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Research Article

SCREENING AND IDENTIFICATION OF MEDICINAL PLANTS FOR L-ASPARAGINASE PRODUCTION

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ABSTRACT

L-Asparaginase a therapeutic protein is widely used in medical sector to diagnose and treat leukemia. It has been obtained from various sources like bacteria, fungi, yeast and plants. Microbial L-asparaginase has been found to be associated with toxicity and sensitivity due to its low specificity to asparagine. The side effects caused by microbial L-asparaginase bought medicinal plants as source into focus. In this study, L-asparaginase was screened in various medicinal plant parts in different seasons. Among all the screened plants, *Phyllanthus emblica* is identified as potential source for L-asparaginase production. The highest enzyme activity (20.3 IU/ml) and specific activity (5.2 IU/mg) was obtained in *Phyllanthus emblica* leaves during the non fruiting season. Further other medicinal plants like *Aegle marmelos* and *Citrus nobilis* contained appreciable amount of L-asparaginase. L-asparaginase from *Phyllanthus emblica* illustrated maximum activity at 37°C and 8.5 pH. Kinetic parameters, Km and Vmax of enzyme, were found to be 6.09mM and 88.12 μM/min, respectively. *Phyllanthus emblica* is recognized as novel source for L-asparaginase production.

INTRODUCTION

Over 5000 years back, plants were recognized as source for extraction of various naturally present biologically active compounds (Sumner and Judith, 2000). Among the biologically active compounds enzymes have evolved as a potential therapeutic agent and have also shown its importance in food, cloth and leather industries due to its various properties like high affinity, specificity and catalytic efficiency.

L-asparaginase, a tetrameric protein, belongs to the amidohydrolase family. The enzyme catalyzes the breakdown of L-asparagine to L-aspartic acid and ammonia. The primary structure of L-asparaginase was recognized and confirmed by Maita and Matsuda in 1980. On the basis of amino acid sequences and biochemical characterization, L-asparaginase enzyme can be categorized into microbial and plant type L-asparaginase (Borek and Jaskolski 2001). Various crystal structures of L-asparaginase have been obtained from a variety of organisms (Lubkowski *et al.,* 2003) and the conserved amino acid motifs were identified to be responsible for activity of the enzyme (Palm *et al*., 1996; Kozak and Jaskolski, 2000; Lubkowski *et al*. 2003). L-asparaginase from plants differs structurally from microbial L-asparaginase and has a different evolutionary origin. Plant L-asparaginase is found to be of two types, K^+-dependent asparaginase and K^+-independent asparaginase (Sieciechowicz *et al*., 1988; Michalska *et al.,* 2006).

Bacteria, fungi, yeast, actinomycete and plants have been identified as source of L-asparaginase. Since the time the antineoplastic acitivity of *E.coli* was demonstrated in guinea pig serum, microorganisms became highlighted as source for L-asparaginase (Broome, 1961; Roberts *et al*., 1968). Microorganisms such as bacteria, fungi, yeast, actinomycetes and algae have been observed as efficient sources of L-asparaginase. Commercially available L-asparaginase is obtained from *Escherichia coli* and *Erwinia carotovora* (Marlborough *et al*., 1975).

Plants have evolved as efficient source of enzymes as plant enzymes are easy to handle, have lesser chances of pathogenicity and can be used crude to develop drug formulations which saves a lot of cost and time. In 1970, Lees and Blakeney studied the distribution of L-asparaginase in *Lupinus leuteus* and *Dolichos lab lab* seedlings. L-asparaginase was extracted from soybean (*Glycine max* L.) leaf blades and root nodules using Na-phosphate (pH-7.3), Tris-HCl (pH-8) and Tricine (pH- 8) buffers. The extractions with different

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buffers were compared to optimize L-asparaginase extraction protocol. The accuracy of assay was checked with two-dimensional Thin Layer Chromatography (TLC) and Paper Chromatography (Streeter, 1977). Chilies (*Capsicum annum*) and tamarind (*Tamarindus indica*) were reported with high amounts of the enzyme when extracted using 3 volumes of 0.15 M KCl, while screening for different plant sources for L-asparaginase (Bano and Sivaramakrishnan, 1980). In another study, L-asparaginase was extracted from cotyledons and testae of *Pisum sativum* by employing extraction buffer containing Tris-HCl (pH 8.0), mercaptoethanol, Phenylmethylsulfonyl fluoride (PMSF), KCI, and glycerol (Sodek *et al*., 1980). *Withania somnifera* was identified as a potential source of L-asparaginase and its different cytotypes were compared (Oza *et al*. 2009; Verma *et al*., 2012). Germinating seeds of *Egyptian cowpea* cultivars had also been reported with high L-asparaginase specific activity (Ali, 2009). L-asparaginase was also found in *Citrus lemon* (Kumar *et al*., 2013) and *Solanum nigrum* (Kataria *et al*., 2015).

