ACS OMEGA

Open Access

http://pubs.acs.org/journal/acsodf

Article

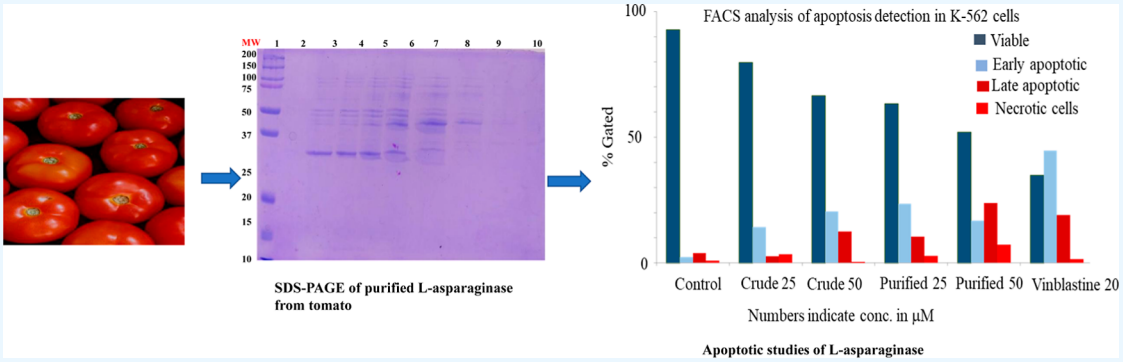
L-Asparaginase from *Solanum lycopersicum* as a Nutraceutical for Acute Lymphoblastic Leukemia

Sarina P. Khabade, Divijendra Natha Reddy Sirigiri,* and Anshu Beulah Ram

Cite This: *ACS Omega* 2024, 9, 3616−3624

Read Online

ACCESS | Metrics & More | Article Recommendations



ABSTRACT: L-Asparaginase (E.C. 3.5.1.1) is an indispensable analeptic anticancer enzyme used as an amalgam with additional cancer medicines for the cure of acute lymphoblastic leukemia (ALL). The presence of LAparaginase in tomato was confirmed byWestern blotting and DNA sequencing. The L-Asparaginase gene from tomato has been deposited in the NCBI database with accession number: OR736141. Crude enzyme was extracted from the fruit pulp of *Solanum lycopersicum,* and the activity was determined by the Nesslerization method. Further, the crude extract was subjected to purification, and kinetic parameters were studied. The percentage yield was calculated to be 6.457, and the purification fold was 0.086. The enzyme showed maximum activity at optimum pH 7.0, optimum temperature 37 °C, and incubation time of 05 min. The Michaelis constant "K_m" and maximum velocity "V_{max}" values were determined by the Lineweaver−Burk plot, which showed a low K_m value of 0.66 and V_{max} of 3.846 IU. Cytotoxic studies were carried out for crude and purified L-asparaginase. Purified L-Asparaginase has exhibited anticancer activity against the ALL model system, K-562 cell line, comparable to that of the anticancer compound vinblastine. Hence, L-Asparaginase from the fruit extract of tomato could be used as a nutraceutical to support cancer treatment in acute lymphoblastic leukemia.

1. INTRODUCTION

L-Asparaginase (E. C. 3.5.1.1) catalyzes the hydrolysis of L-asparagine into L-aspartate and ammonia. It reduces the bioavailability of asparagine to eliminate the profusely proliferating cancer cells.[1] The metabolism of tumor cells is disturbed as they are deprived of L-asparagine, leading to their rapid depletion.[2] L-Asparaginase is therefore an effectual antineoplastic enzyme used in synergic chemotherapy for various cancers, particularly acute lymphoblastic leukemia (ALL). Five various preparations of L-Asparaginase are currently in commercial use. Erwinase and rylase are derived from *Erwinia chrysanthemi* and three others derived from *Escherichia coli*. Among the three, one is a native and the other two are pegylated formulations presently used in pediatric ALL.[3−7] The overall survival rate of ALL with this treatment is reported to be 90%, with the complete remission in standard risk pediatric ALL.[8] The global L-Asparaginase market in 2021 was reported to be around US$ 564.01 million and is expected to grow up to US$ 1575.62 million by 2028.[9] Though commercial L-Asparaginase is a powerful drug in the treatment of ALL, it is associated with unpleasant side effects that include hepatic dysfunction, pancreatitis, thrombohemorrhagic complications related to the depletion of coagulation factors, hyperglycemia, cerebral dysfunction, and hypersensitivity reactions.[10,11] Thus, bioprospecting for new sources of L-Asparaginase with anticancer activity and less immunogenicity is required. L-Asparaginase from edible plant sources can circumvent hypersensitivity reactions and thereby can be preferred over microbial L-Asparaginase from edible plant sources can circumvent hypersensitivity reactions and thereby can be preferred over microbial L-asparaginases.[12] Oza et al.

Received: October 2, 2023
Revised: November 25, 2023
Accepted: December 27, 2023
Published: January 5, 2024

Table 1. Primers Used for the Amplification of L-Asparaginase from Tomato

I set	forward primer	GAGCTTCGTGAGCCAAGAGA	994 bp
	reverse primer	TGACCAGCCTAGCCAGAGAT	
II set	forward primer	GCAGGTGCGGGAACTAGAAA	1021 bp
	reverse primer	GACTTGAGTTGCCAAGAAGGG	
III set	forward primer	AGCCTCAAGGGTTCTTTTTCAGA	1053 bp
	reverse primer	TGTGTGGAGTTTATAGTTATGGGA	

cloned the gene L-Asparaginase from *Withania* into *E. coli* that showed a high specific activity of 17.3 IU/mg.[13,14] Additionally, L-asparaginase is found in the leaves and rhizome of *Asparagus racemosus* and also in the leaves of *Chromolaena odorata*.[15,16] About 35,000 plant species have been researched for anticancer activity like taxol, vinblastine, and vincristine.[17] Solanaceae members have been documented to have powerful anticancer activity against various cancer cell lines.[18]

Tomatoes of the Solanaceae family are natural nutraceuticals that decrease the threat of many types of cancer such as lung, prostate, pancreatic, cervical, breast, oral, colorectal, esophageal, and stomach.[19] However, how tomatoes work against cancer is still not known. In this context, the objective of the present study is isolation, purification, biochemical characterization, and cytotoxic study of L-asparaginase from the fruit pulp of tomatoes. In this investigation, we have successfully isolated L-Asparaginase, studied its biochemical properties, and assessed its effect on the myelogenous leukemic cancer cell lines.

