



## RESEARCH ARTICLE

### Purification of Robust L-Asparaginase from *Capsicum annum* and its Therapeutic Efficacy in Leukemia

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#### ABSTRACT

Medicinal plants used for the treatment of chronic diseases, have attracted the interest of scientists. L-Asparaginase is an extracellular enzyme with biomedical significance. The present article discusses the extraction, purification and characterization of L-asparaginase. Enzyme activity was estimated from Chillies (plant of *Solanaceae* family). Enzyme titer of 112 U/mL was attained from crude extract of Chillies (*Capsicum annum*) fruit while purified enzyme after gel filtration provided 61.99% yield, activity of 69.43 U/mL and specific activity of 234 U/mg. L-Asparaginase stability was envisaged at optimal pH of 8.6 in the presence of alkaline buffer. Optimal substrate concentration for L-Asparaginase activity was 200 mMOL. The efficacy of purified enzyme was tracked *in vivo* model. Normal healthy rats were used as control, which were compared with leukemic rats after treatment with enzyme L-Asparaginase. Eventually after treatment with enzyme extract provided best recovery in leukemic model due to high titer of L-Asparaginase, which bears high antineoplastic activity. It may be employed for chemotherapy in tumors such as acute lymphocytic leukemia.

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#### INTRODUCTION

L-Asparaginase (EC 3.5.1.1) belongs to a group of homologous amidohydrolases family. L-Asparaginase is key component of diverse recipes of chemotherapeutic agents that are employed for the treatment of acute lymphoblastic leukemia (ALL) (Sundaramoorthi, 2012). ALL is one of malignant diseases of bone marrow and lymphoid precursors of white blood cells (lymphocytes). This type of cancer replaces the normal hematopoietic cells of the bone marrow (Seiter, 2017). Numerous drugs are synthesized from medicinal plants, since old times *Solanaceae* (night shades) diverse family members play important role in treatment of different ailments (Shah, 2013).

At present in pharmaceuticals, enzymes are used as drugs (Kumar and Sobha, 2012). The L-asparaginase is well reputed in the chemotherapeutic combinations used for cancer management especially in treatment of leukemia. L-Asparaginase catalysis hydrolysis of asparagine to aspartic acid and ammonia. Healthy cells escape unaffected as L-asparagine synthetase de novo synthesizes asparagine (Ibrahim *et al.*, 2018). Currently, bacterial asparaginases from *Corynebacterium glutamicum*,

*Bacillus* sp, *Pseudomonasstutzeri*, *Erwiniach- ryanthemi*, *Erwiniacaroida* and *E. coli* (Yadav, 2014) are used for treatment purpose which offers immunogenicity complications including thrombosis, pancreatitis, hyperglycemia and hepatotoxicity. Researchers have now focused on alternative sources of L-asparaginase such as plants and fungi. Emergence of low hypersensitive enzyme will dampen the chances of side effects, which will enhance its commercial production (Kumar and Sobha, 2012). It is used as anticancer agent because it is biodegradable and non-toxic (Yadav *et al.*, 2014).

L-Asparaginase is widely employed in food industry as it also reduces the formation of acrylamide from the baked product, frozen fries and preserved juices. It is hypothesized that plants of family *Solanaceae* are novel source of L-asparaginase which possess considerable concentration of L-asparaginase. Secondly, the enzyme derived from edible plant source will exhibit least toxicity as it had been used conventionally. The objective of the present study is production and purification of commercially high titer of enzyme L-asparaginase from inexpensive plant source to meet its therapeutic need in cancer treatment.

## MATERIALS AND METHODS

**Materials:** L-Asparagine (Sigma-Aldrich), Sephadex G-100 (Sigma Aldrich Chemical Co. USA), DEAE cellulose resin (Sigma Aldrich Chemical Co. USA), Dialyzing membrane with pore size 3.2 micron (Shenzhen Taoshi Co., Ltd) were used in this study.

**Selection of plant parts for enzyme extraction:** *Capsicum annum* fruit were purchased from the local market of Faisalabad, Chillies (*Capsicum annum*) were homogenized with 0.15 M KCl solution and partially purified with 60% ammonium sulphate and then 30% sodium sulphate following the method of Bano and Sivaramakrishnan (1980). Crude extracts were filtered and centrifuged at 12000 to 15000 rpm for 15 min using refrigerated centrifuge (Sigma 2K15). The extract was preserved in a solution of 0.2 M phosphate buffer at pH 8.6.