The detection of enzyme in plants parts can be assayed on the principle that L-asparaginase acts on L-asparagine and hydrolyzes it to L-asparatate and ammonia. Jayaram *et al*. (1974) reported three methods for the measurement of L-asparaginase which promised to be sensitive, speedy, easy and with acceptable precision. These include method based on Nesselrization of ammonia (Meister *et al.,* 1956), Calorimetric (Sheng *et al.,* 1993) and Fluorimetric assay (Ytinkangas and Mononen, 2000). Although Microbial sources have been proved as more efficient commercially available source for L-asparaginase production, but they are associated with side effects like anaphylaxis, pancreatitis, diabetes, neurological seizures, leucopoenia and coagulation abnormalities (Haskell *et al*., 1969). In comparison plant enzymes are predicted to be safer but the plant-type enzymes have been studied less thoroughly and thus an attempt has been made to search for novel sources of L-asparaginase among plants. In this paper, we report the identification of three novel plant sources for L-asparaginase production.

MATERIAL AND METHODS

Screening of Plants

Various medicinal plant species were screened for L-asparaginase enzyme activity and total protein content. Different plant parts like leaves, stem and fruits of screened plants were collected in different seasons in sterile polythene bags from Punjabi University, Patiala, Punjab, India. These plants are cultivated and are well documented by the Department of Botany, Punjabi University, Punjab. Selected medicinal plants for L-asparaginase screening are depicted in table1.

Extraction of L-asparaginase

Different parts from selected plants were collected and washed with distilled water. The parts were crushed and homogenized with 0.1M KCl buffer (pH 8.6). It was then centrifuged at 8000 RPM for 20 minutes at 4^{o}C and filtered. The supernatant thus obtained was taken as crude enzyme (Bano and Sivaramakrishnan, 1980).

Table 1 Season and Pharmacological relevance of screened Medicinal plants

S.No.	Plants	Flowering/Fruiting Season	Pharmacological Significance	Reference
1	Phyllanthus emblica	December -March	Acts as Antioxidant, Immune modulatory, Antipyretic, Analgesic, Cytoprotective, Anti ulser, Anti microbial, Immune modulatory, Anti inflammatory and gastroprotective. Plays active role in treatment of peptic ulcer, dyspepsia, jaundice, Pradara, diabetes	Jain *et al*., 2015
2	Aegle marmelos	May-June	Acts as Antidiarroheal, Antidysentric, Antipyretic, Antibacterial, Antiviral, Antioxidant, Radioprotective Activities and Anti Inflammatory activities. Posses biological potential against several diseases like Diabetes, Gastric ulcer and Hyperlipidaemia	Gupta *et al*., 2011
3	Citrus nobilis	December-January	Acts as Antimutagenicity and Anticancer agent	Entezari *et al*., 2014
4	Lagerstroemia speciosa	April-July	Plays active role in treatment of diarrhoea and abdominal pain, high blood pressure, diabetes and kidney ailments (eg. dissolving kidney stones), as health supplements, purportedly effective for blood sugar control and weight loss.	Chan *et al*., 2014
5	Tinospora cordifolia	September-October	Acts as Anti-Inflammatory, Anti-Arthritic, Anti-Cancer, Anti-HIV, Anti-Allergic, Anti-Malarial, Anti-Diabetic Anti-Impotency and Anti-Endotoxic agent	Saha and Ghosh, 2012
6	Dalbergia sisso	March-June	Acts as Anti-Inflammatory, Antipyretic, Analgesic, Anti-Oxidant, Anti-Diabetic and Antimicrobial Agent. Also involved in Gastro protective and Neuroprotective action	Bijauliya *et al*., 2017
7	Coriandrum sativum	January-February; June-December	Acts as Anxiolytic, Antidepressant, Sedative-Hypnotic, Anticonvulsant, Memory Enhancement, Neuroprotective, Antibacterial, Antifungal, Anthelmintic, Insecticidal, Antioxidant, Cardiovascular, Hypolipidemic, Anti-Inflammatory, Analgesic, Antidiabetic, Mutagenic, Antimutagenic, Anticancer, Gastrointestinal, Deodorizing, Dermatological, Diuretic, Reproductive, Hepatoprotective, Detoxification agent	Al-Snafi, 2016
8	Spinacia oleracea	March-April; November-February	Acts as Anti-Oxidant, Ant Proliferative, Anti-Inflammatory, Antihistaminic, CNS Depressant, Hepatoprotective agent. It is good for the heart and circulatory system and has energy-boosting properties	Metha and Belemkar, 2014
9	Hibiscus rosa-sinensis	May-October	Posses anticonvulsant property and is involved in lowering of blood pressure	Siddiqui *et al*., 2006
10	Eriobotrya japonica	March-May	Acts as Anti-Inflammatory , Anti-Diabetic , Anti-Cancer and Antioxidant agent	Liu *et al*., 2016
11	Capsicum annum	May-November	Antioxidant, Antimicrobial, Antiviral, Anti-Inflammatory and Anticancer.	Khan *et al*., 2014