2. EXPERIMENTAL SECTION

2.1. Extraction, L-Asparaginase Assay, and Effect of Various Parameters on its Activity. One gram of tomato pulp was weighed, washed, and ground with two volumes of 0.1 M phosphate buffer of pH 7.5 at 3 °C containing 5 mM dithiothreitol (DTT) and 1 mM ethylenediamine tetra acetic acid (EDTA). The homogenate was centrifuged at 12,000 rpm for 30 min at 2−3 °C, and the supernatant served as the crude enzyme extract.[20]

L-Asparaginase assay was carried out by adding 0.5 mL of 100 mM asparagine, 0.5 mL of 50 mM potassium phosphate buffer of pH 8.0, and 0.5 mL of enzyme extract to a "Test" tube, and a "Blank" tube was also prepared without adding the enzyme. The tubes were incubated for 15 min at 37 °C, and 0.25 mL of 20% trichloroacetic acid (TCA) was added to stop the reaction. Clarified by centrifugation, 3 mL of distilled water and 1 mL of Nessler's reagent were added to the supernatant. Read A_{460} of "Test" versus the "Blank" and the amount of ammonia released were determined by the ammonium sulfate standard graph,[20] and the total protein was estimated by Bradford's method.[21]

L-Asparaginase assay by the modified Nesslerization method was performed at various temperatures ranging from 4°C to 100 °C, at the incubation time over a narrow range of 5−30 min, with pH stretching from 5 to 10, and the effect of NaCl, $MgCl_2$, and $AgNO_3$ on L-Asparaginase activity was studied. The Michaelis constant K_m and maximum velocity V_{max} were calculated using the Lineweaver−Burk plot with the substrate concentration range of 1−5 mM.[22] All the experiments were done in triplicates, and the standard error was calculated using MS excel.

2.2. Purification of L-Asparaginase. The crude enzyme extract was subjected to a series of purification steps. The crude extract was salted out using 100% ammonium sulfate saturation. The pellet obtained was suspended in 10 mM Tris HCl at pH 8 and further dialyzed in a dialysis membrane (8 kDa cutoff) activated using sodium bicarbonate. Dialysis was performed for about 3 h, and water was changed every half an hour to avoid equilibration establishment. The dialyzed sample was further purified by ion exchange chromatography. Positively charged diethylamino ethyl (DEAE)-cellulose resin was used as a matrix because L-Asparaginase is a negatively charged protein. The mobile phase used was the elution buffer consisting of six different concentrations of NaCl solution (25, 50, 75, 100, 125, and 150 mM) and 25 mM Tris-HCl. The dialyzed sample was introduced into the column, followed by elution buffer for protein separation. The eluted sample was then subjected to gel filtration chromatography. Sephadex G75 was used as the gel bead matrix, and the eluted sample was loaded after the matrix settled. 100 mM phosphate buffer (pH 7.0) was used to stabilize the protein, and the samples were collected every 5 min.[23,24] Total protein was estimated by Bradford's method,[21] and enzyme assay was carried out by the Nesslerization method for all the samples to determine both the enzyme and specific activity.

2.3. Western Blotting. Polyclonal L-asparaginase antibodies were procured from Thermo-Fisher Scientific, and Western blot was carried out with chemiluminescence detection. The crude protein was purified by acetone precipitation, followed by gel filtration chromatography, and the purified samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).[25]

12% SDS-PAGE gel was cast, and 100 μg lysates was loaded on the SDS-PAGE system. The gel was run at 10 mA for good separation of lysates. After the gel run was complete, the required size of the poly(vinylidene fluoride) (PVDF) membrane was cut and activated first by immersing the membrane in carbinol/methanol for 2 min. The membrane was then immersed in transfer buffer (10% 10× Tris-Glycine solution, 20% methanol, and 70% water) to remove the upper oily layer. An appropriate size of blotting paper was also cut and immersed in the transfer buffer. The transfer was set up by sandwiching the membrane and the gel between the wet blotting papers. The gel was placed on the negative electrode side, while the membrane was placed on the positive electrode side. The transfer was carried out for 3 h at 150 mA in a cold room. The blot was taken out and blocked with 5% BSA for 90 min at room temperature, followed by incubating the membrane with a primary antibody overnight at 4 °C (1:2500 for IL33 antibody). The blot was washed thrice with Tris-buffered saline with 0.1% Tween 20 detergent (TBST) with 10 min intervals. The blot was incubated with a secondary antibody (1:5000) for 1 h, followed by TBST washing thrice. The blot was then developed by enhanced chemiluminescence (ECL kit; cat no.: 32132; Thermo Fisher Scientific) as per the manufacturer's protocol.

2.4. DNA Isolation, Amplification, and Sequencing of L-Asparaginase Gene. Five grams of tomato pulp was

https://doi.org/10.1021/acsomega.3c07633
ACS Omega 2024, 9, 3616−3624

ground, and the homogenate was preheated at 65 °C with DNA extraction buffer [1 M Tris HCl(pH-8); 0.5 M EDTA (pH-8); 10% SDS; 5 M NaCl; 0.2% mercaptoethanol] for 30 min. 5 mL of 5 M potassium acetate was added, mixed, and an equal volume of chloroform: isoamylalcohol (24:1) was added, spun at 3000 rpm for 20 min, and this step was repeated. An equal volume of chilled ethanol was added to the supernatant, washed, and DNA was precipitated and incubated overnight at 4 °C, centrifuged at 12,000 rpm for 40 min, washed with 70% ethanol twice, and air-dried the pellet overnight. The DNA pellet was reconstituted with Tris-EDTA (TE) buffer. Three sets of primers were designed and synthesized using NCBI Primer tool, as shown in Table 1. Genomic DNA isolated from tomato pulp was used for amplification, and the amplicons were sequenced by Sanger's method using an Applied Biosystems (ABI) 3730 xl DNA analyzer.[26,27]

2.5. MTT Assay. K-562 cell lines were procured from American type culture collection (ATCC); stock cells were cultured in Rosewell Park Memorial Institute (RPMI) supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 μg/mL) in a humidified atmosphere of 5% CO_2 at 37 °C until confluent. The cell was dissociated with a cell-dissociating solution [0.2% trypsin, 0.02% EDTA, and 0.05% glucose in phosphate-buffered saline (PBS)]. The cell viability was checked, and 50,000 cells/well were seeded in a 96-well plate and incubated for 24 h at 37 °C in a 5% CO_2 incubator. The monolayer cell culture was trypsinized, and the cell count was adjusted to 5.0×10^5 cells/mL. 100 μL of the diluted cell suspension (50,000 cells/well) was added to each of the 96-well microtiter plate. After 24 h, a partial monolayer formed was washed with the medium, and 100 μL of different test concentrations of drugs was added. The plates were then incubated at 37 °C for 24 h in 5% CO_2 atmosphere. After incubation, the test solutions were discarded, and to each well, 100 μL of MTT (5 mg/10 mL of MTT in PBS) was added. The plates were incubated for 4 h at 37 °C in 5% CO_2 atmosphere. Then, the supernatant was flicked off, and 100 μL of dimethyl sulfoxide (DMSO) was added, and the plates were shaken gently to solubilize the formazan formed. The absorbance was read at 590 nm using a microplate reader. The growth inhibition percentage was calculated by using the following formula, and the test drug concentration required to inhibit cell growth by 50% (IC_{50}) value was generated from the dose−response curves for the cell line.[28,31]

$$\% \text{ Inhibition} = ((\text{OD of control} - \text{OD of sample})/\text{OD of control}) \times 100$$

