



Anticancer Activity of L-Asparaginase Produced from *Vigna Unguiculata*

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Abstract

The present study aimed to production of purified L-asparaginase from *Vignaunguiculata*. Different physiological parameters, such as pH, temperature and incubation period, were optimized for growth and maximum L-asparaginase production. The optimum parameters were 37°C, 30 min and pH 8.5. Maximum L-asparaginase was 886.4U/ml with a specific activity of 1140.7 U/ml (31 fold purification with 28 %yield) were obtained at optimum conditions. The purified L-asparaginase produced from *Vignaunguiculata* was used for characterization and general properties. The effect of pH and temperature on L-asparaginase activity as well as stability at different pH and temperature were determined. The optimum pH 8.5 and 37°C temperature on L-asparaginase showed 100% residual activity. Stability of pH around 8.5 and temperature 70°C showed 90 and 78 % residual activity at 30 and 60 min respectively. The L-asparaginase showed high stability at alkaline pH (pH 8.5) when incubated for up to 60h. The molecular weight of the produced L-asparaginase was close to 68.5 kDa. Cytotoxic activity of L-asparaginase was examined in vitro using four carcinoma cell lines. L-asparaginase has higher effective in growth inhibition against HEPG2 and HCT-116 but lower against HELLA and MCF7 carcinoma cell lines. The data show that L-asparaginase has a higher cytotoxic activity against HEPG2 and HCT116, revealed higher percentage of cell death, indicating antitumor properties, and demonstrate direct effect on cancer cell proliferation of HEPG2 and HCT116. Therefore, *Vignaunguiculata* was considered to be a suitable source for production of L-asparaginase has higher activity and good stability. Purified L-asparaginase obtained from *Vignaunguiculata* could be employed in drug chemotherapy and treatment of cancer.

Keywords: Seed, L-asparaginase, Cytotoxicity.

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Ethical: This study follows all ethical practices during writing.

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1. Introduction

L-Asparaginase is widely distributed in both prokaryotic and eukaryotic cells and has been intensively studied over the last decades. Legume seeds have played a significant role as protein source in the human diet of the peoples in many parts of the world. Enzymes are proteinaceous in nature in various organs produced by many cells in different unicellular and multicellular organisms. Different types of enzymes are produced based on the adaptation to the environment where the living organisms live to tune the metabolic pathways according to their adapted changes. L-asparaginase enzymes (E.C.3.5.1.1) are widely distributed among plants, animals and microorganisms [1-4]. *Escherichia coli*, *Erwinia carotovora*, *Bacillus sp.* were found to be the most commonly organisms used for production of L-asparaginase [5-7]. Different animal organs were also used for production of L-asparaginases [1, 8]. L- asparaginase was detected in different plant species used as a source of protein for human food [4, 9]. Recently, several investigators search some plant sources for L-asparaginase production to meet the increase continuous need for L-asparaginases [10-13]. L-asparagine is the most abundant metabolite for the storage and transport of nitrogen that is utilized in protein biosynthesis [14-16]. The L-asparaginase (E.C.3.5.1.1) an aminohydrolase catalyses effect involved asparagine hydrolysis to yield L-aspartate and ammonia [2, 14, 17]. L-glutamine to L-glutamate as well as L-asparagine transamination [17, 18]. Several investigators [8, 14, 19] reported an internal and external L-asparaginases were biochemically and genetically distinct in different plants. Moreover, plant source L-asparaginases are evolutionarily and structurally distinct from the bacterial L-asparaginases [7, 20] and Kishor, et al. [21]. Recently, L-asparaginase has attracted much attention in both pharmaceutical and industrial applications [8, 22, 23]. L-asparaginase used as chemotherapeutic agent for treatment of human cancer and acts as a catalyst in the breakdown of asparagine, as nutritional requirement for both normal and cancer cells, to aspartic acid in vitro and in vivo [21, 24, 25]. L-asparaginase from plants origin leads to the death of leukaemic and tumor cells due to catalyses the hydrolysis of asparagine to aspartic acid [6, 11, 23]. L-asparaginase was used to treat hepatocellular cell lines in vitro and in vivo to examine the therapeutic efficacy [22, 26, 27]. L-asparaginase is a therapeutically important protein used in combination with other drugs in the treatment of acute lymphocytic leukemia, lymphosarcoma, melanosarcoma and different kinds of cancer [6, 28-30]. Cancer cases are gradually increasing and leading the cause of death in both developed and developing countries [27, 31, 32]. Among the most common types of cancers are lymphomas, leukemias, colorectal, liver, breast and prostate [6, 13, 32]. [33]. Currently, the most commonly use cancer chemotherapy includes mainly alkylating agents, antimetabolites and natural anticancer agents [23, 34-36]. The microbial L-asparaginases have only been produced commercially as a drug in the treatment of acute lymphoblastic leukemia [21, 37, 38]. Different studies [22, 39] discovered plant natural products have more toxic to cancer cells than normal cells in screening and developed chemotherapy drugs [27, 28, 35, 40]. Important progress has been made in cancer chemotherapy include plant derived drugs with cytotoxic activity as the treatment of cancer [25, 26, 31]. Other workers [32, 41]. Established natural products as anti-cancer properties in vivo or in vitro. However, the continuous need of therapeutic enzymes in biotechnology and medicine for cancer treatment leads to the present study was done aimed to production and purification of a commercially L-asparaginase from plant seeds as inexpensive and suitable sources. Cytotoxic activity of the purified L-asparaginase produced from *Vigna unguiculata* was examined against four different carcinoma cell lines in vitro. Moreover, seed is a member of *Leguminosae* family grown in different parts of the world and used as protein source for human food.

2. Materials and Methods

2.1. Materials

2.1.1-*Vigna unguiculata* seeds were obtained from the local market, Cairo, Egypt. *Vigna unguiculata* seeds were dried and ground to a very fine powder, sifted through a 16 mesh sieve, packed in bags, and stored till used immediately.

2.1.2-TCA and DEAE-Sepharose was purchased from Sigma Chemical Co. (USA).

2.1.3- Sephacryl S-200 and standards protein were purchased from Pharmacia, Uppsala, Sweden.

Preparation of crude extract

A known weight of *Vigna unguiculata* seeds powder (50g) were homogenized in 20mM Tris-HCl buffer (pH8.0) containing 10% glycerol, 50mM KCl, 12.5mM β -mercaptoethanol, and 1mM phenylmethylsulfonyl fluoride (PMSF), homogenized and shaken at 200 rpm using rotary shaker for 2h at 30°C. The samples were stand for one hour, then filtered and centrifuged at 15000 for 30 min using cooling centrifuge (Sigma 2K15). The supernatant was collected and designated as crude enzyme extract. Different factors were studied on crude L-asparaginase activity [42].

