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L-ASPARAGINASE, ACRYLAMIDE QUENCHING ENZYME PRODUCTION FROM LEAVES OF *TAMARINDUS INDICA* AND SEEDS OF *VIGNA RADIATA–FABACEAE*

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Abstract

Current study aims at high yield production of L-Asparaginase from family *fabaceae.* Leaves of *Tamarindus indica,* seeds of *Vicia faba, Phaseolus vulgaris, Pisum sativum, Cicer arientinum, Vigna radiata* are screened for L-Asparaginase yield and activity. Prepared crude extracts are purified through ammonium sulfate precipitation, dialysis, ion exchange chromatography and gel filtration. When incubated with L-Asparagine, crude extracts of *Pisum sativum* leaves and seeds of *Cicer arientinum, Phaseolus vulgaris* and *Vicia faba* extracts showed 24.93 U/mL, 10.38 U/mL, 11.81U/mL enzymatic activity, respectively. Crude extract of *Tamarindus indica* leaves exhibited highest enzyme activity of 64.38 U/mL, with a yield of 67% while *Vigna radiata* seeds crude extract revealed 53% yield with enzymatic activity of 56.34 U/mL.L-Asparaginase enzyme purified from *Tamarindus indica* has activity of 54.260±0.847 U/mL with specific activity of 95.67 U/mg. L-Asparaginase purified from *Vigna radiata* seeds exhibit 45.140±0.302 U/mL activity and specific activity 135.25 U/mg. The purified enzyme exhibits maximal activity at 37℃, stability at optimal pH 8.6 in the presence of alkaline 0.2 M potassium phosphate buffer. Optimal substrate concentration for L-Asparaginase activity is 3.5 ug/mL from *Tamarindus indica.* Results shows that *Vigna radiata* and *Tamarindus indica* can be a better choice as high source of L-Asparaginase that can be employed to reduce acrylamide cancer provoking element in food industry.

Key words: L-Asparaginase, L-asparagine, Characterization, *Fabaceae*.

Introduction

L-asparaginase is widely distributed in microbial, animal and plant sources. It has been observed that prokaryotic microorganisms like yeast, bacteria i.e., *Esherichia coli*, *Erwinia carotovora*, *Bacillus* sp. and fungi as *Aspergillus terrus* have potential for asparaginase production (Moharam *et al*., 2010; Rajesh *et al*., 2011). L-Asparaginase is the (EC.3.5.1.1) asparagine amido hydrolase, which basically catalytically hydrolyses L-asparagine, amino acid and liberates ammonia and aspartic acid (Moharib, 2018) (Fig. 1).

The potassium dependent L-asparaginase is maximum in legumes bearing plant species which resembles microbial L-asparaginase (Khalaf *et al*., 2012). *Fabaceae (Leguminosae*) The pea family has nearly 17,000 species which are distributed world widely (Judd *et al*., 2002). The protein synthesis in these plant species requires high level of amino acids asparagine and glutamine. Synthesis of certain polypeptides comprising of L-asparagine, in germinating seeds of family (*Fabaceae*) is ruled by an enzyme asparagine synthetase. Asparagine synthetase has been isolated from the legume plants only as other plant organs lack it (Credali *et al*., 2012). Seeds of legumes have gained a considerable importance as protein source in the human diet of the peoples in many parts of the world. Moreover Fabaceaefamily seeds are grown in various parts of the world as dietary protein source (Moharib,2018), the reason plants were selected from the family of *Fabaceae*. Where potassium dependent asparaginase activity is maximum in the tissues requiring nitrogen for their growth as in seeds, rippen fruits and young leaves (Mohamed *et al*., 2017). Thus keeping in view the distinctive character of L-asparaginase activity was put forth in the selection of plants. Among six selected plants the enzyme derived from in expansive edible plant source will exhibit least toxicity as they had been used conventionally. In food industry the safety of processed food products from acrylamide contaminant can be assured by pretreatment with L-Asparaginase prior to baking (Miskiewicz *et al*., 2012). It degrades L-asparagine, the precursor of acrylamide, thus it protects the vital membranes, proteins, DNA from alkylating agent like acrylamides. The drastic effects of alkylation may lead to neurotoxicity, reproductive toxicity and carcinogenicity (Friedman, 2003).