2.6. Apoptosis Assay. Cells were seeded at a concentration of 1×10^6 per 35 mm dish and incubated at 37 °C, 5% CO_2 for 24 h. The confluent cells grown after 24 h of incubation were treated with a crude sample and purified L-asparaginase with concentrations of 25, 50 μg/mL, and control. After treatment, the cells were trypsinized and collected by spinning at 2000 rpm for 5 min. The cell pellet was suspended in 0.5 mL of lysis buffer (pH 7.8) (Tris-HCl 10 mM, pH 8; EDTA 20 mM, pH 8.0; TritonX-100 0.2%, 4 M NaCl), vortexed vigorously, and incubated at 50 °C for 5 min. To the lysate, 0.5 mL of phenol−chloroform−iso-amyl alcohol was added, mixed, and centrifuged at 10,000 rpm for 15 min at 4 °C. Two volumes of cold 100% ethanol and 3 M sodium acetate were added to the upper aqueous layer. It was centrifuged at 10,000 rpm for 15 min, and the DNA pellet was washed in 70% ethanol and spun at 5000 rpm for 10 min. The DNA pellet was air-dried and was dissolved in TE buffer (Tris-HCl 10 mM, pH 7.4, EDTA 1 mM, pH 8.0) and separated by 2% agarose gel electrophoresis at 100 V for 50 min.[29]

2.7. Gene Regulation of Caspase 3 Genes in K562 Cells Treated with the Sample. K562 cell line was obtained from ATCC, and cells were cultured in RPMI-1640 supplemented with 10% inactivated fetal bovine serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, and penicillin (100 IU/ml) in a humidified atmosphere of 5% CO_2 at 37 °C until confluent.

2.7.1. Treatment for Gene Expression. Total RNA from K562 cells was extracted using trizol reagent. K562 cells were washed twice with PBS and centrifuged at 2000 rpm for 5 min. To the cell pellet was added 1 mL of trizol (per P_{35} dish), and the resulting lysate was collected. To the reaction mixture, 0.2 mL of chloroform was added and vigorously mixed for 15 s, centrifuged at 10,000 rpm for 15 min at 4 °C. The upper aqueous phase was treated with 0.5 mL of isopropanol. The resultant mixture was mixed gently and incubated at room temperature for 5 min. Samples were centrifuged at 10,000 rpm for 10 min at 4 °C. The RNA pellet was washed by adding 1 mL of 70% ethanol and centrifuged for 5 min at 14,000 rpm at 4 °C. The supernatant was discarded, and the pellet was dried by incubating in a dry bath for 5 min at 55 °C. The pellet was resuspended in 25 μL of diethyl pyrocarbonate (DEPC)-treated water (Table 2).[30]

Table 2. Concentration of Samples for Treatment

cells	treatment	concentration
K562	control	
	vinblastine	20 μM
	crude sample	25 μg/mL
	crude sample	50 μg/mL
	purified sample	25 μg/mL
	purified sample	50 μg/mL

2.7.2. RT-PCR Method. A semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using Techno Prime system to determine the levels of Caspase 3 and beta-actin mRNA expressions. The cDNA was synthesized from 2 μg of RNA using the Verso cDNA synthesis kit with oligo dT primer. The reaction volume was set to 20 μL, and cDNA synthesis was performed at 50 °C for 30 min, followed by reverse transcriptase inactivation at 85 °C for 5 min (Table 3).

Table 3. Primer Details for Caspase3 and Beta-Actin

caspase 3	forward	TGGAATTGATGCGTGATGTT	166 bp
	reverse	CAACGATCCCCTCTGAAAAA	
beta-actin	forward	GTCCAGTTAATTTCTGACCT	154 bp
	reverse	GCTTTGTACATGGTATTCAC	

2.7.3. PCR Analysis. The PCR mixture (final volume of 20 μL) contained 1 μL of cDNA, 10 μL of Red Taq Master Mix 2× (Amplicon), and 1 μM of each complementary primer specific for caspase 3 and beta-actin (internal control) sequence. The samples were denatured at 94 °C for 5 min and amplified using 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. Renaturation for caspase 3 was set to 51

https://doi.org/10.1021/acsomega.3c07633
ACS Omega 2024, 9, 3616−3624

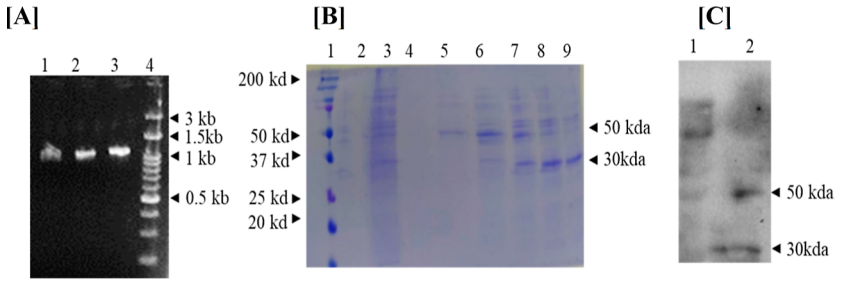


Figure 1. Confirmation and purification of L-Asparaginase from tomato. (A) PCR amplification of L-asparaginase from tomato gDNA, Lane 1. Amplicon of primer set 1, Lane 2. Amplicon of primer set 2, Lane 3. Amplicon of primer set 3, Lane 4. DNA ladder. (B) SDS-PAGE purification profile of L-asparaginase, (1) ladder, (2) crude, (3) acetone ppt, (4–9) fraction 8–13 of gel filtration chromatography. (C) Western blot of L-asparaginase from tomato pulp, lane 1—protein molecular weight marker, lane 2—purified gel filtration fraction.