2.2. Effect of Substrate Concentration

Effect of substrate concentration on the activity of crude L-asparaginase was determined by incubation crude enzyme with different substrate concentrations (50, 100, 150, 200, and 250mM), then L-asparaginase activity was determined as previous.

2.3. Effect of Reaction Time

The optimization of reaction time is needed to determine the highest amount of L-asparaginase production [20]. Effect of reaction time for L-asparaginase was determined by incubation the reaction mixture for different periods of time (10-90 minutes) at 37°C and pH 8.5, then L-asparaginase activity was determined as previous.

2.4. Effect of pH

The effect of pH value on L-asparaginase activity was determined by assaying the enzyme activity at different pH values ranges from 3.0 to 11.0 under the standard conditions [42].

2.5. Effect of Temperature

The effect of temperature on L-asparaginase activity was determined by assaying the enzyme activity at different temperature range from 10 to 90°C. Like most chemical reactions, the rate of an enzyme-catalyzed reaction increases as the temperature is raised. A ten degree Celsius rise in temperature will increase the activity of most enzymes by 50 to 100%. Variations in reaction temperature as small as 1 or 2 degrees may introduce changes of 10 to 20% in the results [43].

3. Purification of L-Asparaginase

3.1. Precipitation with Ammonium Sulfate

L-asparaginase was precipitated by addition of $(\text{NH}_4)_2\text{SO}_4$ at 0-80% saturation and kept overnight at 4°C. The solutions were allowed to stand at room temperature and centrifuged at 15000 rpm for 30 min. The precipitates were re-suspended in 25mM phosphate buffer (pH 8.5) and extensively dialyzed against the same buffer (pH 8.5) for 48h. The dialyzed samples were centrifuged using cooling centrifuge. The collected and concentrated supernatant was used for L-asparaginase studies. L-asparaginase was purified and used for characterization studies according to the methods of Palmieri, et al. [44].

3.2. Anion Exchange Chromatography

The $(\text{NH}_4)_2\text{SO}_4$ precipitated samples were concentrated and applied to a DEAE-Sepharose column (1.6x15cm) previously equilibrated with 25mM Tris-HCl buffer (pH 8.5). The $(\text{NH}_4)_2\text{SO}_4$ precipitated samples were washed with three column volumes of the same buffer and eluted with 25mM Tris-HCl buffer (pH 8.5). The column was washed at a flow rate of 60ml/h with 100ml of the same buffer, and a 0-0.1 M KCl was applied. Two column volumes of eluted fractions containing high L-asparaginase activity were collected and concentrated. Two peaks of protein were eluted with two L-asparaginase activity fractions (F1 and F2).

3.3. Gel Filtration Chromatography

Gel filtration was performed according to Aworh and Nakai [45]. The concentrated eluted fractions (F1 and F2) were pooled and applied onto a Sephacryl S-200 column (1.8x85 cm) and eluted with 25mM Tris-HCl buffer (pH 8.5) containing 0.15M KCl. The eluted fractions were assayed for L-asparaginase activities as described by Dharmsthiti and Luechai [46]. The active samples were pooled and dialyzed against 25mM Tris-HCl buffer (pH 8.5) containing 0.1M KCl. After concentration, the solutions were applied onto a Sephacryl S-200 column and eluted with the same buffer at a flow rate of 30ml/h and 3ml fractions were collected as purified products.

3.4. Protein Determination

Protein concentration was estimated according to the methods of Bradford [47] using bovine serum albumin as standard.

3.4. L-Asparaginase Assay

The activity of L-asparaginase was measured based on L-asparaginase catalyzes L-asparagine to L-asparatate and Ammonia using Nesslerization method [48]. L-asparaginase activity from *Vigna unguiculata* seed extract was determined by adding 0.5ml of crude enzyme to 2 ml of 200 mM prepared L-asparagine, then 2ml of potassium phosphate buffer (0.05M, pH 8.0) was added, mixed gently and incubated at 37°C for 30 minutes. The reaction mixture was stop by 1ml of 1.5M trichloroacetic acid (TCA) and mixture was centrifuged at 15000 rpm for 10 minutes using cooling centrifuge to remove the precipitate. The ammonia concentration was determined in the supernatant by adding Nessler's reagent and left at room temperature for 10 min. The blank was prepared by adding enzyme after TCA addition and the developed color was measured at 450nm [46]. One unit of L-asparaginase activity is defined as the amount of the enzyme that liberates 1µmol of ammonia per min under assay conditions.

4. Characterization

4.1. Determination of Molecular Weight

The average molecular weight of L-asparaginase was determined by gel filtration chromatography [5, 49]. Using Sephacryl S-200 column (1x100). The sample was applied through a glass column packed with pre calibrated Sephacryl S-200 column (1x100) equilibrated and developed with 25mM standard buffer. Fractions of 0.5 ml were collected at a flow-rate of 0.05 ml/min. The following known molecular weight standards were used for the molecular weight determination under optimum conditions. The known molecular weight markers were used for calibration of the column of dehydrogenase (150kDa), phosphorylase (97kDa), bovine serum albumin (67kDa), pepsin (35.5kDa), carbonic anhydrase (29kDa), trypsin (24kDa), myoglobin (17.8kDa), lysozyme (14kDa) and cytochrome C (12,4kDa).

4.2. Effect of pH on L-Asparaginase Activity

This can be achieved using different pH (3.0-11.0), incubated in a water bath at 37°C for 30 minutes, then the activity of L-asparaginase activity was assayed.

4.3. Effect of pH on L-Asparaginase Stability

Purified enzyme was incubated at different pH values ranging between 3.0 and 11.0 at 37°C for one hour. The L-asparaginase activity and residual activity were measured after assaying enzyme activity [50].

4.4. Effect of Temperature on L-Asparaginase Activity

The effect of temperature on L-asparaginase activity was determined by assaying the enzyme activity at different temperature range from 10 to 90°C. Like most chemical reactions, the rate of an enzyme-catalyzed

reaction increases as the temperature is raised. A ten degree Celsius rise in temperature will increase the activity of most enzymes by 50 to 100%.

4.5. Effect of Temperature on L-Asparaginase Stability

A purified enzyme was incubated in a water bath for different temperatures (20, 25, 30, 35, 40, 45, 50, 55 and 60°C) for one hour, then immediately transferred into an ice bath. L-asparaginase activity was assayed for each treatment, then activity and the remaining activity was determined.

4.6. Cytotoxicity and Anticancer Activity in Vitro

Different four cancer cells, colon (HCT-116), liver (HEPG2), breast (MCF-7) and cervical (HELLA). Breast cancer (MCF-7) and cervical cancer were used in the present study in vitro. Cytotoxicity was done according to the method of Itharat, et al. [51]. IC50 was calculated by analysis between surviving fraction and concentration [52]. In vitro anticancer activity was determined [36].