In response to continuous demand of therapeutic enzymes in biotechnology and biological therapy for cancer treatment, the recent study is conducted to enhance L-Asparaginase production. It is desirable to extract from edible sources rather than from microbial and fungal source (Kumar & Sobha, 2012; Moharib, 2018). L-Asparagine (non-essential amino acid) is basic food of immature lymphocytes for their proliferation. Plant L-asparaginasecatalyses the hydrolysis of asparagine to aspartic acid leading to the apoptosis of leukemic and tumor cells (Ebeid *et al*., 2008; Oza *et al*., 2010; Parkash *et al*., 2013).

Materials and Methods

Materials: Plants (*Tamarindus indica*, *Vicia faba, Phaseolus vulgaris, Pisum sativum*), *Cicer arientinum*), *Vigna radiata*), Sephadex G-100 (Sigma Aldrich Chemical Co. USA), DEAE cellulose resin (Sigma Aldrich Chemical Co. USA), Dialysing membrane (Diapes), L Asparagine (Sigma-Aldrich),

Selection of plant parts for enzyme extraction: Seeds and leaves were selected as a plant part which exhibit high enzymatic activity on early growth. Seeds were purchased from the local market of Faisalabad and leaves were obtained from botanical garden of University of Agriculture Faisalabad (Pakistan).

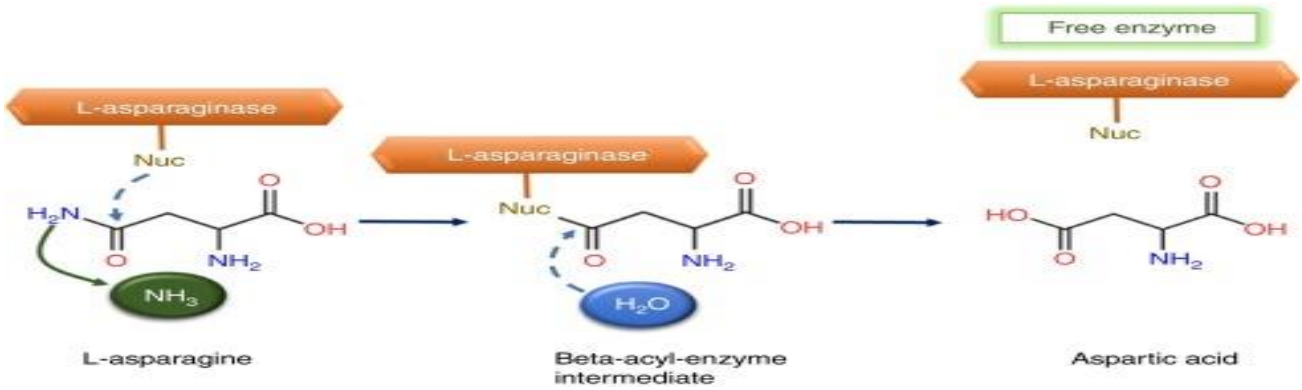


Fig. 1. Enzymatic mechanism of action of L-asparaginase on L-asparagine to yield aspartic acid (Shrivastava *et al*., 2016).

Crude extracts preparation from seeds and leaves

Seeds: Seeds of *Vicia faba*, *Phaseolus vulgaris*, *Pisum sativum*, *Cicer arientinum*, *Vigna radiata*) (5g each) were ground to give mixture of 25 ml crude extract (in distilled water). Mixture was brought to 0-80% saturation by solid ammonium sulfate. The crude extract was desalted *via* dialyzing membrane.

Leaves: The 5 grams leaves of *Tamarindus indica* were taken in powdered form and mixed with doubled volume of 0.05 M Potassium phosphate buffer at pH 8.6 containing 1.5 M sodium chloride, 1 mMphenylmethyl sulfonylfloride (PMSF) and 1 mM EDTA, whole mixture was homogenized (Nimkande *et al*., 2015**)**.The crude extracts were dialysed through dialysis membrane against distilled water.

