $p \leq 0.05$).

Table 3. Kinetic parameters related to the substrate specificity of L-asparaginase

Substrate	V_{max} **(µmol/min)**	K_m **(mM)**
L-asparagine	111.98	8.11×10^{-2}
L-glutamine	52.32	10.23

3.8. Amino acid analysis

Among the pure protein samples that were isolated following *A. aflatoxiformans* AUMC 16562 fermentation, fifteen amino acids (excluding cystine, methionine, and proline) were identified. The determined amino acids were present at different concentrations, with asparagine (10.72 mg/g) being the most abundant, followed by glutamine (4.41 mg/g), glycine (4.35 mg/g), and arginine (2.44 mg/g). The remaining amino acid concentrations ranged from 0.71 mg/g for histidine to 2.26 mg/g for leucine (Figure 6).

As a result, amino acid analysis of the L-ASNase enzyme is crucial, especially when considering its structure, function, and applications, particularly in medicine and biotechnology, as it helps to determine the amino acid composition of the L-ASNase enzyme, which is essential for understanding its molecular structure and helps to elucidate the primary structure of the enzyme.

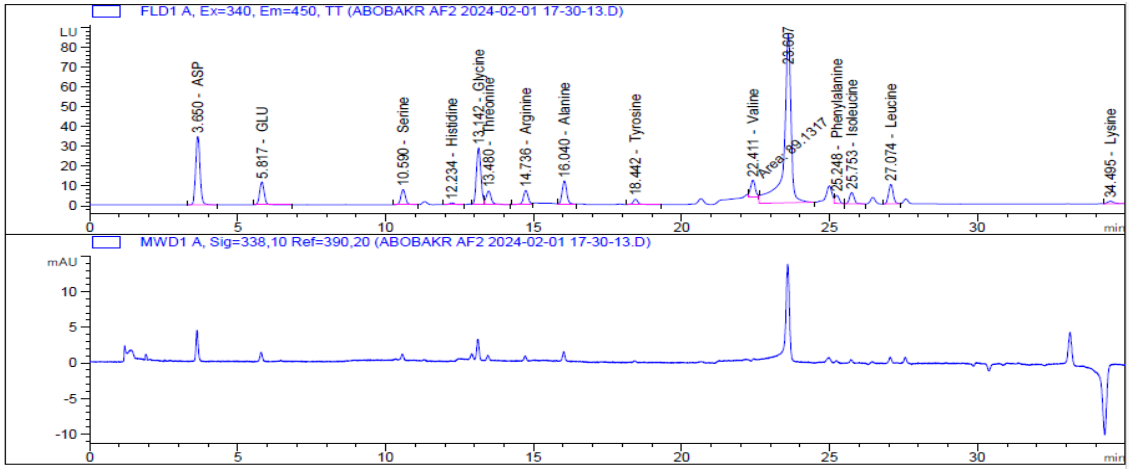


Figure 6. Chromatogram of the amino acid profile of the crude protein sample produced by *A. aflatoxiformans* AUMC 16562.

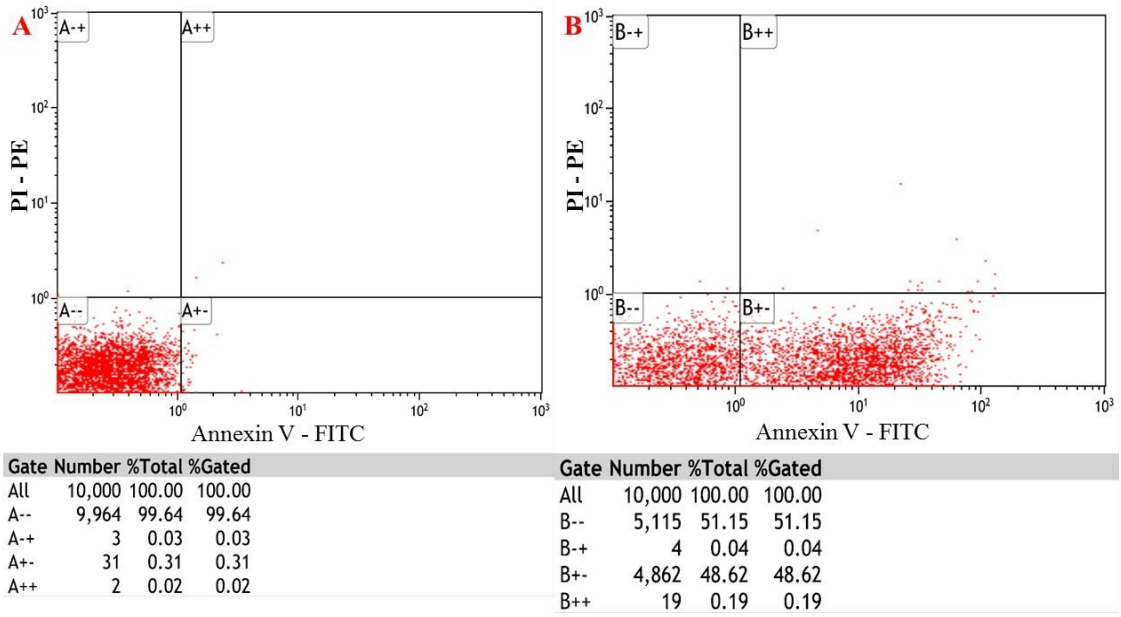
3.9. *In vitro* cytotoxic effect of pure L-asparaginase on HCT-116, HePG-2 and PC-3 human cell lines

In this experiment, HCT-116, HePG-2 and PC-3 were tested. Cell lines that had not been treated were used as the negative control; HCT-116 HePG-2 and PC-3 cells treated with 100 µg/mL of doxorubicin was served as positive control; and HCT-116, HePG-2 and PC-3 cells treated with IC_{50} of *A. aflatoxiformans*' L-ASNase. Cytotoxicity was significantly induced in HCT-116 cells with an IC_{50} of 48.2 µg/mL, Cytotoxicity was induced in HePG-2 cells with an IC_{50} of 65.2 µg/mL and Cytotoxicity was significantly induced in PC-3 cells with an IC_{50} of 66.9 µg/mL.