5. Results and Discussion

5.1. Extraction L-Asparaginase from *VignaUnguiculata* Seeds

L-Asparaginase was extracted from *Vignaunguiculata* seeds using the homogenization method to obtain crude enzyme. Results in Table (1) showed higher total activity of crude L-asparaginase in the extracts of *Vignaunguiculata* seed was 18540U. Results also showed the maximum and specific activity of L-asparaginase were less (309U/ml and 36.8U/mg respectively). According to these results *Vignaunguiculata* seed was used for L-asparaginase production, purification, characterization and applications. It has been reported that plant is a source of protease, therefore phenylmethylsulfonyl fluoride (PMSF) was used in extraction as an inhibitor for serine proteases and alkaline proteases which was active in alkaline pH and may degrade L-asparaginase [20, 21] that was active in alkaline pH (pH 8.5).

Table-1. Purification of L-asparaginase produced from *Vigna unguiculata* seeds.

Ingradients	Total volume (ml)	Protein (mg/ml)	Activity units (U/ml)	Specific activity (U/mg)	Total activity units(U)	Total protein (mg)	Purification fold	Yield (%)
Crude L-asparaginase	60	8.4	309	36.8	18540	504	1	100
(NH ₃) ₄ SO ₄ ppt.	26	6.02	180	72.4	11332.05	156.52	2	61
DEAE-Sephrose								
L-asparaginase (F1)	6.4	2.4	835.7	348.2	5348.5	15.36	10	29
L-asparaginase (F2)	5.2	1.04	462.2	444.4	2403.4	5.41	12	13
Sephacryl S-200	2.4	0.6	886.4	1140.7	5236	1.44	31	28.24

5.2. Optimum Conditions for L- Asparaginase Activity

L-asparaginase enzyme was extracted from *Vignaunguiculata* seed and used for optimum conditions studies for L-asparaginase activity. These conditions include substrate concentration, reaction time, pH and temperature. Kozak and Jurga [53] and Shanmugaparakash, et al. [13] reported the L-asparaginase activity may affect the change of these parameters. Therefore, it is possible to increase the enzyme activity by increasing substrate concentration, reaction, pH and temperature.

5.3. Effects of Substrate Concentration

Different concentrations (10, 50, 100, 150, 200, and 250mM) of substrate (asparagine) for L-asparaginase were used to determine the optimum substrate concentration for L- asparaginase activity. Results in Figure (1) showed that the activity of L- asparaginase was increased gradually with the increase in L-asparagine concentration. Maximum activity of L-asparaginase was obtained at the substrate concentration level was 200mM (332.7 U/ml).

The concentration level (200 mM) of L-asparagine consider as the optimum substrate for L-asparaginase activity. These results were agreed with other workers [10, 54] who found that there is a positive relationship between the enzyme activity and substrate concentration, hence the reaction was increased with the increase of substrate concentration when the L-asparaginase concentration was constant until the maximum rate [55]. In addition, substrate inhibition will sometimes occur when excessive amounts of substrate are present in the reaction mixture [12]. According to the present results (Figure 1), L-asparaginase produced by *Vignaunguiculata* seeds was reached the steady state when the L- asparagine concentration was 200m M. Similar results were reported by other investigators [10, 12, 22] established that the consumption of high substrate concentration indicates high activity of L-asparaginase in the treatment of the late stages of acute lymphoblastic leukemia in the blood of children. However, the optimum substrate concentration of enzyme activity from hot pepper of *Capasciumannum* L. was 33mM using catechol as a substrate [25, 56].

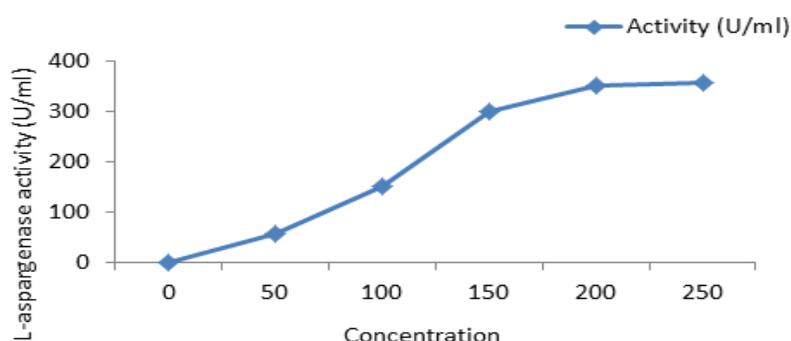


Figure-1. Effect of substrate concentration on the activity of L-asparaginase.

5.4. Effect of Reaction Time

L-asparaginase activity obtained by *seed* was determined at different reaction time (10- 90 minutes). Results in Figure (2) showed that the optimum incubation period was 30 minutes, the enzyme activity was 420U/ml. According to these results, it has been concluded that 30 minutes of incubation was suitable for reaching maximum L-asparaginase activity. Different studies reported that the reaction time is an important factor for determination of the enzyme activity and it was preferred to use methods with short time incubation to determine the enzyme activity [57, 58]. Moreover, there is a linear relationship between enzyme activity and reaction time [8, 55].

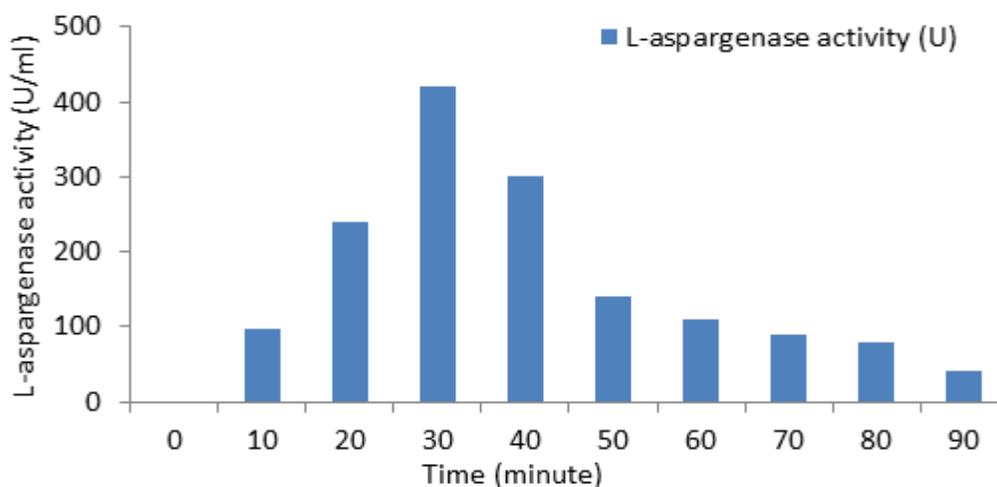


Figure-2. Effect of reaction time on L-asparaginase activity.