Enzyme Assay

Nessler's Method based on estimation of ammonia released on breakdown of asparagine by the enzyme was adopted for L-Asparaginase assay (Meister *et al*., 1956). The reaction between Nessler's reagent (K_2HgI_4) and ammonia leads to production of pale yellow color. The color intensity is directly proportional to the amount of ammonia present. The standard graph of ammonium sulphate was plotted. Further the enzyme activity of crude enzyme was determined by Nessler's method and the intensity of pale yellow color was determined by taking absorbance at 480nm. The micromole of ammonia produced was determined from ammonium chloride standard curve.

Protein Estimation

In order to determine the specific enzyme activity the crude enzyme was subjected to protein estimation by Folin-Lowry's method with bovine serum albumin (BSA) as standard (Lowry *et al*. 1951).

Kinetic Characterization of L-asparaginase

Further L-asparaginase kinetics was studied to determine the effect of experimental parameters like pH, temperature and substrate concentration on the rate of reaction.

In order to determine the effect of pH on enzyme activity, the crude enzyme was incubated in 0.01 M Sodium Borate Buffer of different pH range (5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5). The reaction mixture was incubated at different temperature range (4-80^{o}C) for 15 mins in 0.01 M sodium borate buffer (pH 8.6) to determine the effect of temperature on reaction rate. The determination of effect of substrate concentration was achieved by Lineweaver-Burk's plot (Lineweaver and Burk, 1934) using different L-asparagine concentrations (1.0 to 10.0 mM).

RESULTS AND DISCUSSION

Potential plant source of L-asparaginase

The quantity of L-asparaginase enzyme in different parts of medicinal plants in different seasons is presented in Table 2. Among all plants, maximum L-asparaginase activity was observed in *Phyllanthus emblica* leaves during the non fruiting season. Appreciable amount of L-asparaginase was also observed in *Aegle marmelos, Citrus nobilis* and *Lagerstroemia speciosa, Coriandrum sativum* and *Dalbergia sisso.* However, trace amounts of L-asparaginase concentration was observed in *Tinospora cordifolia, Spinacia oleracea, Hibiscus rosa-sinensis* and *Eriobotrya japonica*. In all the plants, leaves came out as best source for extraction of enzyme in comparison to other plant parts.

Table 2 Comparative screening account of medicinal Plants for L-asparaginase production