3.10. Flow cytometry results

To determine the apoptotic effect of *A. aflatoxiformans*' L-ASNase on HCT-116, HePG-2 and PC-3 cells, the cells were treated with the enzyme for 48 h and followed by Annexin V-FITC/PI-PE application. The data showed that L-ASNase increased the late apoptotic rate in HCT-116 cells from 0.02% to 83.37% (Figures. 7, 8). The effect of L-ASNase on HepG-2 cells, the results were analyzed by flow cytometry as shown in (Figures. 9, 10),

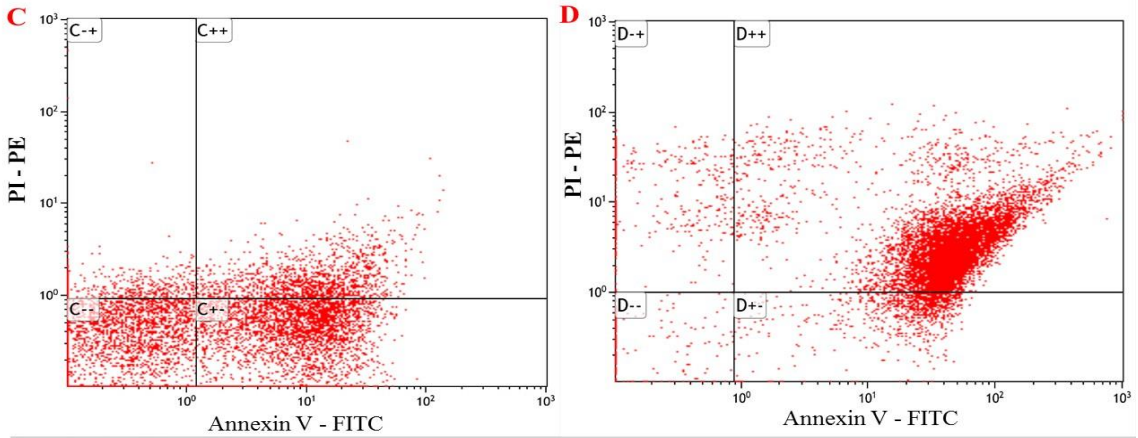
the enzyme increased the late apoptotic rate in HepG-2 cells from 0.10% to 35.01%. The results showed that L-ASNase increased the late apoptotic rate in PC-3 cells from 0.39% to 23.71% (Figures. 11, 12), compared to the control groups.



Gate	Number	%Total	%Gated
All	10,000	100.00	100.00
A--	9,964	99.64	99.64
A-+	3	0.03	0.03
A+-	31	0.31	0.31
A++	2	0.02	0.02

Gate	Number	%Total	%Gated
All	10,000	100.00	100.00
B--	5,115	51.15	51.15
B-+	4	0.04	0.04
B+-	4,862	48.62	48.62
B++	19	0.19	0.19

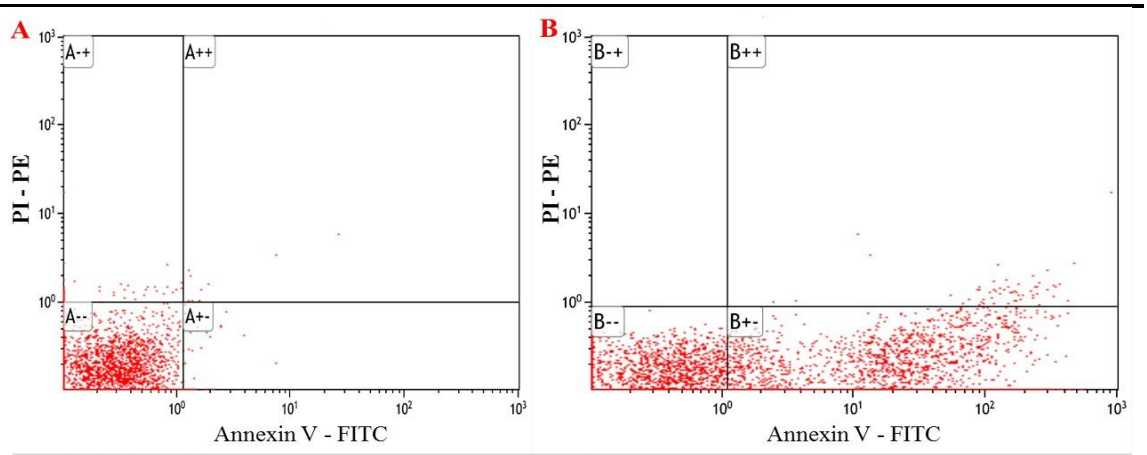
Figure 7. Pure L-ASNase of *Aspergillus aflatoxiformans* AUMC 16562 induce apoptosis, (**A**) Control, (**B**) HCT-116 cells were treated with 25 µg/mL of enzyme for 48 h, and apoptosis was determined by flow cytometry using Annexin V/PI double staining.



Gate	Number	%Total	%Gated
All	10,000	100.00	100.00
C--	4,563	45.63	45.63
C-+	617	6.17	6.17
C+-	3,451	34.51	34.51
C++	1,369	13.69	13.69

Gate	Number	%Total	%Gated
All	10,000	100.00	100.00
D--	258	2.58	2.58
D-+	433	4.33	4.33
D+-	972	9.72	9.72
D++	8,337	83.37	83.37

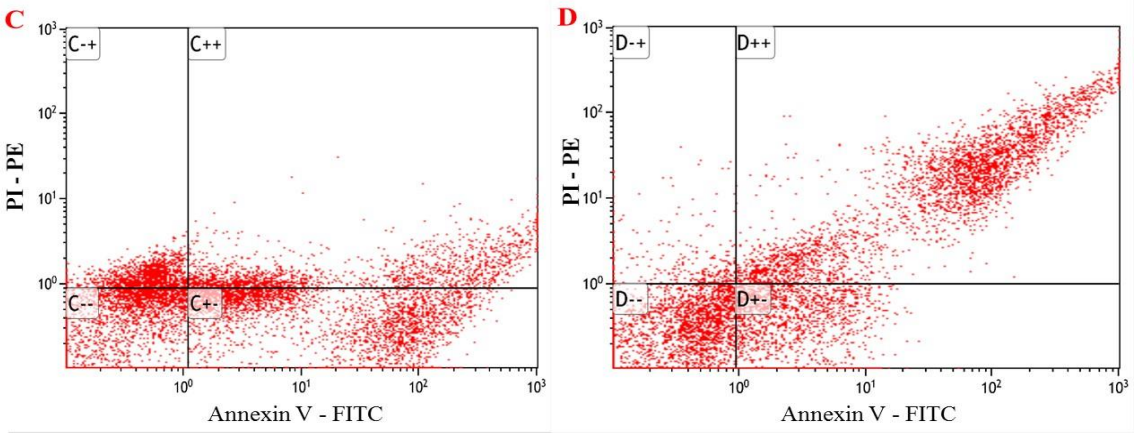
Figure 8. Pure L-ASNase of *Aspergillus aflatoxiformans* AUMC 16562 induce apoptosis, (**C**) HCT-116 cells were treated with 50 µg/mL of enzyme for 48 h, (**D**) HCT-116 cells were treated with 100 µg/mL of enzyme for 48 h, and apoptosis was determined by flow cytometry using Annexin V/PI double staining.