Table 4. Purification Steps of L-Asparaginase

sample	enzyme activity IU	protein (mg/mL)	specific activity (μmol/mg/min)	fold purification	% of yield
crude	40.323	0.126	320.02	1	100
salt precipitation	13.58	0.0224	606.51	1.895	33.69
dialysis	14.11	0.0368	383.41	1.198	34.99
ion exchange	22.15	0.042	527.4	1.648	54.93
gel filtration	2.604	0.094	27.89	0.086	6.457

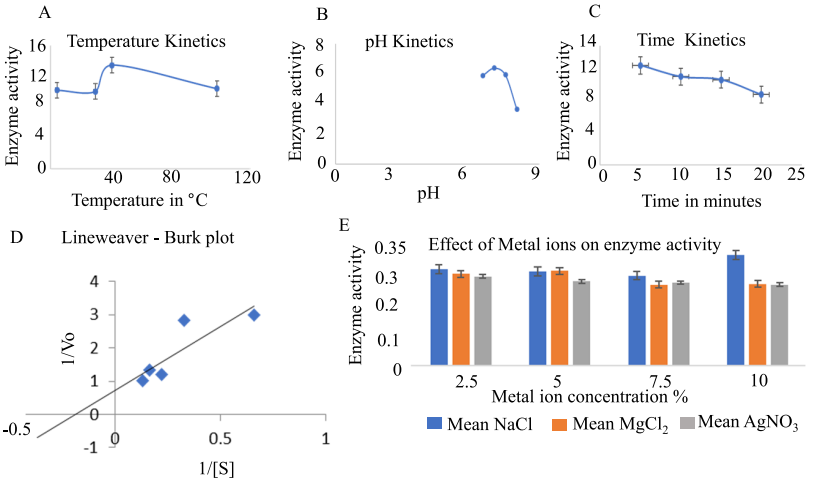


Figure 2. Effect of various parameters on L-asparaginase activity. (A) Effect of temperature, (B) effect of pH, (C) optimum incubation time, (D) lineweaver–Burk plot for L-A catalytic reaction, and (E) effect of salts on L-Asparaginase activity.

°C and that for actin to 55 °C for 30 s, followed by a final elongation at 72 °C for 10 min. The optimal number of cycles selected for amplification of these genes was in the exponential range. Ten microliters of the final amplification product was run on a 2% ethidium bromide-stained agarose gel and photographed. Quantification of the results was accomplished by measuring the optical density of the bands, using the computerized imaging program ImageJ. The values were normalized to beta-actin intensity levels.[31]

2.8. Apoptosis by a Flow Cytometer. The day before the induction of apoptosis, 1×10^6 cells per well were plated in a six-well plate using the respective media with 10% FBS and 1% Pen Strep, incubated overnight at 37 °C at 5% CO_2. The media was replaced with test solutions of different concentrations in the media containing 10% FBS. The treated cells were incubated for 24 h at normal culture conditions. Cells were harvested and transferred to sterile fluorescence-activated cell sorting (FACS) tubes. Cell contents were centrifuged at 2000 rpm for 5 min, and the supernatant was discarded. The cell pellet was washed twice with cold PBS, followed by centrifugation and then resuspended in 1 mL 1× binding buffer at a concentration of ~1×10^6 cells/ml. A volume of 500 μL of the cell suspension (~5×10^5 cells) was transferred to a new FACS tube. Annexin V (5 μL) and PI (10 μL) were added to the tubes, gently mixed, and incubated for 20 min at room temperature (RT) in the dark. Cells were analyzed by flow cytometry within an hour.[32−38]

3. RESULTS AND DISCUSSION

3.1. Confirmation and Purification of L-Asparaginase from Tomato. Although L-Asparaginase is shown to be present in tomato, it was never isolated and purified. In order to confirm the existence of L-Asparaginase in tomato, the total genomic DNA from the tomato sample was isolated, and using L-Asparaginase-specific primers, PCR reaction was carried out, and the resulting PCR products (Figure 1A) were confirmed to

https://doi.org/10.1021/acsomega.3c07633
ACS Omega 2024, 9, 3616−3624

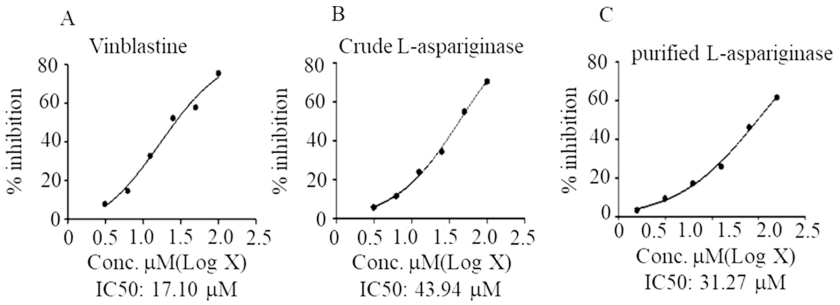


Figure 3. MTT assay using K-562 cells treated with (A) positive control, (B) crude L-A, and (C) purified L-Asparaginase.

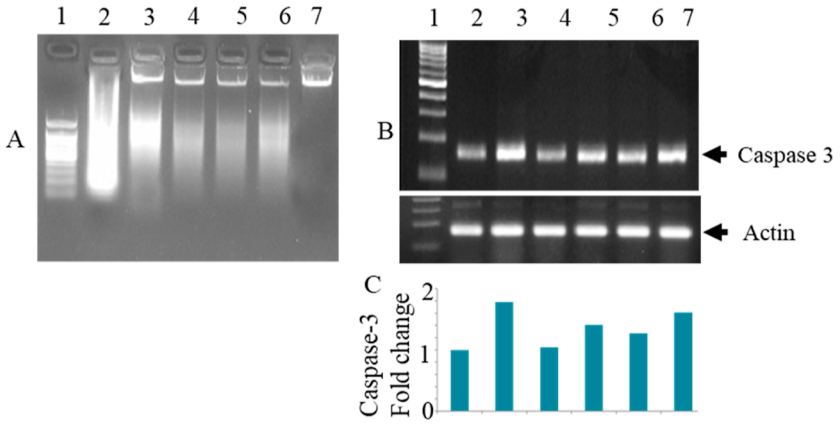


Figure 4. Apoptotic studies by DNA fragmentation and caspase 3 gene expression: (A) DNA fragmentation of K-562 cells: (1) ladder, (2) vinblastine, (3) purified 50 μg/mL, (4) purified 25 μg/mL, (5) crude 50 μg/mL, (6) crude 25 μg/mL, (7) control. (B) Amplification of *caspase 3* gene in K562-treated cells: (1) DNA marker, (2) control, (3) vinblastine-20 μM, (4) crude −25 μg/mL, (5) crude −50 μg/mL, (6) purified −25 μg/mL, (7) purified −50 μg/mL. (C) Bar graph showing caspase 3 gene expression in K-562 cells treated with the standard and samples.

be belonging to L-Asparaginase by sequencing. The reads were assembled into a single contig using the CAP3 sequence assembly program. The sequence was annotated using AUGUSTUS and DAVID tools of Bioinformatics and deposited in GenBank, NCBI, with accession number-OR736141. To establish that tomatoes contain L-Asparaginase, the crude enzyme was extracted from the fruit pulp and was subjected to a series of purification steps that included salt precipitation, dialysis, ion exchange chromatography, and gel filtration chromatography. It was observed that after each purification step, there was some degree of protein loss, and the total protein estimated was about 48 μg. The final percentage yield was 6.457, and purification fold was 0.086 (Table 4). The molecular weight of L-Asparaginase was approximately determined by SDS-PAGE and was found to be around 80 kDa with two subunits of 50 and 30 kDa (Figure 1B).