S.No.	Plants	Parts	Season	Enzyme Activity (IU/ml)±SD
1	*Phyllanthus emblica*	Leaves	Non fruiting Season	20.3±0.42
			Fruiting Season	18.5±0.51
		Stem	Non fruiting Season	9.2±0.21
		Fruit	Fruiting Season	11.5±0.26
2	*Aegle marmelos*	Leaves	Non fruiting Season	19.4±0.65
			Fruiting Season	12.1±0.54
		Stem	Non fruiting Season	9.2±0.23
		Fruit	Fruiting Season	8.9±0.19
3	*Citrus nobilis*	Leaves	Non Fruiting Season	18.5±0.55
			Fruiting Season	12±0.51
		Stem	Non Fruiting Season	10.1±0.31
		Fruit	Fruiting Season	7.4±0.28
4	*Lagerstroemia speciosa*	Leaves	Non Flowering Season	16.2±0.36
			Flowering Season	13.8±0.31
		Stem	Non Flowering Season	5.5±0.11
		Flowers	Flowering Season	3.2±0.08
5	*Tinospora cordifolia*	Leaves	Non Fruiting Season	12±0.37
			Fruiting Season	11.1±0.34
		Stem	Non Fruiting Season	6.01±0.24
		Roots	Fruiting Season	7.3±0.23
6	*Dalbergia sisso*	Leaves	Non Fruiting Season	13.8±0.42
		Stem	Non Fruiting Season	10.2±0.22
7	*Coriandrum sativum*	Leaves	In Season	13.8±0.37
		Stem	In Season	5.01±0.03
		Roots	In Season	6.48±0.11
8	*Spinacia oleracea*	Leaves	In Season	6.9±0.21
		Stem	In Season	3.9±0.09
		Roots	In Season	3.2±0.12
9	*Hibiscus rosa-sinensis*	Leaves	Non Flowering Season	6.48±0.25
			Flowering Season	5.9±0.08
		Stem	Non Flowering Season	5.01±0.07
		Flower	Flowering Season	5.3±0.12
10	*Eriobotrya japonica*	Leaves	Non Fruiting Season	10.08±0.18
			Fruiting Season	7.4±0.22
		Stem	Non fruiting Season	5.5±0.06
		Roots	Non fruiting Season	4.01±0.03
11	*Capsicum annum*	Leaves	Non Fruiting Season	9.3±0.27
			Fruiting Season	7.3±0.22
		Stem	Non fruiting Season	4.9±0.15
		Fruit	Fruiting Season	9±0.11

Each value is represented as means ± SD, Sample Size = 3

Also the maximum enzyme activity in leaves was observed during the non fruiting season. So it could be predicted that L-asparaginase is found in higher concentration in plants before the fruiting season when new proteins are synthesized. L-asparaginase from *Capsicum annum* was extracted as standard.

Protein Estimation

The leaves of screened plants showed maximum enzyme activity. The leaves of *Phyllanthus emblica* showed maximum specific activity of 5.2IU/mg for L-asparaginase enzyme. Appreciable enzyme specific activity was observed in *Aegle marmelos* and *Citrus nobilis* (Table 3).

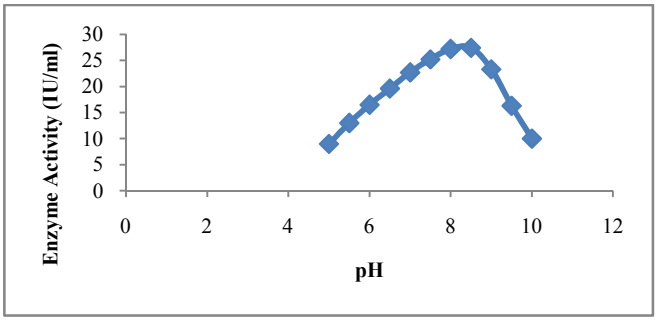

Fig 3 Effect of pH on L-asparaginase activity extracted from *Phyllanthus emblica*

Table 3 Protein content and specific activity of L-asparaginase isolated from Medicinal plants

S.No.	Plants(leaves)	Enzyme Activity (IU/ml)±SD	Protein Content (mg/ml) ±SD	Specific Activity(IU/mg) ±SD
1	*Phyllanthus emblica*	20.3±0.42	3.9172±0.12	5.2±0.21
2	*Aegle marmelos*	19.4±0.65	4.037±0.22	4.8±0.3
3	*Citrus nobilis*	18.5±0.55	4.1372±0.23	4.47± 0.22
4	*Lagerstroemia speciosa*	16.2±0.36	4.1372±0.25	3.91±0.07
5	*Tinospora cordifolia*	12±0.37	3.337±0.14	3.59±0.16
6	*Dalbergia sisso*	13.8±0.42	4.0172±0.18	3.425±0.16
7	*Coriandrum sativum*	13.8±0.37	3.1572±0.09	3.92±0.19
8	*Spinacia oleracea*	6.9±0.21	3.477±0.11	1.984±0.07
9	*Hibiscus rosa-sinensis*	6.48±0.25	1.917±0.05	3.38±0.14
10	*Eriobotrya japonica*	10.05±0.18	3.717±0.08	2.7±0.07
11	*Capsicum annum*	9.3±0.27	3±0.09	3.1±0.12

Each value is represented as means ± SD, Sample Size = 3

Kinetic Characterization of L-asparaginase

The maximum enzyme activity was obtained in leaves of *Phyllanthus emblica*. Therefore, the kinetic parameters of L-asparaginase enzyme extract from *Phyllanthus emblica* was determined by varying the pH, temperature and substrate concentration. Using Lineweaver-Burk plots (Fig 1) Km and Vmax for enzyme were found to be 6.09 mM and 88.12µM/min respectively. Temperature of 37 ° C (Fig 2) and 8.5pH (Fig 3) were found to be optimum for L-asparaginase activity.