Gate	Number	%Total	%Gated
All	10,000	100.00	100.00
A--	9,899	98.99	98.99
A-+	39	0.39	0.39
A+-	52	0.52	0.52
A++	10	0.10	0.10

Gate	Number	%Total	%Gated
All	10,000	100.00	100.00
B--	6,361	63.61	63.61
B-+	0	0.00	0.00
B+-	3,582	35.82	35.82
B++	57	0.57	0.57

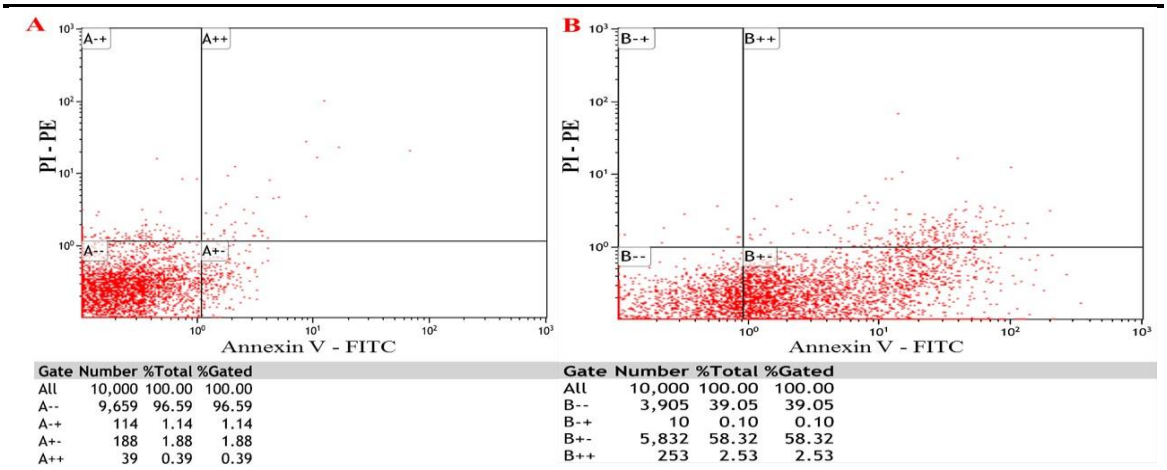
Figure 9. Pure L-ASNase of *Aspergillus aflatoxiformans* AUMC 16562 induce apoptosis, (**A**) Control, (**B**) HepG-2 cells were treated with 25 µg/mL of enzyme for 48 h, and apoptosis was determined by flow cytometry using Annexin V/PI double staining.



Gate	Number	%Total	%Gated
All	10,000	100.00	100.00
C--	3,511	35.11	35.11
C-+	1,443	14.43	14.43
C+-	3,260	32.60	32.60
C++	1,786	17.86	17.86

Gate	Number	%Total	%Gated
All	10,000	100.00	100.00
D--	4,422	44.22	44.22
D-+	286	2.86	2.86
D+-	1,791	17.91	17.91
D++	3,501	35.01	35.01

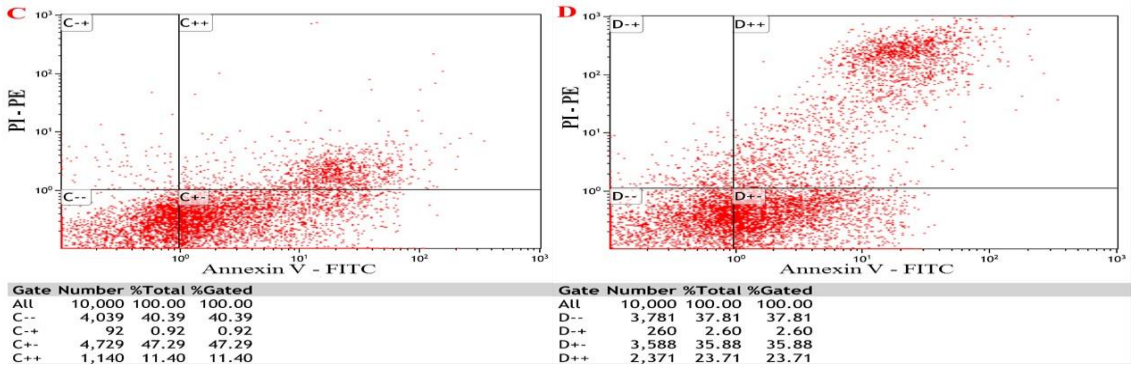
Figure 10. Pure L-ASNase of *Aspergillus aflatoxiformans* AUMC 16562 induce apoptosis, (**C**) HepG-2 cells were treated with 50 µg/mL of enzyme for 48 h, (**D**) HepG-2 cells were treated with 100 µg/mL of enzyme for 48 h, and apoptosis was determined by flow cytometry using Annexin V/PI double staining.



Gate	Number	%Total	%Gated
All	10,000	100.00	100.00
A--	9,659	96.59	96.59
A-+	114	1.14	1.14
A+-	188	1.88	1.88
A++	39	0.39	0.39

Gate	Number	%Total	%Gated
All	10,000	100.00	100.00
B--	3,905	39.05	39.05
B-+	10	0.10	0.10
B+-	5,832	58.32	58.32
B++	253	2.53	2.53

Figure 11. Pure L-ASNase of *Aspergillus aflatoxiformans* AUMC 16562 induce apoptosis, (**A**) Control, (**B**) PC-3 cells were treated with 25 µg/mL of enzyme for 48 h, and apoptosis was determined by flow cytometry using Annexin V/PI double staining.



Gate	Number	%Total	%Gated
All	10,000	100.00	100.00
C--	4,039	40.39	40.39
C-+	92	0.92	0.92
C+-	4,729	47.29	47.29
C++	1,140	11.40	11.40

Gate	Number	%Total	%Gated
All	10,000	100.00	100.00
D--	3,781	37.81	37.81
D-+	260	2.60	2.60
D+-	3,588	35.88	35.88
D++	2,371	23.71	23.71

Figure 12. Pure L-ASNase of *Aspergillus aflatoxiformans* AUMC 16562 induce apoptosis, (**C**) PC-3 cells were treated with 50 µg/mL of enzyme for 48 h, (**D**) PC-3 cells were treated with 100 µg/mL of enzyme for 48 h, and apoptosis was determined by flow cytometry using Annexin V/PI double staining.

Apoptosis study investigates the apoptotic effects of pure L-ASNase extracted from *Aspergillus aflatoxiformans* AUMC 16562 on three human cancer cell lines: HCT-116, HepG-2, and PC-3. Apoptosis was assessed using Annexin V-FITC/PI-PE double staining and analyzed by flow cytometry. HCT-116 cells in control group showed a minimal apoptotic rate of 0.02%, indicating very few cells were undergoing apoptosis under normal conditions.

Treatment with L-ASNase 25 µg/mL resulted in a marked increase in late apoptosis, with the apoptotic rate rising to 83.37%, highlighting the strong apoptotic effect of L-ASNase on these cells. Increasing the enzyme concentration to 50 µg/mL and 100 µg/mL continued to show significant induction of late apoptosis, confirming the dose-dependent apoptotic effects.But in HepG-2 cells, the control cells exhibited an initial apoptotic rate of 0.10%, indicating low spontaneous apoptosis in untreated cells. Treatment with L-ASNase 25 µg/mL caused a significant increase in apoptosis, raising the late apoptotic rate to 35.01%.Further treatment with 50 µg/mL and 100 µg/mL of L-ASNase enhanced

the late apoptotic rate, suggesting that the enzyme induces apoptosis in a concentration-dependent manner, though the effect is less potent than in HCT-116 cells.

