IJHS (Egypt) 2024; 2(2): 84-116

International Journal of Health Sciences (Egypt)

Journal homepage: https://ijhegy.journals.ekb.eg/

Original article

Identification and molecular characterization of arginine deiminase-producing bacteria in Egyptian soil from various soil environments

Mohammed Kassab [*1]

1- *Instructor of microbiology and immunology, Faculty of pharmacy, Cairo university, Egypt..*

ARTICLE INFO

Article history:
Received 2 April 2023
Received in revised form 26 July 2023
Accepted 1 August 2023

Keywords:
L-arginine
Auxotrophic
Cancer
neoplasm

ABSTRACT

Background: Auxotrophic cancers are a leading cause of cancer-related deaths in the world. Because certain cancers are arginine amino acid auxotrophs, Depletion of extracellular arginine using arginine deiminase (ADI) could be exploited to target such cancers. ADI is a potential anticancer agent against arginine auxotrophic cancers. It catalyzed the hydrolysis of L-arginine to L-citrulline and ammonia. **Purpose of research:** Our study aimed to find new sources of bacterial arginine deiminases from different soil environments in Egypt and their production as anticancer agents by recombinant DNA technology. **Methodology:** This study was designed to screen for ADI-producing bacterial species using a simple and convenient method using metallo-arginine agar selective medium (MAA) and the Nesslarization assay. The isolated samples were characterized based on morphological features, biochemical reactions, and DNA probe hybridization. The antitumor activity of ADI was analyzed by MTT cell viability assay using various cancer cell lines. **Results:** Bacillus cereus strain AH173 was the major ADI-producing bacterial isolate from various soil environments in Egypt. The enzyme of interest showed excellent anticancer activity against L-arginine amino acid auxotrophic cancers. The molecular weight of ADI was 48 kDa. The Km and Vmax values of ADI were 8.75 mmol/L and 8.78 µmol/min. The IC50 for bacterial ADI was 2.42 U/ml for liver cancer cell lines, 2.17 U/ml for melanoma cancer cell lines, 4.63 U/ml for colon cancer cell lines, and 4.53 U/ml for lung adenocarcinoma cell line (Calu-3), 3.12 vs PANC-1 pancreatic cancer cell line, 3.77 vs RCC4 renal cancer cell line and 4.37 U/ml against a heart cancer line (HL1). Selectivity index of bacterial ADI against cancer cell lines was as the following: 3.93 vs (JHH4) liver cancer cell lines, 3.24 vs (IM1215) colon cancer cell lines, 3.61 vs (UPMM3) melanoma cancer cell lines, 3.18 vs (Calu-3) lung adenocarcinoma cell lines, 3.07 vs PANC-1 pancreatic cancer cell line, 3.81 vs RCC4 renal cancer cell line and 3.75 vs (HL1) heart cancer cell line. **Conclusion:** In this study, a new bacterial source for the production of ADI as an anticancer agent from various soil environments in Egypt was characterized.

DOI: 10.21608/IJHEGY.2023.203602.1020

* *Corresponding author:* Mohammed Kassab
E-mail address: Mohammed.Kassab676@gmail.com.

Introduction

Auxotrohic cancers for L-arginine amino acid such as melanoma, hepatic carcinoma, lung, heart and colorectal cancers are a leading cause of death globally.1 Auxotrophic cancer cells are deficient in argenine biosynthesis:2Auxotrophic cancer cells, such as liver cancer cells and melanoma cancer cells, cannot synthesize the arginine amino acid, which is essential for their survival and growth.3 Normal cells can synthesize it.5 Deprivation of L-arginine from these cancer cells by bacterial arginine deiminases is a necessary process for eradication of arginine-requiring cancer cells.6 Physiological importance of arginine in the cell biology of cancer cells: Arginine is required for the biosynthesis of proline,7 the production of glutamate amino acids essential for their growth,8 polyamines that prevent their apoptosis,9 and the production of nitric oxide by nitric oxide synthase, a major vasodilator.10 About 70% of cancers are auxotrophic for L-arginine.11 Degradation of the L-arginine amino acid : It is Broken down by the arginase enzyme during urea cycle with the production of urea and ornithine amino acid as secondary metabolites12 or by the arginine deiminase enzyme(ADI) (or known a dihydrolase enzyme) of the dihydrolase pathway.13 Arginine degrading enzymes showed greater antineoplastic activities against solid tumors more than liquid tumors.14Lack of L-arginine leads to inhibition of nucleic acid and protein biosynthesis in auxotrophic cancer cells for arginine, ultimately causing programmed cell death(apoptosis) of cancer cells.15 Deprivation of L-arginine evoked oxidative stress mediated caspase-mediated cell death in auxotrophic cancer types for L-arginine such as renal cancer and lung cancers.16 ADI demonstrates no antioxidant activities.17 Simple carbon origins such as starch, glycerol and glucose intensify the output of the ADI enzyme aside utilizing Bacillus cereus; while negative determinant on ADI production was demonstrated via more complicated origins of carbon such as molasses and whey with the overflowing enzyme production was displayed with greater starch levels 9.5 g/l.18 The present study aimed at screening and isolation of L-arginine degrading enzymes(Arginine deiminase) bacteria from various soil environments in Egypt, followed by the determination of the physiological and environmental factors governing the growth of some selected ADI producing baterial isolates with the characterization of the ADI production and its anticancer activity.