5.5. Effect of pH

Effect of pH on the activity of L-asparaginase activity produced by *Vignaunguiculata* seed was studied. Result illustrated in Figure (3) showed that maximum l-asparaginase activity was obtained when pH of the reaction mixture was adjusted to 8.5, at this value, the enzyme activity was 400 U/ml. Similar results were obtained by several investigators [5, 13]. The present observation was also reported by other investigators [12, 59]. Indicated that many L-asparaginase from plant origin had higher activities at pH ranged from 8.0 to 8.5. Moreover, alkaline pH (8.0–10) showed optimum pH for most bacterial L-asparaginases activity [60, 61]. It has been reported that most plant L-asparaginases have its maximum activity in alkaline pH, and was determined in pH range of 7.5, 8.0 and 8.5 due to the balance between L-aspartic acid and L-aspartate [11, 58]. In general most plants show maximum enzyme activity at or near neutral pH [60]. The decrease or increase in pH value will be changes in the reaction mixture, resulting enzyme denaturation [27]. On the other hand, the effect of pH on enzyme activity resulted from its effect on the substrate ionization Abdel-Fattah and Olama [57]. Khalaf, et al. [12] found favorable balance for the connection with the substrate L-asparagine and they reported that the optimum pH was 7.0. In addition pH 8.0 was optimum for enzyme obtained from *Irvingiagabonnensis* [42].

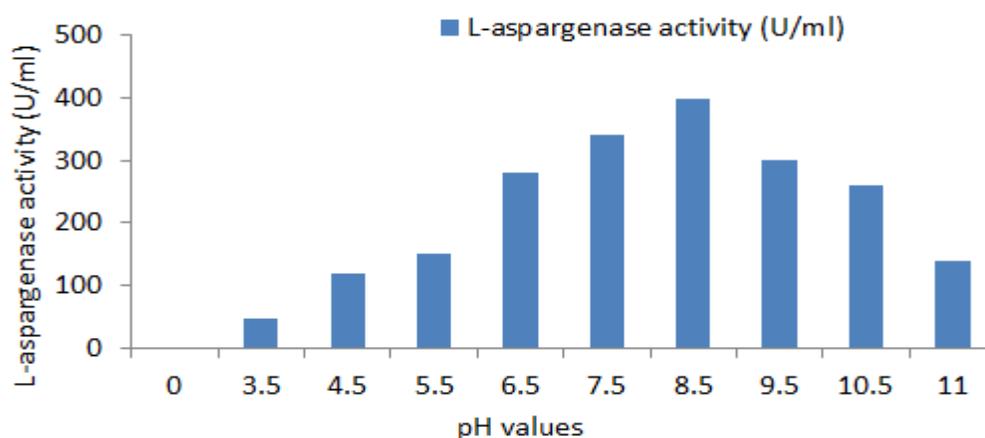


Figure-3. Effect of pH on L-asparaginase activity

5.6. Effect of Temperature

Reaction mixture of L-asparaginase obtained from *Vignaunguiculata* seed was assayed at different temperatures (25, 30, 35 and 40°C). Other investigators reported that these range of temperature was the most suitable for enzyme activity [57, 58]. Results illustrated in Figure (4) showed that the maximum activity of L-asparaginase was obtained at 37°C of the reaction mixture. The enzyme activity was increased to 490 U/ml at this temperature. Moreover, L-asparaginase isolated from most organisms has maximum activity at 37°C [37, 46, 58]. The increase or decrease in reaction temperature above or below the optimum temperature cause a decrease in enzyme activity. L-asparaginase from most organisms have its maximum activity at 37°C [55, 61]. While the increase or decrease in the incubation temperature above or below the optimum temperature cause a decrease in enzyme activity. These variations in the activity of L- asparaginase proved that the suitable temperature for asparaginase reaction to have maximum activity was 37°C, while the activity was decreased when the temperature was above optimum or below the temperature. Other investigators [58, 62] found that the temperature at which polyphenol oxidase from *Thymus logicaulis* showed highest activity was in range of 25-30°C and then decreased at temperature above 40°C. while peroxidase was highly active at 40°C and cost its activity at higher temperature [63].

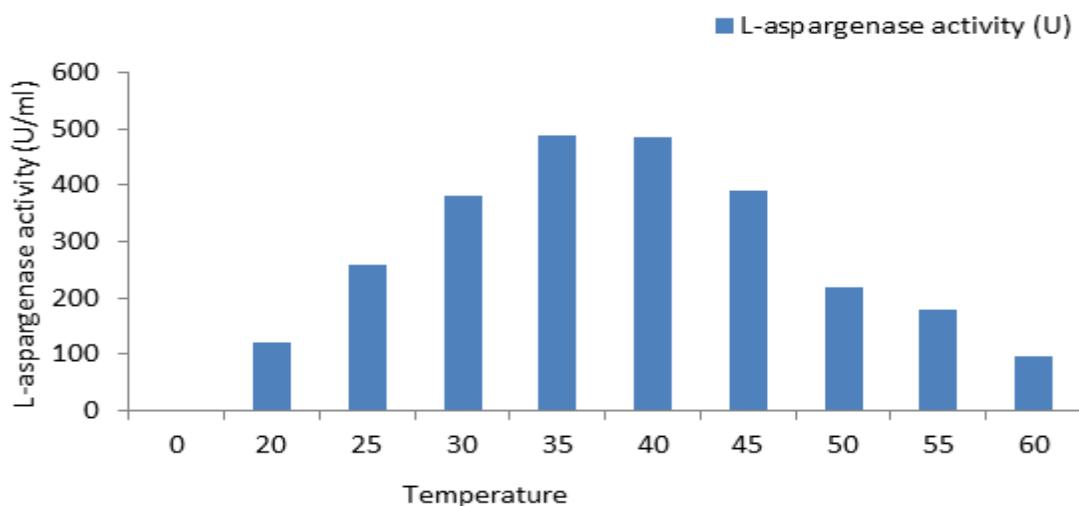


Figure-4. Effect of temperature on L-asparaginase activity.

5.7. Purification of L-Asparaginase Extracted from *Vigna Unguiculata* Seeds

Crude L-asparaginase extracted from seeds was precipitation from *Vigna unguiculata* seeds crude extracts using ammonium sulfate (20%, - 80% respectively) as mentioned in Table (1). L-asparaginase was purified in two steps, ion exchange chromatography by DEAE-Sephacryl and gel filtration chromatography by Sephacryl S-200. The results of purification steps of L-asparaginase from *Vigna unguiculata* seeds are summarized in Table 1. The elution profile of the chromatography on DEAE-Sephacryl column (Figure 5) showed two peaks (F1 and F2) of proteins (15.36 and 5.41 mg respectively) with L-asparaginase activity. Peak one with the highest L-asparaginase activity (5348.5U) was applied onto a Sephacryl S-200 column (Figure 5). L-asparaginase I was purified 10-fold with a specific activity 348.2 units/mg protein. L-asparaginase I was proved to be pure after Sephacryl S-200 column (Figure 5). Specific activity of purified L-asparaginase was 1140.7U/mg with purified 31-fold. Similar results were obtained by other investigators [10, 64].