In case of PC-3, the control group for PC-3 cells exhibited an apoptotic rate of 0.39%, similar to the baseline rates observed in HepG-2 cells. Treatment with L-ASNase 25 μg/mL increased the late apoptotic rate to 23.71%, indicating a moderate apoptotic response to the enzyme. Increasing the enzyme dose to 50 μg/mL and 100 μg/mL showed progressively higher apoptotic rates, reinforcing the dose-dependent action of L-ASNase on PC-3 cells.The results clearly demonstrate that L-ASNase from *Aspergillus aflatoxiformans* induces apoptosis in a dose-dependent manner across all three cell lines tested (HCT-116, HepG-2, and PC-3). The HCT-116 cells were the most sensitive, with apoptosis rates reaching over 83% at higher enzyme concentrations. HepG-2 and PC-3 cells also showed significant apoptosis, though to a lesser extent compared to HCT-116. These findings suggest that L-ASNase has a potential therapeutic effect in inducing apoptosis in cancer cells, with varying efficacy depending on the cell type.

3.11. *In vivo* cytotoxic effects of pure L-ASNase in an animal model

The biochemical profiles revealed no effects on glucose, other electrolytes, the liver, or the kidneys. On the other hand, the total bilirubin level and the activities of aspartate aminotransferase (AST) and alanine transaminase (ALT) were slightly elevated. These results suggested that *A. aflatoxiformans* AUMC 16562' L-ASNase had relatively little effect on liver function (Figure13) (liver impairment was most likely indicated by AST and ALT markers). All the hematological parameters were within normal ranges during the experiment; however, compared with those in the control group (pre-injection), the white blood cell (WBC), platelet, hemoglobin, and red blood cell counts were slightly lower after the injection of L-ASNase for 15 days. Additionally, during the experimental periods, the rats were alive (Figures 13-14). The treatment appears to induce liver stress, as indicated by consistently elevated AST, ALT, and bilirubin levels, potentially due to toxicity or metabolic overload. Changes in total protein and albumin levels suggest alterations in protein metabolism, with signs of recovery at 45 days. Renal function was mostly unaffected, with urea levels only increasing at 45 days, while creatinine remained stable, indicating no severe impairment. Hematological parameters showed only a slight decrease in RBC counts at 15 days, with no long-term effects. Overall, the data suggest early toxicity followed by potential adaptation, highlighting the need for further long-term safety evaluation.

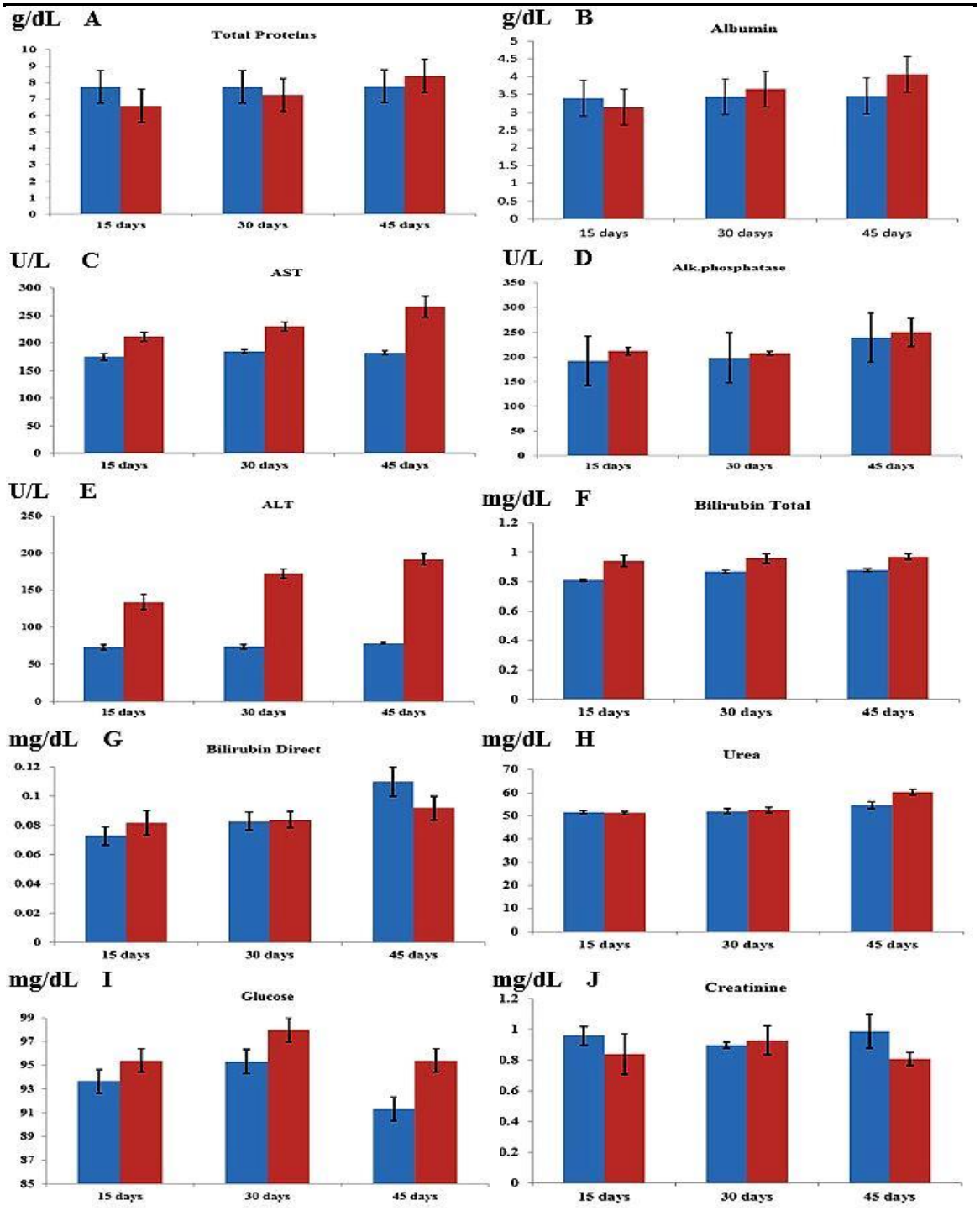


Figure 13. Biochemical parameters of the experimental and control animal models at 15, 30 and 45 days: (**A**) total protein, (**B**) albumin, (**C**) AST, (**D**) alkaline phosphatase, (**E**) ALT, (**F**) total bilirubin, (**G**) bilirubin direct, (**H**) urea, (**I**) glucose, and (**J**) creatinine.