**Enzyme purification:** Enzyme was partially purified by dialysis process. The partially purified enzyme was subjected to Ion exchange chromatography with DEAE cellulose column (Borah *et al.*, 2012). Almost 50 fractions were collected. The fractions possessed high protein content and enzyme activity, were loaded on gel filtration column to obtain purified enzyme for therapeutic application. Analytical grade Sephadex G-100 was used as porous material which was buffered by 0.1 M Tris-HCl buffer to equilibrate column at pH 8.6. The fractions were collected with the flow rate of 30 ml/hour (Jamil *et al.*, 2007).

**Enzyme and protein estimation:** Protein content of enzyme in crude form, partially purified form, dialyzed and purified form was determined by Biuret method Gornall *et al.* (1949) using BSA as standard. Absorbance (O.D) of protein was obtained at 540 nm. Enzyme was assayed based on the conversion of L-asparagine to ammonia and L-aspartate at 37°C and pH 8.6 under specific condition. The absorbance was measured at wave length (450 nm) (Ren *et al.*, 2010).

**Kinetic Characterization of Enzymes:** The effect of pH at (5.5, 6.0, 6.5, 7.0 and 7.5) was recorded on the activity of enzyme. Reaction mixture of L-asparaginase obtained from *Capsicum annum* (fruit) was assayed at different temperatures (15°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C and 55°C) and asparagine concentration (10 to 300 mM) were studied. The optimization of reaction time required for the maximal quantity of L-asparaginase to stay active for the conversion of L-asparagine to L-aspartate was noted from 10 to 90 minutes at optimal temperature and pH (Moharib, 2018).

**Animal model:** Albino rats (*Rattus norvegicus*) were purchased from the local market and kept in the animal facility of the Institute of Pharmacy, Physiology and Pharmacology, University of Agriculture Faisalabad. Leukemia was induced by chemical carcinogen 7,12-Dimethylbenz[a]anthracene (DMBA). The dosage regimen was adopted following Huggins dosage system. Rats were chosen from average age of 50 days. Rats were housed at 26±1°C and divided into three groups (n=5) Efficacy of purified enzyme was tested in albino rats. T1

group had positive control (leukemic rats), T2 contained leukemic individuals which were treated with L-Asparaginase while T3 had normal rats which acted as negative control for leukemia (Oliveira *et al.*, 2015). After 20 days of trial, blood smears were taken on glass slides and stained by Giemsa stain (WHO, 2016). Blood smears were examined histo-logically. The recovery from leukemia was determined by observing rats physically and histologically.

## RESULTS

L-Asparaginase was extracted from fruit part of *Capsicum annum* using the homogenization method. Results portrayed in (Table 1) indicate activity of fruit extract at various purification steps i.e. crude, ammonium sulfate precipitation, dialysis, ion exchange and gel filtration. *Capsicum annum* exhibited pronounced enzyme activities after every step as shown in Table 2. Likewise, the specific activity of L-Asparaginase from *C. annum* was 234U/mg after gel filtration purification. *Capsicum annum* showed high activity of 112 U/mL bearing protein amount of 1.24 mg /mL in crude form, as shown in Table 1. The purification summary of *C. annum* is expressed in (table 2). The partially purified enzyme after ammonium sulphate precipitation and dialyzing against buffer was loaded to porous resin for ion exchange chromatography which exhibited activity of 105.12 U/mL, 80.44 U/mL and 76.45 U/mL respectively. Sample of 500 µL was used for Gel filtration by sephadex G-100 which resulted in purified therapeutic grade enzyme with 69.43U/mL activity for 0.296 mg/mL portion of protein. The percentage yield was 71.81%, 68.29% and 61.99% for dialysed, ionexchange purified and gel filtration purified enzyme, respectively. Enzyme purified after gel filtration from (*Capsicum annum*) fruit provided 61.99% yield and specific activity of 234 U/mg (Table 2).

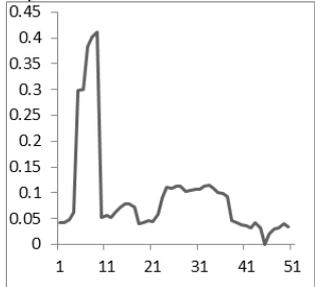
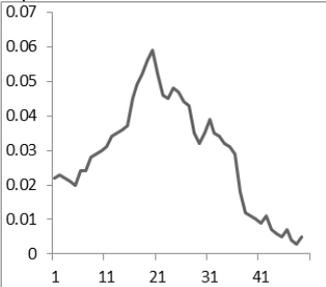
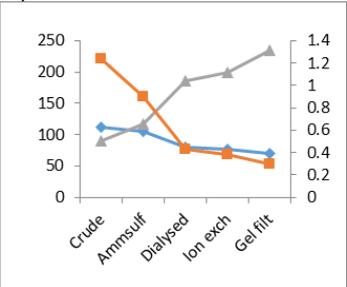
**Effect of temperature on L-Asparaginase Activity:** Results illustrated in Fig. 3b showed that the maximum activity of asparaginase was obtained when the temperature of the reaction mixture was 37°C. The incubated enzyme with substrate at various desirable temperature yielded maximum activity of 48.92 U/ml at 37°C.