5.8. Ion Exchange Chromatography.

L-asparaginase extracted from *Vigna unguiculata* seeds of seeds was purified by ion-Exchange chromatography using DEAE- Sepharose. DEAE- Sepharose was used for purification due to their easy to simplicity prepare, multiple use and has high bioseparation capacity [45, 65]. According to results obtained from this study, optimum pH for L-asparaginase activity which was pH 8.5 [12, 55]. According to these findings, ion exchanger DEAE-Sephacryl was used for purification of Figure (5):

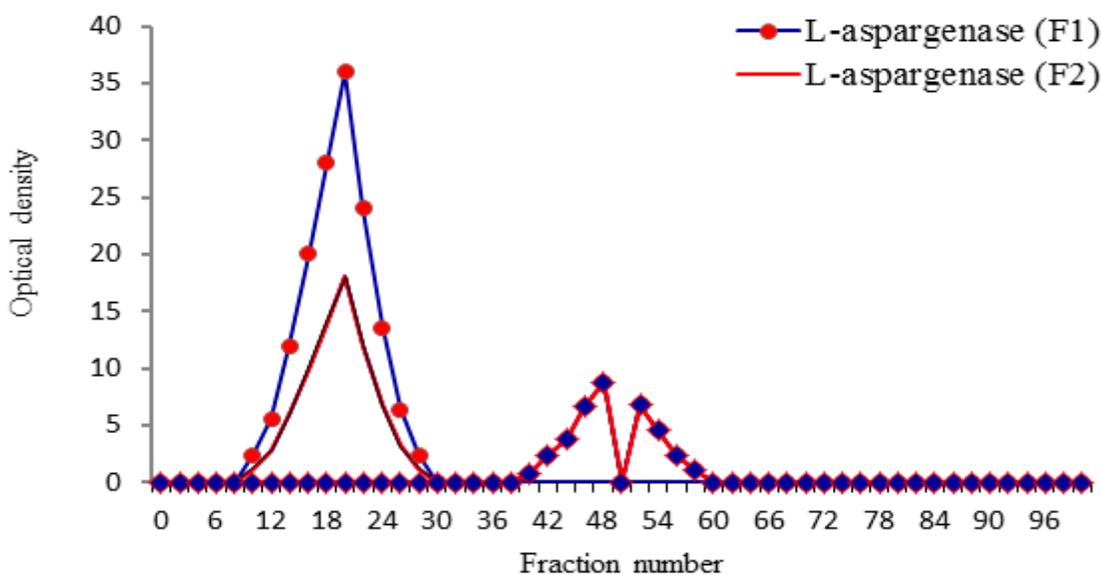


Figure-5. Ion exchange chromatography of L-asparaginase

Ion exchange chromatography for purification of L-asparaginase produced from seeds using DEAE- Sepharose column (2×23 cm) with a flow rate of 20 ml/hour. Results illustrated in Figure (5) showed that the third peak that eluted in fraction numbers 20 to 48 has L-asparaginase activity that reaches 1297.9.0 U/ml. Fractions represents L-asparaginase active were collected and pooled, then protein concentration, L-asparaginase activity, specific activity was estimated. Results indicated in Table (1) showed that the maximum L-asparaginase activity and specific activity in the L-asparaginase concentrate were 886.4 U/ml and 1140.7 U/mg respectively, with 31- fold of purification. Ion exchange chromatography was applied for purification of L-asparaginase by many investigators [9, 27, 64]. Partially purified L-asparaginase obtained from the ion exchange chromatography was further purified by gel filtration chromatography.

5.9. Gel Filtration Chromatography

Gel filtration chromatography was used in the purification of L-asparaginase produced by *Vigna unguiculata* seed after purification by ion exchange chromatography. 5 ml of partially purified L-asparaginase was applied on Sephacryl S-200 column (1.8x85 cm) which previously equilibrated with 0.1 M potassium phosphate buffer (pH 8.0). which allows ability of separation with high degree of purification [7, 21, 65]. Furthermore, gel filtration is the

simplest and separates molecules [66]. Proteins were eluted at a flow rate of 20 ml/hour. Protein peaks were determined. Results in Figure (6) showed that only one peak represents L-asparaginase activity was appeared after elution with potassium phosphate buffer. Fractions representing L-asparaginase activity were pooled, then protein concentration, L-asparaginase activity and specific activity were measured in 20 ml of enzyme concentrate Figure (6). Results in Table (1) showed that there is an increase in both activity of purified enzyme (886.4 U/ml) and specific activity (1140.7 U/mg) with a purification fold 31 with an increase in the yield of L-asparaginase (28-.24%). Partial purification of L-asparaginase from chicken liver using Sephadex G-200 (20-80 ammonium sulfate precipitation) gives specific activity of 158.11U/mg with 17% yield [8]. Purification of L-asparaginase produced by *Streptomyces* using precipitation with ammonium sulfate 45-85% and Sephadex G-200 gel filtration gave a maximum specific activity of 63.0 U/mg with 82.9 fold of purification with 2.18 % yield demonstrated [3]. On the other hand, L-asparaginase from *Enterobacter aerogenes* was purified by gel filtration with Sephacryl S-100 gives 10-fold of purification with a specific activity of 55 IU/mg and recovery of 54% [67].