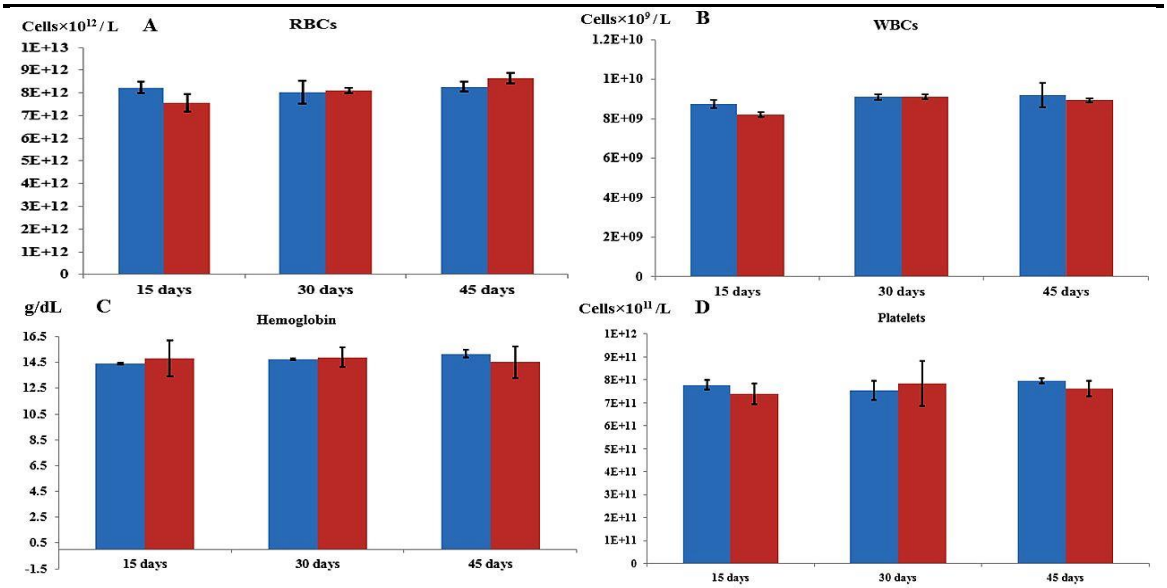


Figure 14. Hematological parameters of the experimental and control animal models at 15, 30 and 45 days: (**A**) RBCs, (**B**) WBCs, (**C**) hemoglbin and (**D**) platelets

4. DISCUSSION

L-ASNase is a chemotherapeutic agent that is used to treat a range of lymphoproliferative disorders and lymphomas, including ALL. For nearly 30 years, it has been a backbone of combination chemotherapy protocols used in the treatment of pediatric ALL [17]. Because they are simpler to cultivate and easier to extract and purify L-ASNase from, microbes are thought to be a better source of the enzyme, allowing for large-scale production [46]. For pharmaceutical purposes, L-ASNase is acquired from *E. coli* and *E. carotovora* [15]. Nonetheless, allergic reactions could be triggered on by the bacterial source of this enzyme [47].

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has declared that fungal L-ASNase s are considered safe; this has been the case since 2007. However, the amount of L-ASNase produced does not match demand, thus new methods to boost yield, like the use of statistical tools, must be developed [48].

In previous studies, several *Fusarium* spp. isolated from either plants, soil and marine algae were found to produce L-ASNase [49]. An isolate of *F. proliferatum* was found to have the highest L-ASNase enzyme activity in a research that evaluated the L-ASNase activity of 84 distinct fungal endophytes [50, 51]. Previous research investigated the L-ASNase produced by *F. solani* along with the purification and characterization of the enzyme to obtain maximum yield [52-54]. The characterization and optimization of production conditions were proved in many studies to possess significant impact on the enzyme production [53, 55, 56]. In this study, experimental L-ASNase activity was analyzed to investigate the effect of various factors including: incubation period, incubation temperature, the effect of using different nitrogen sources as well as different pH values on production of L-ASNase by *A. aflatoxiformans* AUMC 16562.

Since enzymes have an inherent fragile nature, the difference between their native and denatured structures limits their applicability. In the industrial field, enzymes that are stable throughout a wide range of temperatures are of great demand. Our findings showed

that the enzyme was stable at 39 °C, and it maintained up to 54% of its activity at 42 °C. Additionally, the activity of the enzyme preparation from *A. aflatoxiformans* AUMC 16562 increased gradually over temperatures ranging from 20 to 50 °C showing maximum activity at 30 °C. However, the activity declined dramatically at 45 °C where it achieved 24%. These findings came in agreement with the previously published study on *B. licheniformis* where the activity of the test isolate's enzyme preparation increased gradually from 20 to 35 °C and then decreased by approximately 27.7% at 45 °C, Its activity peaked at 40 °C [57]. Similar findings were obtained with *Streptomyces gulbargensis* where the enzyme was observed to be maximally active at 40 °C [58]. Almost all isolated and purified L-ASNase from microbial sources were reported to operate in a basic medium with pH ranging from 8 to 10, whereas, any further increase in the alkalinity of the medium will lead to a dramatic decrease in enzyme activity [59, 60]. The optimum alkaline pH of the enzyme is attributed to the fact that the aspartate liberated by asparagine hydrolysis has lower affinity to the active catalytic site of the enzyme. This allows more asparagine to bind to the enzyme. At acidic pH, however, the enzyme's breakdown of asparagine results in the production of aspartic acid, which has a high affinity for the enzyme's catalytic site, preventing asparagine from binding to the enzyme [57]. These reported data confirmed our results where the best enzymatic activity was achieved at alkaline pH (pH 9) and any further increase in the alkalinity of the medium caused a decline in the enzyme activity. Similar results were obtained in *A. fumigatus* [19], *Phaseolus vulgaris* [61], while in other *Fusarium* sp. the maximum activity was achieved at pH 9 [62].

On analyzing the results obtained from studying the optimum incubation time for L-ASNase production by *A. aflatoxiformans* AUMC 16562, differences in enzyme level produced were observed with different incubation periods. Quantitative analysis of the enzyme showed that its peak activity was achieved on the 7^{th} day of incubation. Incubation beyond that time period results in a decline in enzyme production, which might be caused by the inactivation of the enzyme due to the presence of proteolytic activity or by the organism's growth reaching a point where it is no longer able to maintain a stable growth in balance with the availability of nutrient resources. The enzyme activity declined with extended incubation times, which may be related to nutritional depletion or accumulation of hazardous end products [63].

Short incubation periods are generally preferred for commercial enzyme manufacture as they are more economical and reduce the decomposition liability of the produced enzyme [57]. Similar results were obtained from a study conducted on *F. solani*, where five days was the ideal incubation period [64]. Microorganisms produced L-ASNase at different incubation periods, as revealed by studies on *A. terreus* and *A. niger* [63] which indicated that incubation beyond 96 h showed a decline in enzyme production, 48 h for *E. nidulans* [65] and 120 h for *Fusarium* spp. [66].Studying the effect of incubation temperature is considered one of the most significant factors affecting the enzyme productivity for

maximum yield. The significance of the incubation temperature is due to the fact that it could determine the effects of inhibition, cell viability and death. Because it controls microbial proliferation and subsequently enzyme secretion, it is regarded as a crucial environmental element for L-ASNase synthesis by microbes. The incubation temperature assay was done at different temperatures viz., 25, 30, 35, 40, 45 and 50 °C to examine their effect on enzyme production. Results revealed that the incubation temperature had a major impact on the production of L-ASNase with the maximum enzyme yield occurring at 30 °C.