Enzyme purification: After dialysis, the partially purified sample of 500µL was loaded to Ion exchange chromatography DEAE cellulose column at 25℃. Further 50 fractions of 2 mL each were eluted with the flow rate of 30 ml/hour (Ambreen *et al*., 2019), which were further analyzed for protein content at 280 nm. The fraction containing active protein content and enzyme activity were pooled and whole volume was subjected for lypholization for removal of extra water from protein. Lypholized sample of 500 µL was loaded on Sephadex G-100 gel filtration column. Column was calibrated with 0.1M Tris HCL buffer, to purify enzyme for further applications. Sephadex G-100 was used as porous material in column of (1.5 x 90 cm dimensions), and 0.1 M Tris-HCl buffer was loaded to equilibrate the column at pH 8.6 (Al-Zobaidy *et al*., 2016). The fractions collected were also analyzed spectrophotometrically at 540nm (Ambreen *et al*., 2019).

L-Asparaginase quantification: L-Asparaginase was assayed according to Nesslerization based on the rate of hydrolysis of asparagine by measuring released ammonia; one unit releases one micromole of ammonia per minute at 37°C and pH 8.6 under specific condition. The absorbance was measured at wave length (450 nm) (Ren *et al.*, 2010). Protein concentration in plant extracts and purified enzyme was determined by Biuret method of (Gornall & Nardwall, 1949). Bovine serum albumin was used as standard and absorbance was recorded at 540 nm.

$$\text{U/mg} = \frac{\text{Micromole of ammonia released}}{\text{10 min X mg enzyme in reaction}}$$

$$\text{Specific activity (unit/mg)} = \frac{\text{Activity (unit/ml)}}{\text{Protein concentration (mg/ml)}}$$

Results

Among the Fabaceae family, seeds and leaves from different of plant species were chosen. Leaves of *Tamarindus indica* were used for enzyme extraction also. The ion exchange chromatography revealed two to three peaks from individual plant extract (Table 1). The fractions bearing peaks at 280 nm also showed increased protein amount and maximal enzyme activity when analysed. After sephadex Gel filtration purified enzyme with maximum titer was elucidated from the singlet peak (Table 1).