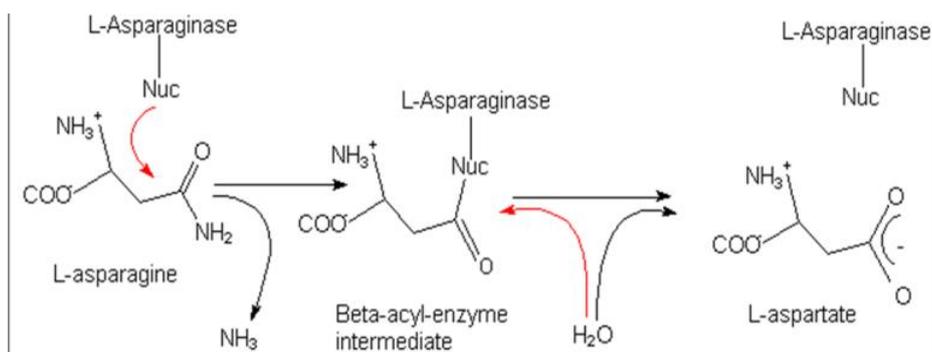
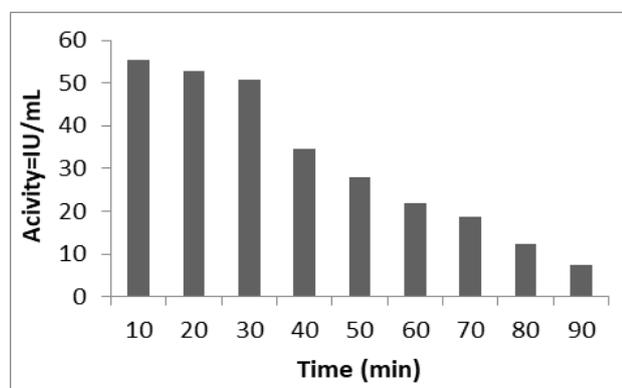
**Effect of pH on L-Asparaginase Stability:** L-Asparaginase was incubated at different physiological pH to obtain the optimal milieu for enzyme efficacy. At pH 8.6 enzyme showed enhanced activity of 51.23 U/mL (Fig. 3a).

**Enzyme response on substrate concentration:** Different substrate concentrations were incubated with enzyme, amongst them highest activity of enzyme achieved was, 16.57 IU/mL for 200mM concentration of substrate L-Asparagine (Fig. 3c).

Microscopic examination of blood smears indicated infiltration pattern similar to benign looking leukemia and lymphocytosis, as portrayed in Fig. 5, which have very less number of RBCs. Rats were irritable with skin rashes similar to allergic response after DMBA administration. The site of injection of (DMBA) caused skin lesions on tail region Fig. 4. These skin lesions specially developed on the dorsum of tail that subsided after treatment with L-Asparaginase as shown in Fig. 4.

**Table 1:** Extraction and purification summary of *Capsicum annuum*

Plant part	Purification tools		Enzyme activity (U/mL) and specific activity(U/mg), protein(mg) = y-axis
	Ion exchange chromatography	Gel filtration	
<i>Capsicum annuum</i>	<i>Capsicum annuum</i> –fruit	<i>Capsicum annuum</i> –fruit	<i>Capsicum annuum</i> –fruit
			
	*Horizontal axis title=Fractions *Vertical axis title=Absorbance	*Horizontal axis title=Fractions *Vertical axis title=Absorbance	Y1=Activity(U/mL), Sp activity(U/mg), Y2= Protein (mg/mL)

**Fig. 1:** Schematic representation of enzymatic action of L-Asparaginase on L-Asparagine.**Fig. 2:** Effect of reaction time on the activity of L-Asparaginase.**Table 2:** Purification summary of (*Capsicum annuum*) fruit