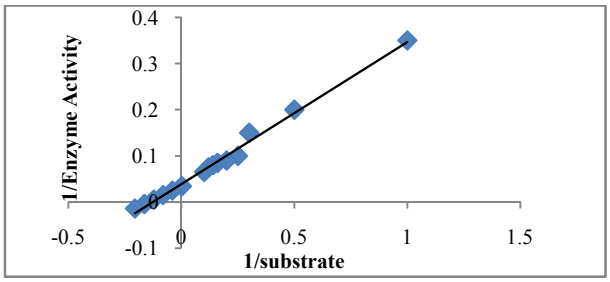

Fig 1 Lineweaver–Burk Plot for the determination of Km and Vmax for L-asparaginase from *Phyllanthus emblica*

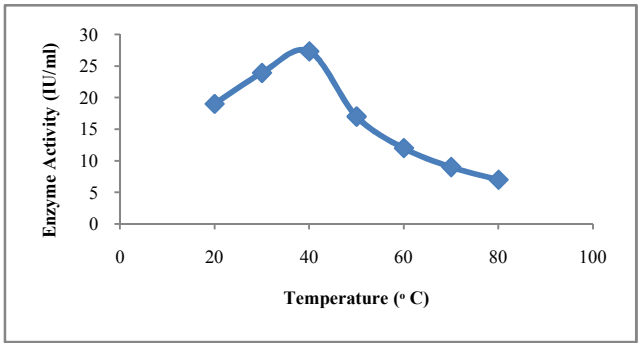

Fig 2 Effect of Temperature on L-asparaginase activity extracted from *Phyllanthus emblica*

DISCUSSION

L-asparaginase is an enzyme of high therapeutic value due to its use in leukemia treatment (Kumar *et al*., 2013). Bacteria, plants, actinomycetes, yeast and fungus have been recognized as source of L-asparaginase. Various medicinal plants have been reported to show anti cancerous properties. Therefore, different medicinal plants were screened for the presence of L-asparaginase. Among all the screened plants maximum quantity of L-asparaginase is found in *Phyllanthus emblica.* The maximum L-asparaginase activity was found to be 20.3 IU/ml in *Phyllanthus emblica*. Anticancer properties of fruit extract of *Phyllanthus emblica* have been reported by Zhao *et al*. (2015). Considerable concentration of L-asparaginase was recorded in *Aegle marmelos* and *Citrus nobilis.* This is the first report on presence of *L-asparaginase* in these medicinally important plants. Further in order to determine the enzyme purity specific activity of screened plants were determined. Among all the highest specific activity of 5.2 IU/mg was obtained in *Phyllanthus emblica.*

The extract from *Phyllanthus emblica* showed maximum activity at 37°C and 8.5 pH. The optimum pH and temperature were found to be comparative with that of L-asparaginase extracted from various microorganisms (Kamble *et al*., 2006; Kumar *et al*., 2011; Makky *et al*., 2006). Similar optimum pH and temperature have been reported for L-asparaginase extracted from various plants (Ali, 2009; Mohammad *et al*., 2015).

The Km value for enzyme was found to be 6.09 mM with 88.12 µM/min Vmax. Comparable Km value of 6.6 and 7.0mM has been reported for *L. arboreus* and *L. angustifolius*, respectively (Chang and Farnden, 1981). The Km value was also found to be comparative with Km value of L-asparaginase from *Escherichia coli* (3.5 mM) and *Erwinia carotovora* (7.14 mM) (Willis and Woolfolk, 1974; Kamble *et al*., 2006). L-

asparaginase from *Phaseolus vulgaris* seeds also showed similar Km and Vmax values of 6.72mM (Mohammad *et al*., 2015).

CONCLUSION

L-asparaginase extracted from *Phyllanthus emblica* showed comparative similarities with bacterial L-asparaginase. The enzyme catalyzed reactions depends on various parameters like temperature, pH and substrate concentration. Optimum temperature of 37^{o}C and 8.5 pH lies within the conditions required for medically useful asparaginase. Also, a low Km value of 6.09 mM with 88.12 µM/min Vmax illustrates high affinity of enzyme towards the substrate. Moreover, plant enzymes have lesser chances of pathogenicity and side effects, thus L-asparaginase extracted from *Phyllanthus emblica* could act as potential therapeutic protein.

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