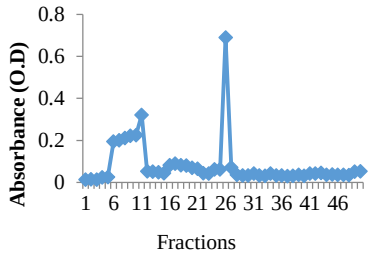
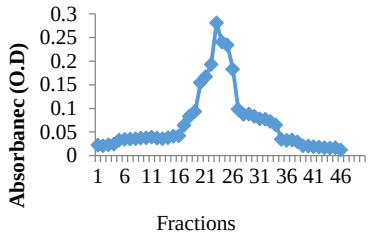
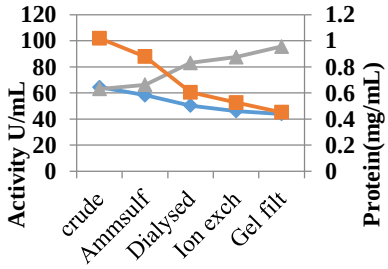
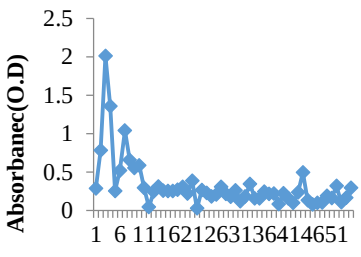
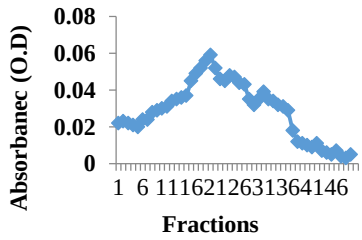
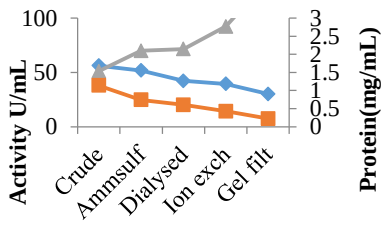
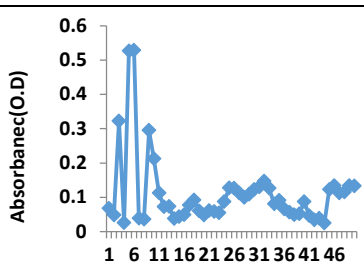
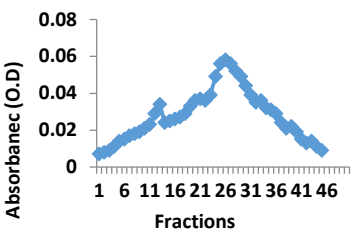
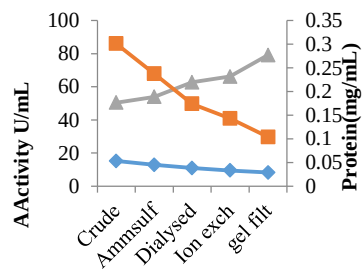
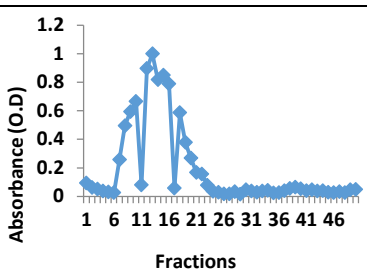
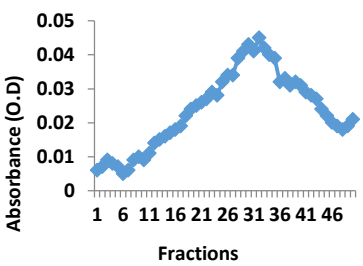
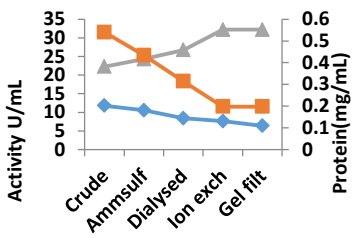
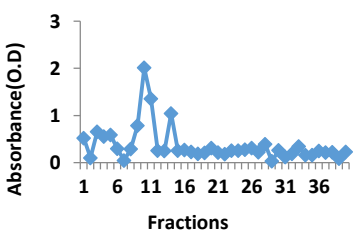
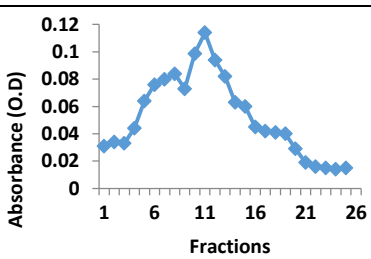
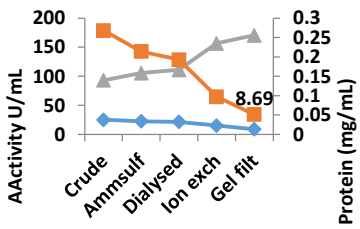
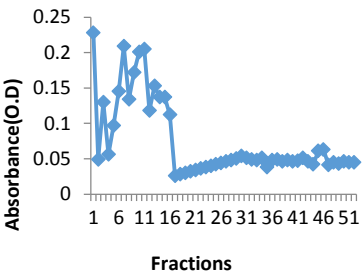
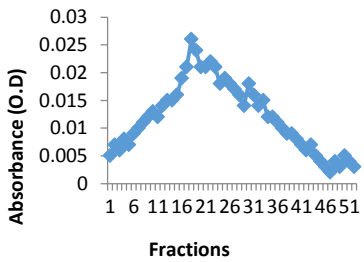
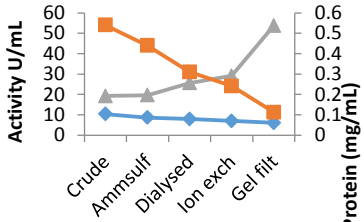
The results were expressed in terms of activity U/mL and specific activity U/mg of L-Asparaginase. All the plants activities were compared at every step of purification i.e. crude, ammonium sulfate precipitation, dialysis, ion exchange etc. (Fig. 2). *Tamarindus indica* showed highest activity followed by *Vigna radiata* (Fig. 2).