Steps in purification	Activity (U/mL)	Protein (mg)	Specific activity (U/mg)	Fold purification	% Yields
Crude extract	112	1.24	90.51	1	100
Ammonium sulfate	105.12	0.899	116.83	1.29	93.85
Dialysis	80.44	0.433	185.66	1.39	71.81
Ion exchange chrom	76.45	0.385	198.67	2.19	68.29
Gel filtration	69.43	0.296	234	2.85	61.99

## DISCUSSION

High enzyme amount is obtained from *Capsicum annuum*, which belong to family *Solanaceae*. Globally pepper is known for its high nutritional value, health benefits and medicinal properties. It is rich in vitamins and minerals, has antimicrobial and anticancer properties (Saleh *et al.*, 2018). The specific activity of crude enzyme in (*Capsicum annuum*) was 90.51 U/mg (Table 2), while on purification specific activity of 234 U/mg was achieved. The enzyme was 2.85 fold purified after

homogeneity and 61.99% yield was obtained. Enzyme purity is comparable to that reported by Bano and Sivaramakrishnan, (1980), where L-asparaginase from *C. annuum* had specific activity of 128 U/mg, with 320 times purified enzyme and 26% recovery. Shanmugaparakash, *et al.* (2015) recovered 567% purified enzyme from *C. annuum*, which was 6.83 times purified in an optimized system of parameters i.e temperature, pH and substrate concentration. The L-asparaginase obtained from green chilli resembles more with bacterial enzymes than mammalian. Asparaginases from *E. coli K 12* (Mashburn and Wriston, 1964; Schwartz *et al.*, 1996) and *Acinetobacter calcoaceticus*, both exist in two forms, but only one have antitumor potential (Oza, 2010).

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. 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 have maximum activity at 37°C, while the activity was decreased when the temperature was above optimum or below the optimal temperature, similar results as reported by other investigators that high substrate concentration indicates high activity of L-asparaginase in the treatment of the late stages of childhood acute lymphoblastic leukemia (Khalaf *et al.*, 2012). The studies revealed that maximal activity and stability of the enzyme is at 37 to 40°C. According to Khalaf *et al.* (2012) the proteins remained active under certain range of temperature. Shanmugaparakash *et al.* (2015) reported stability of enzyme L-Asparaginase till 45°C.

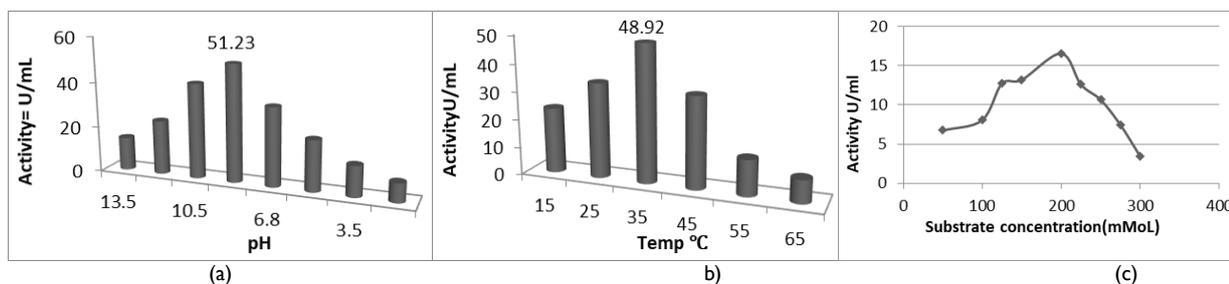


Fig. 3: Effect of a; pH, b; Temperature and c; Substrate concentration on the activity of L-Asparaginase.



Fig. 4: Condition of leukemic rat tail region before and after treatment with L-Asparaginase.