Further, to confirm that the purified product is L-Asparaginase, Western blotting was carried out using L-asparaginase-specific antibody. The appearance of bands at 50 and 30 kDa, as shown in Figure 1C, demonstrated the occurrence of L-asparaginase in tomato (Table 4).

3.2. Determining Optimum Conditions for L-Asparaginase. The optimum conditions related to the temperature, pH, time, and effect of metal ions on L-Asparaginase were studied. The enzyme showed maximum activity at the temperature of 37 °C, pH 7, and the optimum incubation time for the high enzyme activity was found to be 5 min (Figure 2A−C). K_m and V_{max} were determined by the Lineweaver−Burk plot, and K_m was found to be 0.66 and V_{max} 3.846 IU (Figure 2D). The enzyme activity was found to be stable with different salt concentrations, and NaCl was reported to enhance the enzyme activity when compared to the other salts (1E).

3.3. Anticancer Activity of L-Asparaginase. As the enzyme is known to possess anticancer activity, we explored whether L-Asparaginase from tomato would have similar anticancer properties. We used the K-562 cell line, a model system for chronic myelogenous leukemia, for testing anticancer properties of L-Asparaginase from tomato. L-Asparaginase from the fruit pulp of tomato showed significant anticancer activity against the K-562 cell lines. The crude sample has shown the IC_{50} value of 0.556 μM (43.94 μg/mL) inhibition in K-562 cells, and the purified sample has reported the IC_{50} value of 0.395 μM (31.27 μg/mL) when compared to the IC_{50} value of standard vinblastine 17.1 μM in the same cell line (Figure 3A−C).

In order to test whether L-asparaginase induces DNA fragmentation, a hallmark of apoptosis/cell death, K-562 cells were treated with standard vinblastine, crude, and purified L-asparaginase enzyme. DNA fragmentation was observed in K562 cells when treated with vinblastine and purified L-asparaginase. The purified sample has shown better DNA fragmentation when compared to the crude sample. Overall, these results suggest that tomato L-asparaginase induces the fragmentation of DNA, indicating apoptotic activity (Figure 4A).

Since L-Asparaginase induces apoptosis, pro-apoptotic molecules such as caspase 3 should show a higher expression in enzyme-treated cells. Crude sample treatment at different

https://doi.org/10.1021/acsomega.3c07633
ACS Omega 2024, 9, 3616−3624

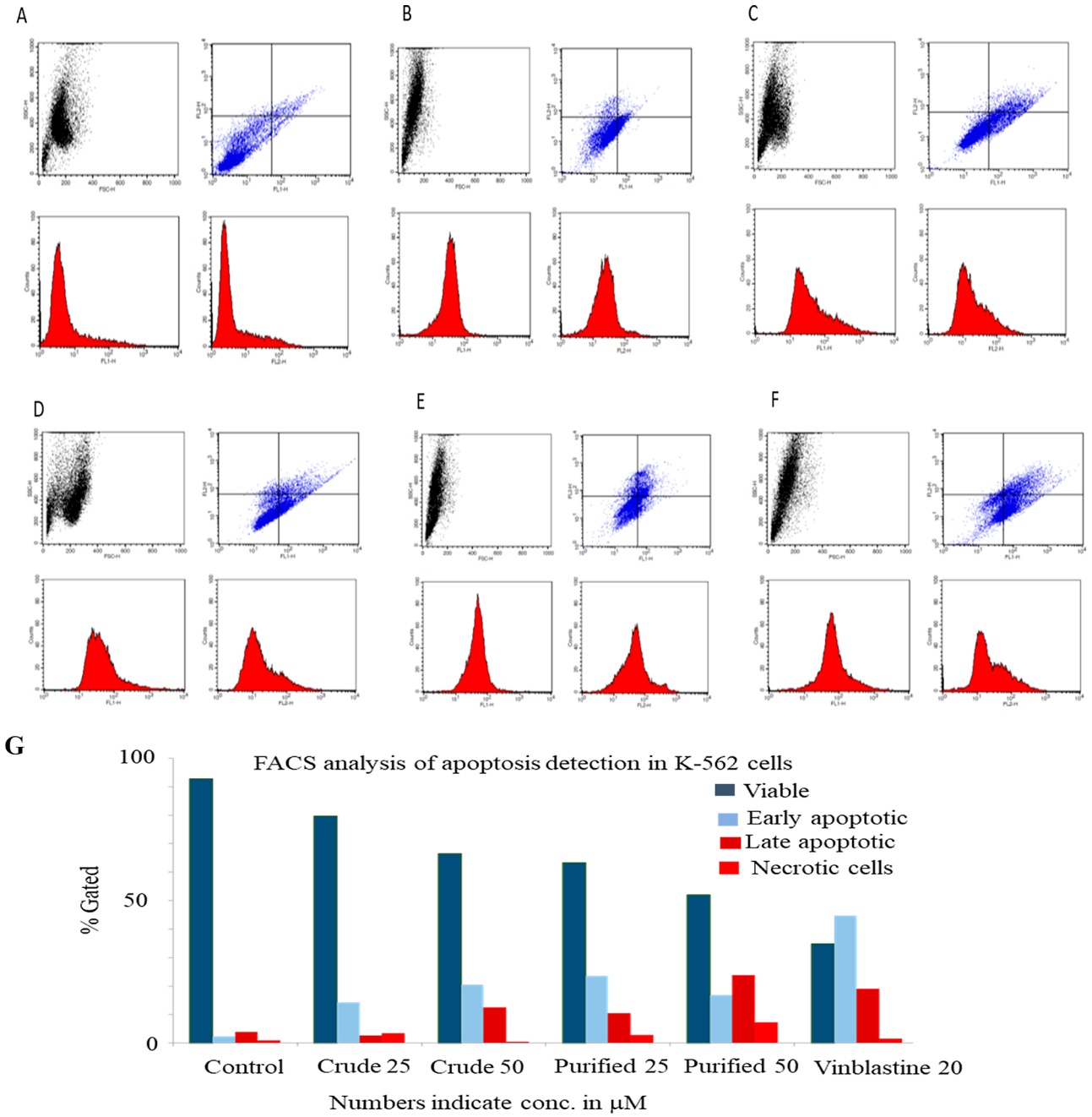


Figure 5. Apoptosis detection of K-562 cells by flow cytometry analysis and graphical representations: (A) K-562-untreated cells, (B) K-562 cells treated with crude sample_25 μg/mL, (C) K-562 cells treated with crude sample_50 μg/mL, (D) K-562 cells treated with purified sample_25 μg/mL, (E) K-562 cells treated with purified sample_50 μg/mL, (F) K-562 cells treated with vinblastine_ 20 μM, (G) bar graph showing apoptosis detection in K-562 cells by FACS analysis.