The order of mean activity among plants purified for L-asparaginase was (*Tamarindus indica)* 54.26 ± 0.847U/mL > (*Vigna radiata*) 45.14 ± 0.302 U/mL >(*Pisum sativum*) 14.38 ± 0.401 U/mL > *(Phaseolus vulgaris)* 11.795 ± 0.743 U/mL > *(Vicia faba)* 9.2825 ± 0.393 U/mL > *(Cicer arientinum*) 8.365 ± 0.743 U/mL as explained in Table 2.

By applying Tucky test the plants with highly significant value of activity are determined. The p value < 0.05 is rendering the results to be significant (Table 2). *Tamarindus indica* and *Vigna radiata* have delivered the highest yield as compared to the other family members of plants which can be commercially employed for the L-Asparaginase production. Statistical Analysis of plant L-Asparaginase activity was conducted by two way analysis of variance (ANOVA) followed by Minitab 13 version (Dielman, 2002) to identify any statistically significant differences in the L-Asparaginase enzyme activity.

The characterization of optimal parameters was conducted on the enzyme obtained from *Tamarindus indica*. Enzyme activity appeared to be maximum at 8.6 with the concentration of substrate to be 3.5 ug/mL. The optimal temperature for enzyme activity was elucidated as 37℃ (Fig. 3).

Table 1. Extraction and purification summary of under study plants.

Sr. No.	Purification tools		
	Ion exchange chromatography	Gel filtration	Enzyme purification summary at each step (*Blue = protein content, Red= Activity, Green= Specific activity)
1-*Tamarindus indica*			
2-*Vigna radiata*			
3-*Phaseolus vulgaris*			
4-*Vicia faba*			
5-*Pisum sativum*			
6-*Cicer arientinum*			

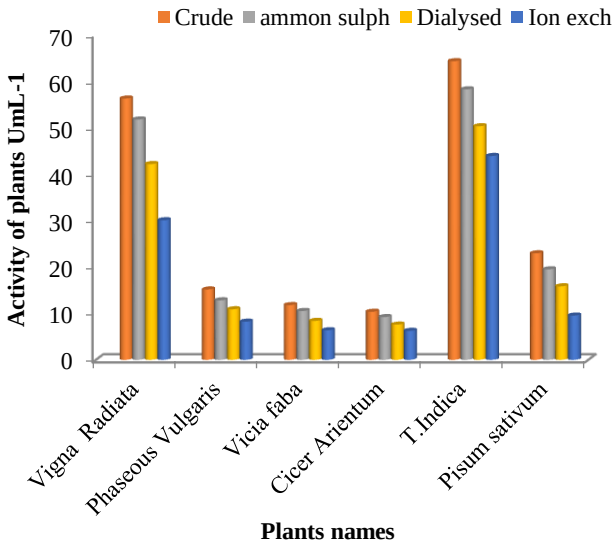


Fig. 2. Comparison of activities of selected plants at various steps of purification.

Discussion

Animal and human body can absorb the acrylamide through gastrointestinal tract GIT and lungs. Monomeric form of acrylamide is lethal for human body. Almost 25% of acrylamide dosage was absorbed through the skin, when studied for the effects in animal model. These acrylamide moieties when absorbed by body are converted to the epoxy derivatives by cytochrome P450 (Miskiewicz *et al.*, 2012). Enzyme L-asparaginase is reliable and efficient contender for acrylamide prevention to produce healthier and safe food (Mahajan *et al*., 2014). Many researchers have analysed that among starchy foods acrylamides are formed on frying. So in food processing industry a suitable agent should be employed to inhibit the acrylamide initiation reactions (Meghavarnam & Janakiraman, 2018).