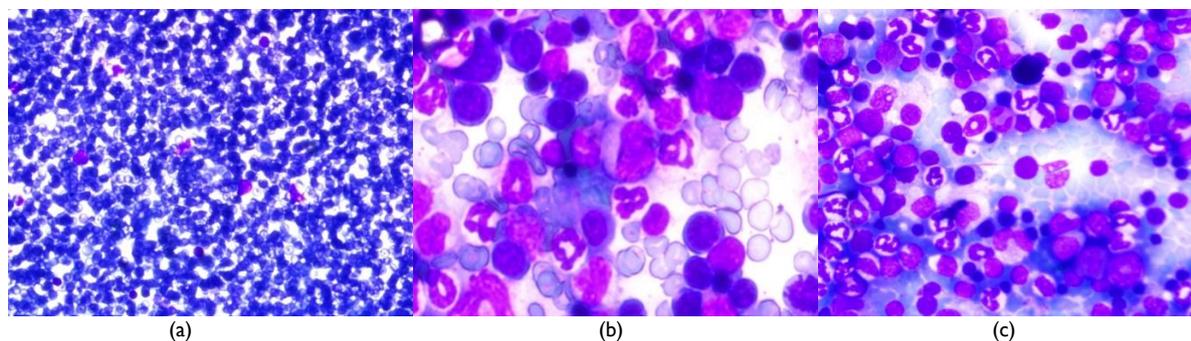


Fig. 5: Illustration of blood smears among three treatment groups after treatment (a) T3=normal non leukemic rats, (b) T2=L-Asparaginase administered leukemic rat,(c) T1=leukemic rat).

Effect of reaction time for L-asparaginase was determined by incubating the reaction mixture for different periods of time (10-90 minutes) at 37°C and 8.5 pH (Fig.2). The enzyme sample incubated till 30 min showed optimal enzyme activity. After 30 min the activity of enzyme depleted. The findings are in close comparison with Moharib (2018) who revealed 30 min optimal reaction time for L-asparaginase extracted from *Vigna unguiculata* seed.

Substrate concentration hold equal importance since the minimal concentration of substrate to the optimal concentration defines the enzyme activity. Substrate concentration of 200mMol was required for maximum L-Asparaginase activity. Different concentrations have been used by different researchers to determine the optimum substrate concentration for L- asparaginase activity. Results in (Fig. 3c) describe that the activity of L-asparaginase was increased gradually with the increase in L-asparagine concentration, then maximum activity of L-asparaginase 16.57 U/mL was obtained when the substrate concentration was 200mMol. The result agreed with Khalaf (2012), who revealed a proportionate relationship between the substrate concentration and enzyme activity. According to the graphical representation (Fig. 3c) the reaction rate increased with the increase of substrate concentration while the L-asparaginase concentration was

constant until the maximum rate achieved. Sometimes substrate inhibition occur when disproportionate amounts of substrate are added to the reaction mixture (Khalaf *et al.*, 2012). Few investigators (Khalaf *et al.*, 2012) established that high substrate consumption depicts the high enzymatic activity for the treatment of childhood lymphoblastic leukemia. While the substrate optimal concentration determined from *Capascium annum* L was 33mM, where catechol was acting as substrate in the mixture (Arnnok *et al.*, 2010). While Moharib (2018) has revealed 200mMol concentration of substrate for maximal activity of L asparaginase activity derived from *Vigna unguiculata*.

The purified enzyme showed highest enzyme activity 51.23U/mL at 8.6 pH. The pH effect on the activity L-Asparaginase enzyme was studied at 37°C. Enzyme exhibited increased activity between pH7.0 to 8.5, where as the enzymatic efficiency was decreased at low and high pH. Previously it was reported that plant L-Asparaginase optimal pH for high activity was found in the range of 8.2 to 8.5 (Al Zobaidy *et al.*, 2016). The Optimal pH for L-Asparaginase activity may differ subsequently to different strains or species. Shanmugaparakash and his coworkers (2015) envisaged that our therapeutic protein retained its activity over a broad range of pH from 5.0 to 9.0 which

also encompassed present investigation of optimal pH of 8.4 for chillies.

Leukemic group (T1) slide represented infiltration of benign looking leukemia and lymphocytosis, as portrayed in Fig. 4c. A decrease in lymphocyte count was observed in T2 group (Fig. 4b). Decrease in neutrophil level was observed in T2 group which is clear from slide of blood smear in Fig. 4, which is comparable to the blood smear of group T3 (normal rats) (Fig. 4a). After 20 days of study, slides illustrated hemolytic anemia and in some cases neutropenia and thrombocytopenia as sentenced by Moloney (1969). Neutrophils, eosinophils, and basophils have multilobed nucleus and granulation in cell cytoplasm characteristic of leukemia (Raymakers, 2017), are visible in Fig. 5.

**Conclusions:** *Capsicum annum* possess high titer of L-Asparaginase. The young fruit of above mentioned plant holds appreciable amount of L-Asparaginase activity which can be effective therapeutically. *In vivo model* indicated that *Capsicum annum* extract may be used in chemotherapy as anti-tumorigenic agent against tumors and Acute lymphocytic leukemia.

**Authors contribution:** AA and MF had conceived and designed the project and animal study. ZMA contributed in enzyme analysis. AA and MF executed the experiment and discussed the histopathology of tissue samples. All authors chiefly MF contributed critically revised the manuscript for their improvement of contents and approved the final version.

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