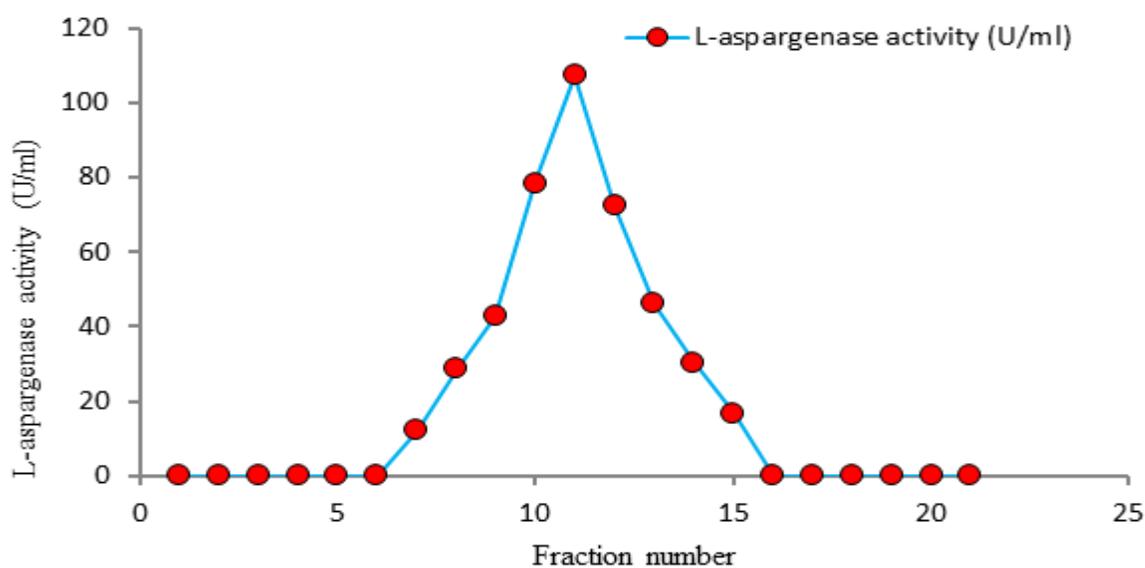


Figure-6. Gel filtration chromatography of L-asparaginase

6. Characterization of Purified L-Asparaginase

6.1. Molecular Weight of L-Asparaginase

Molecular weight of the purified L-asparaginase produced by *Vigna unguiculata* seed was determined by gel filtration chromatography using Sephacryl S-200 and different known molecular weight standard proteins as Pepsin (14000 Dalton), Trypsin (20000 Dalton), Urease (430000 Dalton), Bovine serum albumin (67000 Dalton), phosphorylase (97kDa), dehydrogenase (150kDa), and separately were applied and eluted individually. Results showed that L-asparaginase has a molecular weight of 68,5 KeloDalton. These results are in the range with those obtained by many investigators [43, 58] reported that the molecular weight of L-asparaginase from *P. sativum* seeds was 68 KDa using Sephadex LH-20 and 69 KDa using Sephacryl S-200 chromatography. Other studies found the molecular weight of L-asparaginase produced from *P. sativum* was 58 KDa [12, 13, 68] However, the molecular weight of L-asparaginase by Sephacryl S-200 yielded a value of 79 kDa. *Vigna unguiculata* with a molecular weight of 70000 Dalton using Sephacryl S-200 chromatography [7, 10, 21]. On another hand the molecular weight of L-asparaginase purified from *Withania somnifera* was detected by polyacrylamide-gel electrophoresis revealed dense bands along the gel with purified enzyme [11, 43] and stated to have 72000 Dalton by gel chromatography technique [4] This finding is in agreement with molecular weights for L-asparaginases from *Vigna unguiculata* (70 kDa) and *Lupinus polyphyllus* (75 kDa) as reported by other investigators [10, 21, 69]. A medium molecular weight of 58 kDa was detected for L-asparaginase from pea leaves [70]. Bacterial L-asparaginases, the molecular weight ranged from 140 to 160 kDa [24, 53, 60]. A very low molecular weight of 11.2 kDa was detected for *Streptobacillus* sp. KK2S4 L-asparaginase [58]. However, Molecular weight of L-asparaginase was differed according to the organisms. Results of characterization showed that the molecular weight of L-asparaginase was 68,5 Kelodalton, and the optimum pH for enzyme activity and stability was pH 8.5, while the optimum temperature for enzyme activity and stability was 37°C and 40°C respectively.

6.2. Effect of pH on L-Asparaginase Activity

The pH for L-asparaginase activity was determined at different pH values ranging from pH 3.0 to pH 11.0. Results in Figure (3) showed higher L-asparaginase activity (622 U/ml and 632 U/ml) at pH 8.0 and 8.5 respectively. These effect may be attributed to the hydrolysis and breakdown of asparagine by L-asparaginase [2, 14]. These results are in agreement with those reported by other investigators [9] found the optimum activity of L-asparaginase at pH 8.5. *E. coli* and *S. marssonii* have an optimum activity at pH 8.6 [2, 68]. While the optimum pH for L-asparaginase activity of *Bacillus* spp. was at pH 7.0 [60]. This property of the enzyme make clear that the enzyme produced by plant under the present study has effective carcinostatic property, because the physiological pH is one of the prerequisites for antitumor activity [24]. However, L-asparaginase activity below pH 8.0 was not effective tumor treatment [27, 33]. The purified enzyme would be useful because 80% of the enzyme activity was retained at pH 7.5.

6.3. Effect of Temperature on L-Asparaginase Activity

Temperature is an important factor affects enzyme activity. Therefore optimum temperature for L-asparaginase activity was determined and the residual activity was assayed at different temperatures. Results in Figure (4) showed that 37°C was the optimum temperature for maximum L-asparaginase activity but around this temperature

at lower or higher value, the enzyme activity was decreased. Similar results was obtained by other investigators [61, 64] indicated that optimum activity of L-asparaginase was obtained at 37°C. L-asparaginase of *Aspergillus terreus* was active at 50°C [33] and *Bacillus* sp. was active at 80°C [21].

6.4. Effect of pH on L-Asparaginase Stability

Determination of pH on enzyme stability was important for the maintaining the enzyme conformation. This may differ among the kind of enzyme and the organism. Figure (7): The enzyme was stable at alkaline pH 8.5 and nearly remains 100% activity after incubation for 30 and 60 min at 37°C (Fig. 7). The enzyme remains 55% and 40% activity at pH 11 for 30 and 60 min respectively. The L-asparaginase enzyme obtained from *seed* was more stable at alkaline pH than at the acidic one. The enzyme was maximally stable at pH range from 8.0 to 9.0. Optimum pH for L-asparaginase stability was studied at different pH values (3.0-11.0) and the residual activity was determined. Results in Figure (7) showed that L-asparaginase has maximum residual activity (95% and 96%) and more stable at pH 8.0 and 8.5 respectively [42]. The residual activity was decreased with the pH values increased or decreased than optimum pH values. Enzymes like other proteins, are stable over only a limited pH range. Proteases are stable over detected pH range but around these pH modification and denaturation of protein structure were obtained [14, 33]. L-asparaginase was stable at pH ranged (4.5-11.0) as previous reported [50, 71] but more stable was observed with alkaline pH. L-asparaginase of some bacteria was, however, most stable at the pH range of 5-9 [2, 5, 42]. The enzyme showed stability at alkaline pH (pH 7.5-9.0) as it retained 90% of its original activity when incubated up to 24h (Figure 6). However, These findings are in agreement with those of other studies [5, 12, 13] reported the optimum pH of L-asparaginases from several plants ranged from 8.0 to 8.5.