Asparaginase (EC 3.5.1.1) is predominantly found in developing leaves and seeds where the enzyme catalyzes the enzymatic hydrolysis of asparagine into aspartate and ammonia which are then available for amino acid and polypeptide synthesis together with synthesis of other nitrogenous compounds (Chagas & Sodek, 2001). Use of this enzyme results into lowering of acrylamide content in the food. The *fabaceae* comprises mostly plants containing legumes which is characteristic of these family. They acquire maximally fixed nitrogen in the form of Asparagine which is metabolized by L-Asparaginase (Mohamed *et al*., 2017). Fabaceae includes *Phaseleous vulgaris* (soyabean), *Pisum sativum* (pea), *Glycyrrhiza glabra* (liquorice or Mulethi), *Medicago sativa* (alfalfa), *Arachis hypogaea* (peanut), *Cicer arietinum* (chick peas), lentils, broad beans, black beans, Mung beans and species of *Lupinus.* In germinating seeds and young leaves L-asparagine is the most abundant metabolite for organic nitrogen transport from site to the tissue sink. L-asparaginase action on L-asparagine releases ammonia for nitrogen mobilization (Khalaf *et al*., 2012). The reason why the seeds and young leaves were selected as concerned part of plant from Fabaceaewhichpossess significant quantity of L-asparaginase.

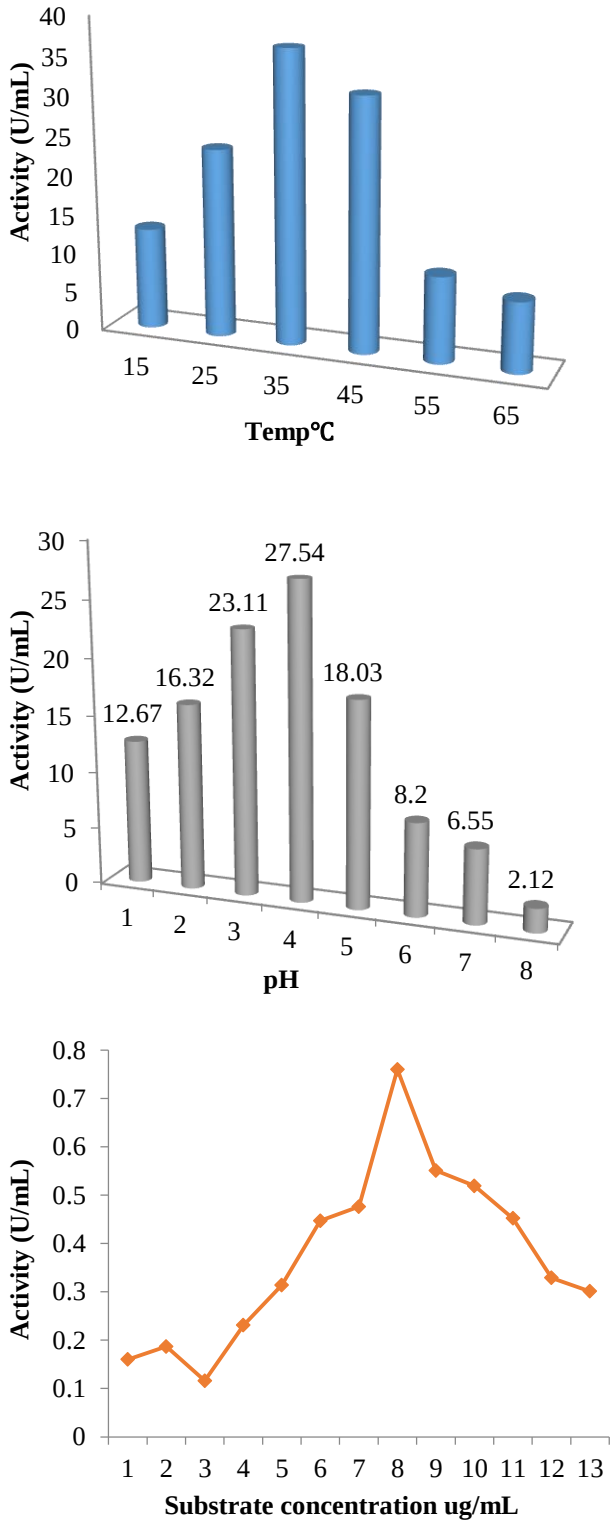


Fig. 3. Effect of a; temperature, b; pH and c; substrate concentration on theactivity of l-Asparaginase from *Tamarnidus indica*.