Higher temperatures showed a decline in enzyme production until it completely stopped at 50 °C. This could be attributed to the deceleration in the microorganism's metabolic activity as previously mentioned by [60]. Similar findings were obtained from a study on *F. solani* where maximum enzyme productivity was obtained at 30 °C [64]. Another study examining the enzyme obtained from *Fusarium* sp. also reported the optimum temperature to be between 30 and 40 °C for L-ASNase activity, whereas higher temperatures resulted in a reduction in enzyme activity, and the activity completely vanished at a temperature from 50 to 60 °C [59]. Our results were also in agreement with other studies done on various microorganisms, where [63] proved that the optimal incubation temperature for *A. terreus* was 30 °C and the enzyme production decreased gradually with additional rise in incubation temperature. Similar findings were observed in *E. nidulans* [65], *Bacillus aryabhattai* [67], and *F. equiseti* [55] studies suggesting that the decline in enzyme production was caused by improper enzyme molecule conformation brought on by the denaturation of mesophilic enzymes at higher temperatures.

It is well known that L-ASNase is a very effective antileukemic agent and for most patients it is usually administered once every two weeks [68-70]. The relative selectivity with regard to metabolism of malignant tumor cell forces us to look for novel L-ASNase compared to existing enzymes [68]. On investigating the *in vitro* antiproliferative activity of L-ASNase on malignant and normal cells, results revealed that the carcinoma cell death percentage reached against HePG-2 with IC_{50} of 65.2 µg/mL, HCT-116 with IC_{50} of 48.2 µg/mL, and PC-3 with IC_{50} of 68.9 µg/mL. Meanwhile, the normal cell death percentage upon treatment with high enzyme concentrations did not exceed 25% with IC_{50} value greater than 300 µg/mL.

These results came in agreement with several previous studies indicating that L-ASNase enzyme obtained from different *Fusarium* spp. showed selective toxicity against various malignant cells while having low toxicity on normal cells. In 2018, a study reported that cell death percentage of carcinoma cell lines was reached between 70 and 80% after treatment with the crude L-ASNase of either *A. sydowii* or *F. oxysporum*, respectively [68]. Purified L-ASNase from *F. equiseti* was isolated in a different study, and demonstrated promising anti-proliferative activity towards different malignant cell lines including: cervical epitheloid carcinoma (Hela), epidermoid larynx carcinoma (Hep-2),

hepatocellular carcinoma (HepG-2), colorectal carcinoma (HCT-116), in addition to breast adenocarcinoma (MCF-7), with IC_{50} values of 2.0, 5.0, 12.40, 8.26 and 22.8 µg/mL, respectively. The enzyme exhibited less cytotoxicity towards normal cells *in vivo* and greater activity, selectivity, and anti-proliferative activity in a dose-dependent manner towards malignant cells [55].

A recent study investigating the cytotoxic influence of L-ASNase from *Fusarium* sp. against RAW2674 leukemic cell lines also revealed antileukemic activity with IC_{50} of 50.1 U/mL [59]. The enzyme's selectivity for malignant cells is primarily due to their high reliance on L-ASNase to maintain malignant growth, as it lacks L-asparagine synthetase. Meanwhile, normal cells were not affected as they were able to synthesize L-asparagine with the aid of L-asparagine synthetase which was present in sufficient amounts [17, 59].

Limitations

The present study has several limitations. There is no structural characterization (such as X-ray crystallography or stability assays) was undertaken. The purification yield was only moderate (~39.8%), indicating potential challenges for large-scale production, and the investigation was limited to a single fungal isolate without comparison to alternative sources. Enzymatic kinetics were studied only with L-asparagine and L-glutamine as substrates, with no exploration of other potential substrates or inhibitors, and the optimization of production was carried out using a one-factor-at-a-time approach rather than more comprehensive statistical models. Finally, cytotoxic effects were evaluated against only three cancer cell lines and apoptosis induction was monitored solely by Annexin V/PI staining without deeper mechanistic analysis.

5. CONCLUSION

In conclusion, endophytic fungus, *A. aflatoxiformans* AUMC 16562, was revealed to be a successful source for production of L-ASNase. Maximization of enzyme production was achieved under suitable nutritional and environmental conditions. To find its path for use in the realm of medicine, more tests are required.

Ethics statement

The animal study was reviewed and approved by the Research Ethical Committee of the Molecular Biology Research and Studies Institute (IORG0010947-MB-21-35-R), Assiut University, Assiut, Egypt. All the experiments were performed in accordance with the relevant guidelines and regulations.

Authors' contributions

AAS and HME: conceptualization; **HME, AAS and AME**: methodology; **AAS, AME** and **RME**: visualization and investigation; **AAS, OAMA, HME, and RM**: data curation and writing, original draft preparation. All authors: final draft writing, reviewing and editing.

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Competing interest

The authors declare that they have no competing interests.

6. REFERENCES

[1] Zuo S, Zhang T, Jiang B, Mu W, 2015. Recent research progress on microbial L-asparaginases. Applied microbiology and biotechnology. 99:1069-1079.

[2] Kumar K, Kataria M, Verma N, 2013. Plant asparaginase-based asparagine biosensor for leukemia. Artificial Cells, Nanomedicine, and Biotechnology. 41:184-188.

[3] Vala AK, Sachaniya B, Dudhagara D, Panseriya HZ, Gosai H, Rawal R, Dave BP, 2018. Characterization of L-asparaginase from marine-derived *Aspergillus niger* AKV-MKBU, its antiproliferative activity and bench scale production using industrial waste. International journal of biological macromolecules. 108:41-46.

[4] Emadi A, Law JY, Strovel ET, Lapidus RG, Jeng LJ, Lee M, Blitzer MG, Carter-Cooper BA, Sewell D, Van Der Merwe I, 2018. Asparaginase *Erwinia chrysanthemi* effectively depletes plasma glutamine in adult patients with relapsed/refractory acute myeloid leukemia. Cancer Chemotherapy and Pharmacology. 81:217-222.

[5] Muneer F, Siddique MH, Azeem F, Rasul I, Muzammil S, Zubair M, Afzal M, Nadeem H, 2020. Microbial L-asparaginase: purification, characterization and applications. Archives of Microbiology. 202:967-981.

[6] Wang Y, Xu W, Wu H, Zhang W, Guang C, Mu W, 2021. Microbial production, molecular modification, and practical application of L-asparaginase: A review. International Journal of Biological Macromolecules. 186:975-983.

[7] Rabelo NG, Simões LA, Fernandes NdAT, Souza AC, Fernandes MLP, Veríssimo LAA, Schwan RF, Dias DR, 2025. Optimization of L-Asparaginase Production from *Aspergillus caespitosus*: Solid-State and Submerged Fermentation Using Low-Cost Substrates and Partial Purification. Applied Microbiology. 5:19.