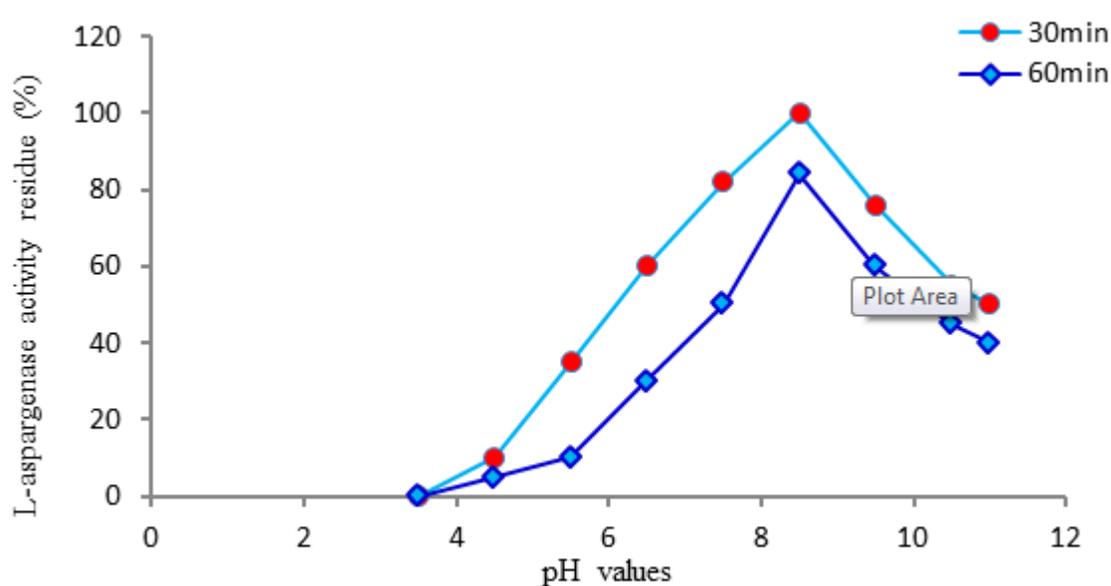


Figure-7. Effect of pH values on L-asparaginase stability.

6.5. Effect of Temperature on L-Asparaginase Stability

The effect of temperature on enzyme stability are present in Fig.(8). Results indicated that the enzyme activity was not lost when incubated at 40°C for 30 and 60 min. The residual activity is nearly 100% at this temperature. At 80°C, 69% and 60% L-asparaginase activity for 30 min and 60 min was remains respectively. L-asparaginase activity and residual enzyme activity were determined at different temperature values (15-90). Results in Figure (9) showed that L-asparaginase was stable at 40°C and maximum residual activity (75%) was observed at this temperature. The residual activity of the enzyme was decreased around lower or higher optimum temperature for enzyme stability (40°C). L-asparaginase of *Aspergillus terreus* KLS2 retained all of its activity through incubation at 70°C for 30 and 60 minutes [33]. Other investigators [3] noticed that L-asparaginase of marine *Vibrio* lost approximately 20% of its activity through incubation at 60°C for 30 minutes. The enzyme activity was stable up to 37°C after incubation for 1 h. L-asparaginase from *V. unguiculata* was stable up to 40°C after incubation for 15min [10, 21, 43]. then remaining activity decreased when the enzyme was incubated at temperature values more than the optimum for stability (40°C), and then L-asparaginase began to lose its activity after incubation at temperature 45°C [36]. The decrease in the enzyme activity with the increase in temperature may lead to the denaturation of enzyme by destructing the three dimensional structure of protein and that cause a change in the active site which leads to inactivation of the enzyme at high temperatures. Crude and partially purified enzymes are more stable than purified enzymes due to the existence of carbohydrates and other proteins protecting them [24, 50]. Moreover, enzyme that work either at extreme pH values or high temperature are now of biotechnological interest, therefore, purification of enzymes with these properties presents genuine commercial opportunities and a valuable contribution to the field of biotechnology. Enzyme that work either at extreme pH values or high temperature are now of biotechnological interest.

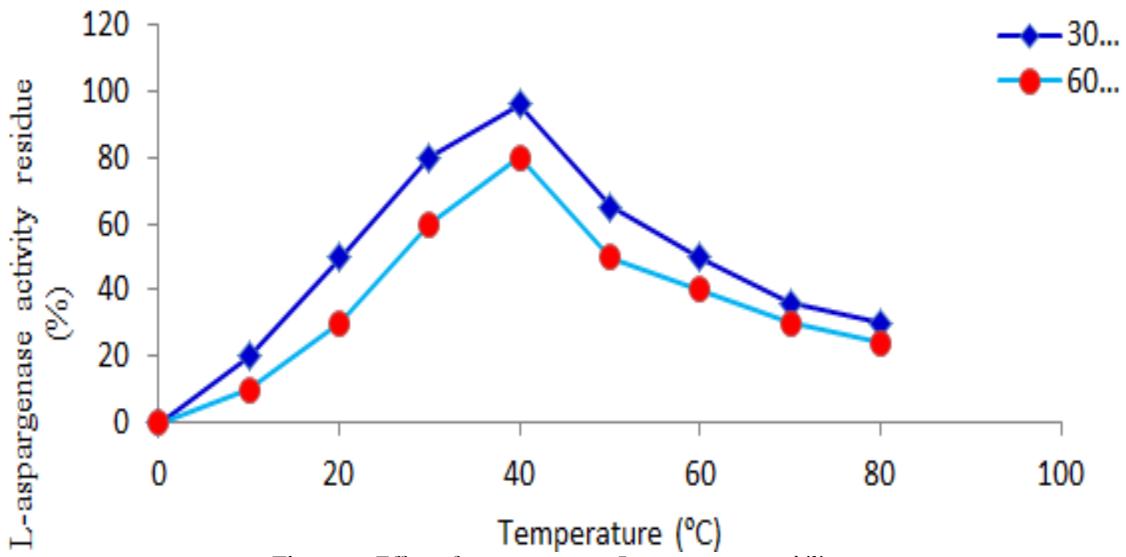


Figure-8. Effect of temperature on L-asparaginase stability.