Among the all selected plants *Tamarindus indica* showed maximum titer of 64.38 U/mL of L-Asparaginase. *Tamarindus indica* is tropical plant mostly found in Southern Asia. Its stem bark, acidic fruit are used for remedial purpose of many ailments like yellow fever, body pain, jaundice, skin tonic and also as blood tonic. Its antimicrobial activity, phytochemical constituents are well established Doughari, (2006).

Our study showed *Tamarindus indica* leaves had activity 43.98U/mL of purified enzyme with protein concentration 0.451 mg/mL and specific activity 95.67U/mg while 67% was possible (Table 4). Kataria *et al.,* (2015) extracted enzyme from same plant which exhibited specific activity 44.68U/mg with 48.97 % recovery. Our results are comparable with the findings of Kataria *et al.,* (2015) as our yield and specific activity are greater than his findings. On the contrary our crude sample had 64.38 U/mL L-asparaginase with protein amount 1.02mg/mL (Table 5), while Kataria *et al*., gained activity of 1020.83 U/mL from crude extract of *Tamarindus indica* with protein content 22.82 mg. Bano & Sivaramakrishnan, (1980) reported 4.57 U/mL of L-Asparaginase.

After *Tamarindus indica*, *Vigna radiata* showed next highest enzymatic activity 56.34 U/mL of L-Asparaginase with protein concentration 1.14 U/mL which is lower than activity determined by (Kataria *et al*., 2015) 85.51U/mL. Nimkande *et al*., (2015) research provided enzyme L-asparaginase activity of 0.17U/mg from *Vigna radiata*. Moharib, (2018) extracted L-asparaginase from *Vigna unguiculata*, by using 60 ml of sample, which had protein content 8.4 mg, activity 309 U/mL and specific activity of 36.8 U/mg. In our study crude extract of *Vigna radiata* had 56.34 U/mL activity and protein content was 1.14 mg and specific activity reached 51.04 U/mg (Table 3). While according to Moharib the purified enzyme had 2.4 protein, 835.7 U/mL enzyme activity, 348.2 U/mg specific activity with fold purification 10 and 29% yield.The variation in the activity may also be contributed to volume of crude sample taken as different researchers have used different volume of extract. As in case of *vigna* purified by Moharib, (2018) which was analysed for enzymatic activity and yielded 835.7U/mL and specific activity 348.2 U/mg finally.

Our finally purified enzyme after using gel filtration chromatography had 30.14 U/mL activity showing protein content to be 0.222 mg and specific activity was 135.25 U/mg which is half of the specific activity in the enzyme purified by Moharib who used 6.4 ml of sample as compared to our study in which 0.5 ml sample was taken for gel filtration. Which finally gave 53% yield purification shown in table 5. L-Asparaginase purified from *Vigna unguiculata* is suitable as anticancer because it exhibited cytotoxic activity against different cancerous cell lines *In vitro* (Moharib, 2018).

Table 2. L-Asparaginase activities after purification (n=6, Replicates =3).

Sr. No.	Plants	Activities of purified enzyme(mean± SE)
1.	*Tamarindus indica*	54.260 ± 0.847^{A}
2.	*Vigna radiata*	45.140 ± 0.302^{A}
3.	*Phaseolus vulgaris*	11.795 ± 0.743^{BC}
4.	*Vicia faba*	9.283 ± 0.393^{BC}
5.	*Pisum sativum*	8.69 ± 0.40^{B}
6.	*Cicer arientinum*	8.365 ± 0.743^{CD}

* = Significant (p<0.05), ^{B,CD} non-significant, ^{A}Significantly important, Means sharing same letter are statistically non-significant

Table 3. Crude activities, specific activities and % yield of selected plants (n=6).