concentrations on K562 cells show a slight upregulation of the caspase 3 gene. At 25 and 50 μg/mL concentrations, caspase 3 gene showed upregulation of 1.04 and 1.41 folds in K562 cells (Figure 4B, lanes 4 and 5). Comparatively, the purified sample has shown upregulation with 1.27- and 1.61-folds increase at 25 and 50 μg/mL concentrations, respectively (Figure 4B, lanes 6 and 7). The positive control vinblastine treatment at 20 μM has shown a better upregulation of caspase 3 gene up to 1.78-fold (Figure 4B, lane 3). The purified 50 μg/mL sample results are comparable to that of the standard vinblastine and hence confirm the apoptotic activity (Figure 4, compare lanes 3 and 7).

K-562 cells were treated with vinblastine, crude, and purified L-Asparaginase enzymes of different concentrations and subjected to FACS analysis. The treatment of 25 and 50 μg/mL of crude samples has induced early and late apoptosis in K-562 with 14.28, 20.51, 2.69, and 12.56% apoptotic cells, respectively; the purified sample treated at 25 and 50 μg/mL has induced early and late apoptosis in K-562 with 23.47, 16.85, 10.46, and 23.85% apoptotic cells. Necrotic cells were found to be 0.5% and 7.25% at the highest concentrations of samples, respectively, in K-562 cells. These results infer that the sample induces apoptosis in K-562 cells (Figure 5).

Plants have emerged as an alternative source of enzymes as they do not manifest pathogenicity and can be used as either

https://doi.org/10.1021/acsomega.3c07633
ACS Omega 2024, 9, 3616−3624

crude extracts or in purified form to treat or use against many human diseases.[39,40] There are many purified compounds from plants such as taxol and vinblastine that are being used as chemotherapeutic drugs to treat many cancers.[41] The edible fruit of *Solanum lycopersicum* has a wide range of biological effects and has been reported to have many health benefits.[42−44] Tomatoes help reduce the risk of cardiovascular diseases, atopic dermatitis, liver inflammatory disease, and inflammatory bowel diseases.[45] The anticancer properties of L-asparaginase are well documented, particularly against ALL.[46] In terms of L-asparaginase synthesis, plant sources have been less investigated. The plant sources listed in the literature as L-asparaginase producers include *Tamarindus indica*, *Capsicum annum*, *Withania somnifera*, *Vicia faba*, *Lupinus angustifolius*, and *Phaseoulus vulgaris*.[47] However, there are very few studies related to L-apsaraginase from tomato and its usage against cancer and ALL in particular. Although it is shown that L-asparaginase is being present in tomato (https://www.ncbi.nlm.nih.gov/nuccore/NC_015440), the enzymatic properties and its anticancer activities are not studied so far to the best of our knowledge. In this study, we showed that L-Asparaginase indeed exists in tomato by elucidating its sequence and matching it with that of L-asparaginase from other sources. The sequence match is similar to other available sources and not with many variations in terms of nucleotide sequence. The sequence similarity with the known L-asparaginase from tomato plants is almost 99% with a single nucleotide polymorphism. This L-asparaginase gene from tomato has been deposited in the NCBI database with accession number: OR736141. In this study, we established a protocol for isolating L-asparaginase from tomato pulp and its purification. The existence of L-asparaginase was further confirmed by using L-asparaginase-specific antibody, and the enzymatic product from tomato has two subunits with molecular weights of 50 and 30 kDa respectively. The optimum conditions for L-asparaginase activity from tomato are shown to vary with other sources.[48−50]

With respect to the anticancer properties when used against a cell line, K-562, that is a model system for ALL, the enzyme performance is comparable to the anticancer activity of the standard drug vinblastine from another plant source. Although crude extract induces apoptosis in the cancer cells used, the purified enzyme shows better apoptotic activity and is comparable to the standard drug vinblastine. In this study, we successfully isolated and characterized L-Asparaginase from tomato. Since tomato is widely available, it may act as a good source for commercial isolation and purification of L-asparaginase.

4. CONCLUSIONS

In this study, we demonstrated the existence of L-asparaginase from tomato and established a protocol for its purification from tomato pulp. We also characterized optimal conditions for enzymatic activity and studied anticancer properties using ALL cancer cell line. Purified L-Asparaginase from tomato has comparable anticancer activity with respect to the standard cancer drug, vinblastine. Currently, bacteria are the primary source of L-Asparaginase for the production of anticancer drug. In the near future, this piece of research investigation offers an alternative source from plants for the commercial production of L-Asparaginase that has advantages in terms of production scalability, economic viability, and reduced immunogenicity.

AUTHOR INFORMATION

Corresponding Author

Divijendra Natha Reddy Sirigiri − *Department of Biotechnology, BMS College of Engineering, Bangalore, Affiliated to Visvesvaraya Technological University, Belagavi, Karnataka 560019, India;* orcid.org/0000-0003-4657-2285; Email: sdnreddy@gmail.com

Authors

Sarina P. Khabade − *Department of PG Studies in Biotechnology, Nrupathunga University, Bangalore 560001, India*

Anshu Beulah Ram − *Department of PG Studies in Biotechnology, Nrupathunga University, Bangalore 560001, India*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c07633

Author Contributions

S.P.K.: Project conceptualization, execution, data analysis, interpretation, and paper writing. D.N.R.S.: Conceptualization, data analysis, and writing paper. A.B.R.: Data interpretation.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The research facilities to carry out the research work were provided by Nrupathunga University, BMSCE, and RVCE, Bangalore, India.

REFERENCES

(1) Van Trimpont, M.; Peeters, E.; De Visser, Y.; Schalk, A. M.; Mondelaers, V.; De Moerloose, B.; Lavie, A.; Lammens, T.; Goossens, S.; Van Vlierberghe, P. Novel Insights on the Use of L-Asparaginase as an Efficient and Safe Anti-cancer Therapy. *Cancers* **2022**, *14* (4), 902.

(2) Patel, P.; Patel, A.; Agarwal-Rajput, R.; Rawal, R.; Dave, B.; Gosai, H. Characterization, Anti-Proliferative Activity, and Bench-Scale Production of Novel pH-Stable and Thermotolerant L-Asparaginase from Bacillus Licheniformis PPD37. *Appl. Biochem. Biotechnol.* **2023**, *195* (5), 3122−3141.