6.7. Cytotoxicity and Anticancer Activity of L-Asparaginase

The present study was carried out to evaluate the potential efficacy of *Vigna unguiculata* seed L-asparaginase against four different carcinoma cell lines in vitro (Figures 9, 10). The effect of L-asparaginase used in vitro cytotoxicity test was done to identify activity of the L-asparaginase in growth inhibition of four different carcinoma cell lines, colon (HCT-116), liver (HEPG2), cervical (HELA) and Brest (MCF7). Results showed that L-asparaginase has higher effective in inhibition against HEPG2 and HCT-116 but lower effective against HELA and MCF7 cancer cell lines. L-asparaginase exhibited more effectiveness on growth inhibition of HEPG2 but less on HCT 116 cancer cell lines (Figure 9). Results also showed that L-asparaginase was effective in inhibition of HELLA but slightly effective against MCF7 cancer cell line (Figure 9). The effect of L-asparaginase on HEPG2 and HCT 116 cancer cell line in vitro revealed that L-asparaginase capable on inhibit cell proliferation of human HEPG2 and HCT-116. Results in Figure (10), illustrate the dose response (IC₅₀) of L-asparaginase on HEPG2, HCT-116, HELA and MCF7. The present results showed the growth inhibitory effect of L-asparaginase on HEPG2, HCT-116 and HELLA. The data show that L-asparaginase have a higher cytotoxic activity against HEPG2 and HCT116 than the other cell lines (Figures 9,10). These results indicated that L-asparaginase have more anticancer effect against HEPG2 and HCT116. The L-asparaginase reduced the survival fraction to 50% where less than 10µg of L-asparaginase killed 50 % of cancer cells, particularly HEPG2, HCT116 and HELLA respectively (Figure 10). Similar results were found by other workers [23, 32, 34, 36]. It can be observed that L-asparaginase inhibits cell proliferation of HEPG2 and HCT-116 (human cancer cell lines) that could arrest the cell cycle and generate apoptosis, which explain the in vitro anti-proliferative effect of L-asparaginase. Cancer cells exhibit rapid growth and cell division, and therefore have an increased nutritional need than normal cell [23, 51]. In most human cells deficiency, L-asparagine is produced from aspartic acid and glutamine by asparagine synthetase. Since tumor cells need asparagines for growing and functioning whereas normal cells can produce enough of this amino acid to meet their requirements with the help of asparagine synthetase [17, 35, 51]. Tumor cells are destroyed by L-asparaginase without significant damage to normal cells [11, 27]. Most of cancer cells are dependent on Lasparagine an amino acid essential for lymphoblasts growth. However, normal cells are able to synthesize L-asparagine and thus are less affected by its rapid depletion due to treatment with L-asparaginase, since lymphoblasts are unable to produce L-asparagine which leads to death of these cells [68]. The data presented in Figure 9,10 show that the L-asparaginase exhibited high cytotoxic activity. The IC₅₀ values for enzyme confirmed the most potent. Several plant species having disease preventive and therapeutic properties. This observation is of particular importance in many vegetables and fruits and the association of vegetable and fruit consumption with reduced cancer risk has been reported [32, 56, 72, 73]. The present results indicate that L-asparaginase produced in the present study may serve as a natural chemotherapy target for the treatment of colon and HCC. L-asparaginase may be reduce the possibility of side effects over anticancer therapy courses.

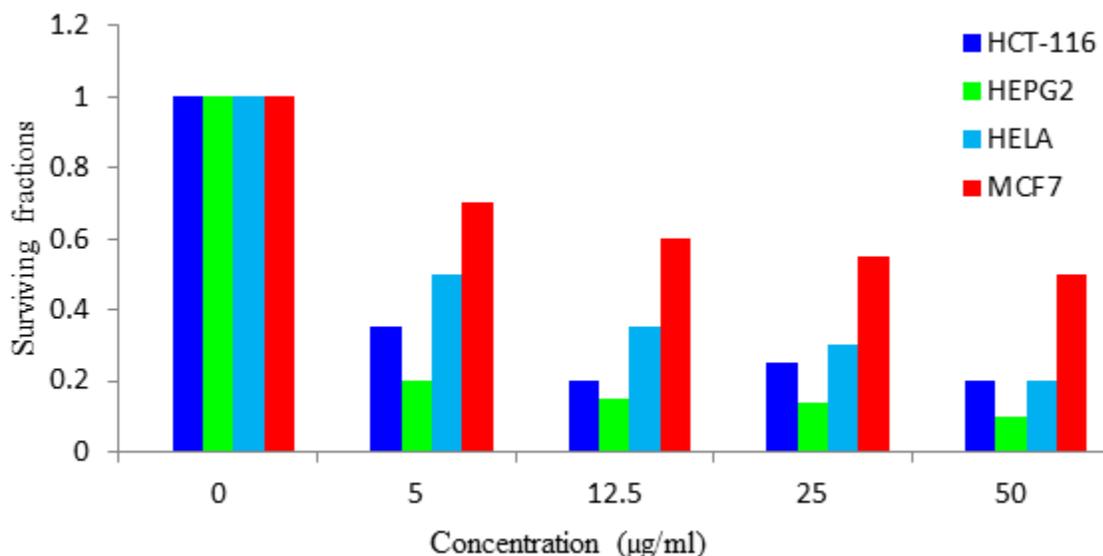


Figure-9. Cytotoxic effects of L-asparaginase on carcinoma cell lines.

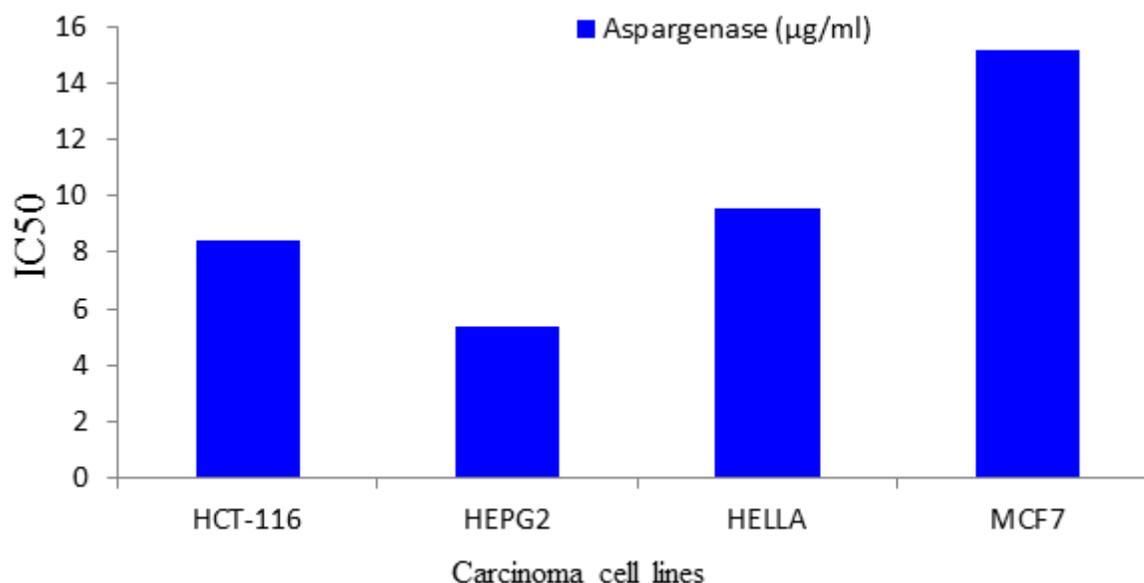


Figure-10. IC50 of L-asparaginase against carcinoma cell lines.

7. Conclusions

Vigna unguiculata seeds consider to be efficient and suitable source for L-asparaginase production under optimum conditions. L-asparaginase extracted from *Vigna unguiculata* seed can easily purified by using ion exchange and gel filtration chromatography techniques. Purified active L-asparaginase has closed molecular weight of 68,5 kDa and was stable and active at pH 8.5 and 37°C for 30min. Purified L-asparaginase produced in the present study has cytotoxic effect against four different carcinoma cell lines. L-asparaginase showed good stability over a wide range of physiological conditions as pH and temperature can be reduce the possibility of side effects of anticancer therapy over course treatment.

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