[8] Srinivasan N, Thangavelu K, Sekar A, Sanjeev B, Uthandi S, 2021. *Aspergillus caespitosus* ASEF14, an oleaginous fungus as a potential candidate for biodiesel production using sago processing wastewater (SWW). Microbial Cell Factories. 20:1-23.

[9] Guimarães L, Júnior A, Jorge J, Terenzi H, Polizeli M, 2007. Purification and biochemical characterization of a mycelial alkaline phosphatase without DNAase activity produced by *Aspergillus caespitosus*. Folia microbiologica. 52:231-236.

[10] Kasirajan L, Kamaraj K, Maupin-Furlow JA, Uthandi S, 2024. Isolation, purification, and identification of novel lignin-degrading *Aspergillus caespitosus* strain S2. Biomass Conversion and Biorefinery. 14:28685-28699.

[11] Alegre ACP, Polizeli MdLTdM, Terenzi HF, Jorge JA, Guimarães LHS, 2009. Production of thermostable invertases by *Aspergillus caespitosus* under submerged or

solid state fermentation using agroindustrial residues as carbon source. Brazilian Journal of Microbiology. 40:612-622.

[12] Vimal A, Kumar A, 2017. Biotechnological production and practical application of L-asparaginase enzyme. Biotechnol Genet Eng Rev. 33:40-61.

[13] Fernandes MLP, Veríssimo LAA, de Souza AC, Schwan RF, Dias DR, 2021. Low-cost agro-industrial sources as a substrate for the production of L-asparaginase using filamentous fungi. Biocatalysis and Agricultural Biotechnology. 34:102037.

[14] Rodrigues D, Pillaca-Pullo O, Torres-Obreque K, Flores-Santos J, Sánchez-Moguel I, Pimenta MV, Basi T, Converti A, Lopes AM, Monteiro G, 2019. Fed-batch production of *Saccharomyces cerevisiae* L-asparaginase II by recombinant *Pichia pastoris* MUT s strain. Frontiers in bioengineering and biotechnology. 7:16.

[15] Cachumba JJM, Antunes FAF, Peres GFD, Brumano LP, Santos JCD, Silva SSD, 2016. Current applications and different approaches for microbial L-asparaginase production. Brazilian journal of microbiology. 47:77-85.

[16] Deshpande N, Choubey P, Agashe M, 2014. Studies on Optimization of Growth Parameters for L-asparaginase Production by *Streptomyces ginsengisoli.* The Scientific World Journal. 2014:895167.

[17] Narta UK, Kanwar SS, Azmi W, 2007. Pharmacological and clinical evaluation of L-asparaginase in the treatment of leukemia. Critical reviews in oncology/hematology. 61:208-221.

[18] Alam S, Pranaw K, Tiwari R, Khare SK, 2019. Recent development in the uses of asparaginase as food enzyme. Green bio-processes: enzymes in industrial food processing:55-81.

[19] Benchamin D, Sreejai R, Sujitha S, Albert C, Rishad K, 2019. Anti-proliferative activity of L-asparaginase enzyme from fungi on breast cancer. Journal of Pharmacognosy and Phytochemistry. 8:407-410.

[20] Abdel-Sater M, Abdel-Latif A, Abdel-Wahab D, Al-Bedak O, 2021. Endophytic mycobiota of wild medicinal plants from New Valley Governorate, Egypt and quantitative assessment of their cell wall degrading enzymes. Studies in Fungi. 6:78-91.

[21] Gulati R, Saxena R, Gupta R, 1997. A rapid plate assay for screening L-asparaginase producing micro-organisms. Letters in applied microbiology. 24:23-26.

[22] Moubasher A, Ismail M, Al-Bedak O, Mohamed R, 2019. *Ramophialophora chlamydospora*, a new species from an alkaline lake of Wadi-El-Natron, Egypt. Asian Journal of Mycology. 2:110-117.

[23] White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications. 18:315-322.

[24] Al-Bedak O, Moubasher A, 2020. Aspergillus gaarensis, a new addition to section Circumdati from soil of Lake El-Gaar in Wadi-El-Natron, Egypt. Studies in Fungi. 5:59-65.

[25] Katoh K, Standley DM, 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Molecular biology and evolution. 30:772-780.

[26] Criscuolo A, Gribaldo S, 2010. BMGE (Block Mapping and Gathering with Entropy): a new software for selection of phylogenetic informative regions from multiple sequence alignments. BMC evolutionary biology. 10:210.

[27] Kumar S, Stecher G, Li M, Knyaz C, Tamura K, 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular biology and evolution. 35:1547.

[28] Felsenstein J, 1985. Confidence limits on phylogenies: an approach using the bootstrap. evolution. 39:783-791.

[29] Posada D, Crandall KA, 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics (Oxford, England). 14:817-818.

[30] Imada A, Igarasi S, Nakahama K, Isono M, 1973. Asparaginase and glutaminase activities of micro-organisms. Microbiology. 76:85-99.

[31] Lowry O, Rosebrough N, Farr AL, Randall R, 1951. Protein measurement with the Folin phenol reagent. Journal of biological chemistry. 193:265-275.

[32] Waterborg JH, 2009. The Lowry method for protein quantitation. The protein protocols handbook:7-10.

[33] Scopes RK, 2013. Protein purification: principles and practice. Springer Science & Business Media. 3rd Edition.

[34] Lineweaver H, Burk D, 1934. The determination of enzyme dissociation constants. Journal of the American chemical society. 56:658-666.

[35] Campanella L, Russo MV, Avino P, 2002. Free and total amino acid composition in blue-green algae. Ann Chim. 92:343-352.

[36] Laurens LM, Dempster TA, Jones HD, Wolfrum EJ, Van Wychen S, McAllister JS, Rencenberger M, Parchert KJ, Gloe LM, 2012. Algal biomass constituent analysis: method uncertainties and investigation of the underlying measuring chemistries. Analytical chemistry. 84:1879-1887.

[37] Jajić I, Krstović S, Glamočić D, Jakšić S, Abramović B, 2013. Validation of an HPLC method for the determination of amino acids in feed. Journal of the Serbian Chemical Society. 78:839-850.

[38] Mosmann T, 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods. 65:55-63.

[39] Thabrew MI, Hughes RD, McFarlane IG, 1997. Screening of hepatoprotective plant components using a HepG-2 cell cytotoxicity assay. Journal of pharmacy and pharmacology. 49:1132-1135.

[40] El-Menshawi BS, Fayad W, Mahmoud K, El-Hallouty SM, El-Manawaty M, 2010. Screening of natural products for therapeutic activity against solid tumors.

[41] Altay A, Caglar S, Caglar B, Sahin ZS, 2019. Novel silver (I) complexes bearing mefenamic acid and pyridine derivatives: Synthesis, chemical characterization and in vitro anticancer evaluation. Inorganica Chimica Acta. 493:61-71.