Sr. No.	Plants	Crude activity (U/mL)	Pure specific activity (U/mg)	Final %Yield
1.	*Pisum sativum*	24.93	82.76	34.85
2.	*Tamarindus indica*	64.38	95.67	67
3.	*Vigna radiata*	56.34	135.25	53
4.	*Cicer arientinum*	10.38	53.83	58.09
5.	*Phaseolus vulgaris*	15.19	79.13	54.18
6.	*Vicia faba*	11.81	32.19	54

Table 4. Purification summary of*Tamarindus indica* leaves.

Steps in purification	Activity (U/mL)	Protein (mg/mL)	Specific activity (U/mg)	Fold purification	% Yields
Crude extract	64.38	1.02	63.117	1	100
Ammonium sulfate ppt	58.3	0.879	66.27	1.05	95.6
Dialysis	50.37	0.605	83.12	1.31	78.25
Ion exchange chromatography	46.15	0.527	87.57	1.36	71.68
Gel filtration	43.98	0.451	95.67	1.51	67

Table 5. Purification summary of*Vigna radiate* seeds.

Steps in purification	Activity (U/mL)	Protein (mg/mL)	Specific activity (U/mg)	Fold purification	% Yields
Crude	56.34	1.14	51.04	1	100
Ammonium sulfate	51.84	0.741	69.95	1.37	92.08
Dialysis	42.24	0.604	71.58	1.41	74.97
Ion xchangechromatography	39.38	0.426	92.44	1.811	69.89
Gel filtration	30.14	0.222	135.25	2.64	53

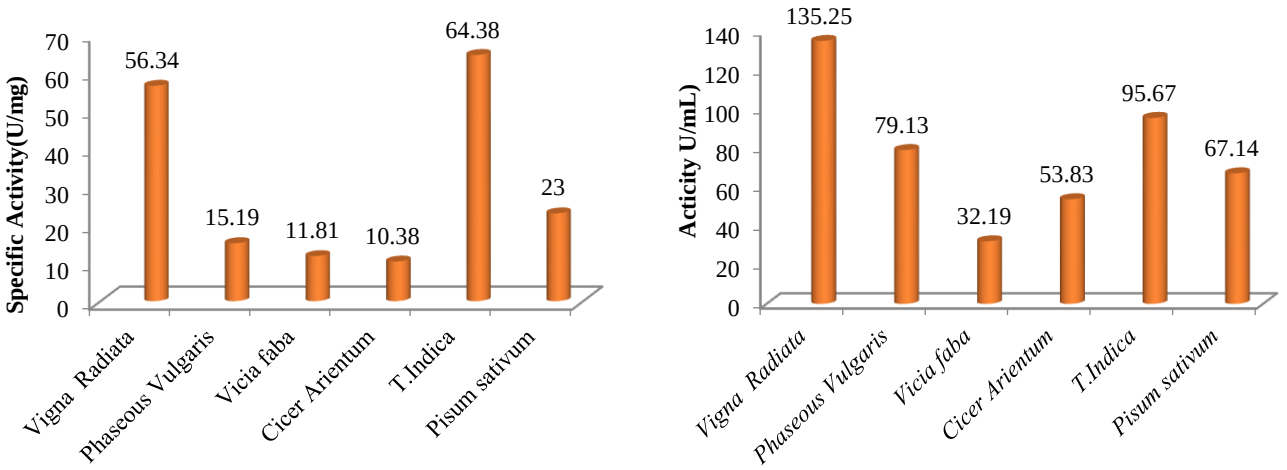


Fig. 4. (a) Activities of crude samples selected plants. (b) Specific activities of pure samples of selected plants.

The specific activities Fig. 4 (a and b) exhibited *Vigna radiate* seeds to be maximal enzyme producing plant. *Tamarindus indica*is next in specific activity.

Conclusion

Among members of Fabaceae family highest concentration of L-asparaginase is produced by *Vigna radiate* and *Tamarindus indica*. 24.9.

Recommendations: The isolated enzyme can be used for the treatment of acrylamides in food products along with control of untreated food.Mechanistic pathways involved in acrylamide production and its toxicity should be further studied *In vivo* models.

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