(3) Pieters, R.; Hunger, S. P.; Boos, J.; Rizzari, C.; Silverman, L.; Baruchel, A.; Goekbuget, N.; Schrappe, M.; Pui, C. H. L-Asparaginase Treatment in Acute Lymphoblastic Leukemia: A Focus on Erwinia Asparaginase. *Cancer* **2011**, *117* (2), 238−249.

(4) Maese, L.; Rizzari, C.; Coleman, R.; Power, A.; van der Sluis, I.; Rau, R. E. Can Recombinant Technology Address Asparaginase Erwinia Chrysanthemi Shortages? *Pediatr. Blood Cancer* **2021**, *68* (10), No. e29169.

(5) Bender, C.; Maese, L.; Carter-Febres, M.; Verma, A. Clinical Utility of Pegaspargase in Children, Adolescents and Young Adult Patients with Acute Lymphoblastic Leukemia: A Review. *Blood Lymphatic Cancer* **2021**, *11*, 25−40.

(6) Medawar, C. V.; Mosegui, G. B. G.; Vianna, C. M. M.; Costa, T. M. PEG-Asparaginase and native Escherichia coli L-Asparaginase in Acute Lymphoblastic Leukemia in Children and Adolescents: A Systematic Review. *Hematol. Transfus. Cell Ther.* **2020**, *42* (1), 54−61.

(7) Mondelaers, V.; Ferster, A.; Uyttebroeck, A.; Brichard, B.; van der Werff Ten Bosch, J.; Norga, K.; Francotte, N.; Piette, C.; Vandemeulebroecke, K.; Verbeke, C.; Schmidt, S.; Benoit, Y.; Lammens, T.; De Moerloose, B. Prospective, Real-Time Monitoring of Pegylated *Escherichia coli* and Erwinia Asparaginase Therapy in Childhood Acute Lymphoblastic Leukaemia and Non-Hodgkin Lymphoma in Belgium. *Br. J. Haematol.* **2020**, *190* (1), 105−114.

(8) Samra, B.; Jabbour, E.; Ravandi, F.; Kantarjian, H.; Short, N. J. Evolving Therapy of Adult Acute Lymphoblastic Leukemia: State-of-