[42] Kalın ŞN, Altay A, Budak H, 2022. Diffractaic acid, a novel TrxR1 inhibitor, induces cytotoxicity, apoptosis, and antimigration in human breast cancer cells. Chemico-Biological Interactions. 361:109984.

[43] El-Naggar NE-A, Deraz SF, El-Ewasy SM, Suddek GM, 2018. Purification, characterization and immunogenicity assessment of glutaminase free L-asparaginase from *Streptomyces brollosae* NEAE-115. BMC Pharmacology and Toxicology. 19:1-15.

[44] Rodrigues MA, Pimenta MV, Costa IM, Zenatti PP, Migita NA, Yunes JA, Rangel-Yagui CO, de Sa MM, Pessoa A, Costa-Silva TA, 2020. Influence of lysosomal protease sensitivity in the immunogenicity of the antitumor biopharmaceutical asparaginase. Biochemical Pharmacology. 182:114230.

[45] St L, Wold S, 1990. Multivariate analysis of variance (MANOVA). Chemometrics and Intelligent Laboratory Systems. 9:127-141.

[46] Asthana N, Azmi W, 2003. Microbial L-asparaginase: A potent antitumour enzyme.

[47] Qeshmi FI, Homaei A, Fernandes P, Javadpour S, 2018. Marine microbial L-asparaginase: biochemistry, molecular approaches and applications in tumor therapy and in food industry. Microbiological Research. 208:99-112.

[48] da Cunha MC, dos Santos Aguilar JG, de Melo RR, Nagamatsu ST, Ali F, de Castro RJS, Sato HH, 2019. Fungal L-asparaginase: Strategies for production and food applications. Food Research International. 126:108658.

[49] Manasa C, Nalini MS, 2014. L-Asparaginase Activity of Fungal Endophytes from *Tabernaemontana heyneana* Wall.(*Apocynaceae*), Endemic to the Western Ghats (India). International scholarly research notices. 2014:925131.

[50] Balbool BA, Abdel-Azeem AM, 2020. Diversity of the culturable endophytic fungi producing L-asparaginase in arid Sinai, Egypt. Italian Journal of Mycology. 49:8-24.

[51] Hatamzadeh S, Rahnama K, Nasrollahnejad S, Fotouhifar KB, Hemmati K, White JF, Taliei F, 2020. Isolation and identification of L-asparaginase-producing endophytic fungi from the *Asteraceae* family plant species of Iran. PeerJ. 8:e8309.

[52] Uzma F, Murthy KN, Srinivas C, 2016. Optimization of physiological conditions for L-asparaginase production by endophytic fungi (*Fusarium solani*) isolated from *Tinospora cordifolia* (Willd.) Hook. F & Thomson. Eur J Exp Biol. 6:37-45.

[53] Zaki RA, Shafei MS, Refai HAE, El-Hadi AA, Mostafa H, 2018. Evaluation of factors affecting L-asparaginase activity using experimental design. Current Trends in Biotechnology and Pharmacy. 12:377-389.

[54] As-Suhbani AE, Bhosale H, 2020. Optimizing L-ASPARAGINASE production by *Fusarium solani* CLR-36: a statistical approach using response surface methodology. Journal of Advanced Scientific Research. 11:365-374.

[55] El-Gendy MMAA, Awad MF, El-Shenawy FS, El-Bondkly AMA, 2021. Production, purification, characterization, antioxidant and antiproliferative activities of

extracellular L-asparaginase produced by *Fusarium equiseti* AHMF4. Saudi Journal of Biological Sciences. 28:2540-2548.

[56] El-Naggar NE-A, Deraz SF, Soliman HM, El-Deeb NM, El-Ewasy SM, 2016. Purification, characterization, cytotoxicity and anticancer activities of L-asparaginase, anti-colon cancer protein, from the newly isolated alkaliphilic *Streptomyces fradiae* NEAE-82. Scientific reports. 6:32926.

[57] Abdelrazek NA, Elkhatib WF, Raafat MM, Aboulwafa MM, 2019. Experimental and bioinformatics study for production of L-asparaginase from *Bacillus licheniformis*: a promising enzyme for medical application. Amb Express. 9:39.

[58] Amena S, Vishalakshi N, Prabhakar M, Dayanand A, Lingappa K, 2010. Production, purification and characterization of L-asparaginase from *Streptomyces gulbargensis*. Brazilian journal of Microbiology. 41:173-178.

[59] Al Yousef SA, 2022. Fusarium sp. L-asparaginases: purification, characterization, and potential assessment as an antileukemic chemotherapeutic agent. Environmental Science and Pollution Research. 29:11243-11254.

[60] El-Hefnawy M, Attia M, El-Hofy M, Ali SM, 2015. Optimization Production of L asparaginase by locally isolated filamentous fungi from Egypt. Curr Sci Int. 4:330-341.

[61] Mohamed SA, Elshal MF, Kumosani TA, Aldahlawi AM, 2015. Purification and characterization of asparaginase from *Phaseolus vulgaris* seeds. Evidence-Based Complementary and Alternative Medicine. 2015:309214.

[62] Asha A, Pallavi B, 2012. Production, purification and characterization of extracellular L-asparaginase having antineoplastic activity from *Fusarium* sp. J Adv Res Biol Sci. 4:293-301.

[63] Varalakshmi V, Raju KJ, 2013. Optimization of L-asparaginase production by *Aspergillus terreus* mtcc 1782 using bajra seed flour under solid state fermentation. Int J Res Eng Technol. 2:121-129.

[64] Isaac G, Abu-Tahon M, 2016. Production of extracellular anti-leukemic enzyme L-asparaginase from *Fusarium solani* AUMC 8615 grown under solid-state fermentation conditions: purification and characterization of the free and immobilized enzyme. Egypt J Bot. 56:799-816.

[65] Jayaramu M, Hemalatha N, Rajeshwari C, Siddalingeshwara K, Mohsin S, Dutt PS, 2010. A novel approach for detection, confirmation and optimization of L-asparaginase from *Emericella nidulans*. Journal of Current Pharma Research. 1:20.

[66] Murali T, 2011. L-asparaginase from marine derived fungal endophytes of seaweeds. Mycosphere:147-155.

[67] Singh Y, Srivastava S, 2012. L-asparaginase production by a new isolate *Bacillus aryabhattai* strain ITBHU02 in solid state culture. In 1st International Conference on Biosciences and Bioengineering: A collaborative Approach. 2012: 150-168.

[68] Ali D, Ouf S, Eweis M, Solieman D, 2018. Optimization of L-asparaginase production from some filamentous fungi with potential pharmaceutical properties. Egyptian Journal of Botany. 58:355-369.

[69] Graham ML, 2003. Pegaspargase: a review of clinical studies. Advanced drug delivery reviews. 55:1293-1302.

[70] Oza VP, Parmar PP, Kumar S, Subramanian R, 2010. Anticancer properties of highly purified L-asparaginase from *Withania somnifera* L. against acute lymphoblastic leukemia. Applied biochemistry and biotechnology. 160:1833-1840.