the-Art Treatment and Future Directions. *J. Hematol. Oncol.* **2020**, *13* (1), 70.
(9) *Asparaginase Market Forecast to 2028*; The Insight Partners, Report ID:6321015, 2022.
(10) Shrivastava, A.; Khan, A. A.; Khurshid, M.; Kalam, M. A.; Jain, S. K.; Singhal, P. K. Recent Developments in L-Asparaginase Discovery and its Potential as Anticancer Agent. *Crit. Rev. Oncol. Hematol.* **2016**, *100*, 1−10.
(11) Burke, M. J. How to Manage Asparaginase Hypersensitivity in Acute Lymphoblastic Leukemia. *Future Oncol.* **2014**, *10* (16), 2615−2627.
(12) Moharib, S. A. Anticancer Activity of L-Asparaginase Produced from Vigna unguiculata. *World Sci. Res.* **2018**, *5* (1), 1−12.
(13) Oza, V. P.; Trivedi, S. D.; Parmar, P. P.; Subramanian, R. B. Withania Somnifera (Ashwagandha): A Novel Source of L-Asparaginase. *J. Integr. Plant Biol.* **2009**, *51* (2), 201−206.
(14) Oza, V. P.; Parmar, P. P.; Patel, D. H.; Subramanian, R. B. Cloning, Expression and Characterization of l-Asparaginase from Withania Somnifera L. for Large Scale Production. *3 Biotech* **2011**, *1* (1), 21−26.
(15) Beulah, K.; Hemalatha, K. P. J. Screening of Medicinal Plants for Potential Source of L-Asparaginase and Optimization of Conditions for Maximum Extraction and Assay of L-Asparaginase from *Asparagus racemosus*. *Iran. J. Sci. Technol. Trans. Sci.* **2019**, *43* (1), 1−6.
(16) Khaerah, N.; Ahmad, A.; Yusriadi; Arfah, R. A.; Karim, A. Purification, Characterization and Anticancer Potential Tests of L-Asparaginase Enzyme from the Leaves Siam Weed (*Chronolaena odorata* Linn). *J. Phys.: Conf. Ser.* **2019**, *1341* (3), 032018.
(17) Mukavi, J. W.; Mayeku, P. W.; Nyaga, J. M.; Kituyi, S. N. In Vitro Anticancer Efficacy and Phyto-Chemical Screening of Solvent Extracts of Kigelia africana. Lam Benth. *Heliyon* **2020**, *6* (7), No. e04481.
(18) Patel, A.; Biswas, S.; Shoja, M. H.; Ramalingayya, G. V.; Nandakumar, K. Protective Effects of Aqueous Extract of Solanum nigrum Linn. Leaves in Rat Models of Oral Mucositis. *Sci. World J.* **2014**, *2014*, 1−10.
(19) Martí, R.; Roselló, S.; Cebolla-Cornejo, J. Tomato as a Source of Carotenoids and Polyphenols Targeted to Cancer Prevention. *Cancers* **2016**, *8* (6), No. E58.
(20) Thimmaiah, S. R. *Standard Methods of Biochemical Analysis*; Kalyani Publication, 2001.
(21) Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72* (1−2), 248−254.
(22) Hames, D.; Hooper, N. *Biochemistry*, 3rd ed.; Taylor & Francis Group, 2005.
(23) Hassan, S. W. M.; Farag, A. M.; Beltagy, E. A. Purification, Characterization and Anticancer Activity of L-Asparaginase Produced by Marine Aspergillus Terreus Produced by Marine *Aspergillus terreus*. *J. Pure Appl. Microbiol.* **2018**, *12* (4), 1845−1854.
(24) El-Ahmady, N. E.-A.; Deraz, S. F.; Soliman, H. M.; El-Deeb, N. M.; El-Ewasy, S. M. Purification, Characterization, Cytotoxicity and Anticancer Activities of L-Asparaginase, Anti-colon Cancer Protein, from the Newly Isolated Alkaliphilic *Streptomyces fradiae* NEAE-82. *Sci. Rep.* **2016**, *6*, 32926.
(25) Bollag, D. M.; Rozycki, M. D.; Edelstein, S. J. *Protein Methods*, 2nd ed.; Wiley-Liss Inc., 2002.
(26) Dhaliwal, A. DNA Extraction and Purification. *Mater. Methods* **2013**, *3*, 191.
(27) Shetty, P. J. The Evolution of DNA Extraction Methods. *Am. J. Biomed. Sci.* **2020**, *8*, 39−45.
(28) Deeparani, U.; Jayakumari, S. In Vitro Anti-Cancer Study of Vitis Viniferae, Ixora coccinea and Piper longum Extract on Human Prostate Carcinoma Cells. *Res. J. Pharm. Technol.* **2019**, *12* (9), 4485−4488.
(29) Li, Z.; Yu, J.; Liu, L.; Wei, Z.; Ehrlich, E. S.; Liu, G.; Li, J.; Liu, X.; Wang, H.; Yu, X. F.; Zhang, W. Coxsackie virus A16 Infection Induces Neural Cell and Non-Neural Cell Apoptosis In Vitro. *PLoS One* **2014**, *9* (10), No. e111174.
(30) *Trizol Reagent Literature*; Invitrogen Life Technologies, 2013.
(31) Dirican, E.; Özcan, H.; Karabulut Uzunçakmak, S.; Takım, U. Evaluation Expression of the Caspase-3 and Caspase-9 Apoptotic Genes in Schizophrenia Patients. *Clin. Psychopharmacol. Neurosci.* **2023**, *21* (1), 171−178.
(32) Vahedi, A.; Bigdelou, P.; Farnoud, A. M. Quantitative Analysis of Red Blood Cell Membrane Phospholipids and Modulation of Cell-Macrophage Interactions Using Cyclodextrins. *Sci. Rep.* **2020**, *10* (1), 15111.
(33) Schloer, S.; Pajonczyk, D.; Rescher, U. Annexins in Translational Research: Hidden Treasures to be Found. *Int. J. Mol. Sci.* **2018**, *19* (6), 1781.
(34) Sambavi, N.; Kumar, N. K.; Balaji, A. A. Evaluation of *Amaranthus roxiburghianus* Linn Plant Extract on Breast Cancer MCF-7 Cells by MTT Assay. *YMER* **2022**, *21* (12), 137−151.
(35) Tsuboi, S.; Jin, T. Dual-Colour (Near Infrared/Visible) Emitting Annexin V for Fluorescence Imaging of Tumour Cell Apoptosis *In-Vitro* and *In-Vivo*. *RSC Adv.* **2020**, *10*, 38244−38250.
(36) Kausar, M. S.; Manjunath, B. K. *In-Vitro* Study of Macelignan as a Potential Anticancer Drug Against Colorectal Cancer Using HCT116 Cell Line. *J. Clin. Diagn. Res.* **2022**, *16* (1), KC01−KC07.
(37) Calderwood, S. K.; Prince, T. L. Cell Death and Survival Assays. *Chaperones*; *Chapter 9. Methods in Molecular Biology*, 2018; Vol. *1709*; pp 107−127..
(38) Worsley, C. M.; Veale, R. B.; Mayne, E. S. Inducing Apoptosis Using Chemical Treatment and Acidic pH, and Detecting it Using the Annexin V Flow Cytometric Assay. *PLoS One* **2022**, *17* (6), No. e0270599.
(39) Singh, A.; Verma, N.; Kumar, K. L-Asparaginase from *Phyllanthus emblica* (Amla): A Novel Source. *IJPSR* **2018**, *9* (12), 5394−5400.
(40) Saleh, M. S. M.; Kamisah, Y. Potential Medicinal Plants for the Treatment of Dengue Fever and Severe Acute Respiratory Syndrome-Coronavirus. *Biomolecules* **2020**, *11* (1), 42.
(41) Shen, F.; Long, D.; Yu, T.; Chen, X.; Liao, Y.; Wu, Y.; Lin, X. Vinblastine Differs from Taxol as it Inhibits the Malignant Phenotypes of NSCLC Cells by Increasing the Phosphorylation of Op18/Stathmin. *Oncol. Rep.* **2017**, *37* (4), 2481−2489.
(42) Delbrouck, J. A.; Desgagné, M.; Comeau, C.; Bouarab, K.; Malouin, F.; Boudreault, P. L. The Therapeutic Value of Solanum Steroidal (Glyco)Alkaloids: A 10-Year Comprehensive Review. *Molecules* **2023**, *28* (13), 4957.
(43) Elizalde-Romero, C. A.; Montoya-Inzunza, L. A.; Contreras-Angulo, L. A.; Heredia, J. B.; Gutiérrez-Grijalva, E. P. Solanum Fruits: Phytochemicals, Bioaccessibility and Bioavailability, and their Relationship with their Health-Promoting Effects. *Front. Nutr.* **2021**, *8*, 790582.
(44) Tam, C. C.; Nguyen, K.; Nguyen, D.; Hamada, S.; Kwon, O.; Kuang, I.; Gong, S.; Escobar, S.; Liu, M.; Kim, J.; Hou, T.; Tam, J.; Cheng, L. W.; Kim, J. H.; Land, K. M.; Friedman, M. Antimicrobial Properties of Tomato Leaves, Stems, and Fruit and their Relationship to Chemical Composition. *BMC Complement. Med. Ther.* **2021**, *21* (1), 229.
(45) Collins, E. J.; Bowyer, C.; Tsouza, A.; Chopra, M. Tomatoes: An Extensive Review of the Associated Health Impacts of Tomatoes and Factors That Can Affect their Cultivation. *Biology* **2022**, *11* (2), 239.
(46) Maese, L.; Rau, R. E. Current Use of Asparaginase in Acute Lymphoblastic Leukemia/Lymphoblastic Lymphoma. *Front. Pediatr.* **2022**, *10*, 902117.
(47) Vimal, A.; Kumar, A. Biotechnological Production and Practical Application of L-Asparaginase Enzyme. *Biotechnol. Genet. Eng. Rev.* **2017**, *33* (1), 40−61.
(48) Chi, H.; Zhu, X.; Shen, J.; Lu, Z.; Lu, F.; Lyu, Y.; Zhu, P. Thermostability Enhancement and Insight of L-Asparaginase from Mycobacterium sp. via Consensus-Guided Engineering. *Appl. Microbiol. Biotechnol.* **2023**, *107* (7−8), 2321−2333.

https://doi.org/10.1021/acsomega.3c07633
ACS Omega 2024, 9, 3616−3624

(49) Shahana Kabeer, S.; Francis, B.; Vishnupriya, S.; Kattatheyil, H.; Joseph, K. J.; Krishnan, K. P.; Mohamed Hatha, A. A. Characterization of L-Asparaginase from Streptomyces koyangensis SK4 with Acrylamide-Minimizing Potential in Potato Chips. *Braz. J. Microbiol.* **2023**, *54* (3), 1645−1654.
(50) Ratuchne, A.; Izidoro, S. C.; Beitel, S. M.; Lacerda, L. T.; Knob, A. A New Extracellular Glutaminase and Urease-Free L-Asparaginase from Meyerozyma guilliermondii. *Braz. J. Microbiol.* **2023**, *54* (2), 715−723.

NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on December 27, 2023 with errors in Figure 2. The corrected version was reposted on January 10, 2024.

https://doi.org/10.1021/acsomega.3c07633
ACS Omega 2024, 9, 3616−3624