Material and Method

Ethical statement:

In the present survey, we preceded All applicable national, international and/or institutional guidelines for the attention and utilization of humans and animals. All processes carried out in study including humans and animals were authorized by the local authorities, Ethical committee for human and animal handling at Cairo university(ECAHCU), at the faculty of Pharmacy, Cairo University, Egypt in agreement with the recommendations of the weathrall report with approval number P-3-7-2021. All efforts were performed to ablate the number of humans and animals utilized and their suffering during study.

Type of the study: Screening experimental study. Place and date of the study: This study was done faculty of pharmacy, Cairo university, Egypt between July 2021 and November 2022. Source of animal models: They were obtained and legalized from the pharmacology and toxicology department of the faculty of pharmacy, Cairo university, Egypt. Inclusion criteria for animal models were : Adult male obese animal models such as mice weighing 40-50 gm and male rabbit animal models weighing about 2kg; can be induced by hepatic carcinoma, pancreatic cancer, melanoma, colorectal cancer, lung cancer, renal cancer and heart cancer; blood ammonia levels can be easily estimated. Exclusion criteria were:

Young animals; Pregnant female animals; Animal blood ammonia levels can not be easily estimated; Non-obese animals.

Material

The cell lines were purchased from Accegen biotic company, USA. In order to standardize our current study, The reference bacteria utilized during the present study was Bacillus subtilis, strain W23.

list of media:

Identification and isolation media

Metallic Arginine Agar(MAA):

A selective media for the isolation and identification of microorganisms having the ability to use L-argenine as the sole carbon and nitrogen source for their growth. This medium was composed of the following ingredients(g/L):

All ingredients were bought from Algomhuria company for chemicals in Cairo, Egypt

Blood agar:

It was an enrichment media for identification of beta- hemolysis of the positive isolates cultured on MAA to aid in identification of arginine deiminase producing bacterial species together with the gram stain, the biochemical tests and the morphology of colonies.

Methods

Sample collection:

Samples were grassland soils collected from the 0-10 cm depth, samples were collected from different areas and chosen randomly. Samples were placed in sterile containers and stored at 4 0C until processed.

Isolation of L-arginine deiminase producing strains:

One gram of each soil sample was suspended in 99 ml of sterile distilled water contained in 250 ml Erlenmeyer flasks and shaken at 400 rpm for two minutes in a gyrator shaker.

The soil suspensions were serially diluted in sterile distilled water and the dilutions from 10-1 to10-6 were plated on mineral L-arginine agar(MAA) medium containing 0.5 g KCl, 0.5 g Mgso4, 1 g KH2po4,0.1 g Feso4, 0.1 g Znso4,1 g L.argenine and 2% agar. The PH of medium adjusted at 7.3 at temperature 37 0C. The plates were incubated at 37 0C for 48 hours. Only the microorganisms having the ability to use L-arginine as the sole carbon and nitrogen source could grow on the MAA medium. Colonies showing growth were purified twice using streak plate technique, then taken on nutrient agar slants and kept at 4 0C. Subculturing of the positive isolated colonies on sheep blood agar was achieved to show if there was either beta-hemolysis or not.

Identification of arginine deiminase producing strains:

Gram stain:

It separated bacteria into two classifications according to the composition of their cell walls. a specimen on a microscope slide was treated with a solution of crystal violet and then iodine, the bacterial cells stained purple. The stained cells were then treated with a solvent such as alcohol or acetone; Gram positive organisms retained the stain, where as Gram negative species lost the stain and became colorless. Addition of counter stain safranin stained the clear, gram negative bacteria pink.

Motility test under the microscope:

It separated the bacteria into either motile or non motile.

Biochemical tests:

Lecithinase test:

A loopful of the test organism was taken and was streaked on the plate. Incubation was at 35-37 0C for 24 hours; then the plate was examined for opalescent halo surrounding the inoculums(precipitation around the streak of bacteria(+ve)).

Methyl red test:

MRVP broth was Fitted out in test tubes then the broth was Inoculated aseptically with two loopfuls of individual bacterial culture. The test tubes were incubated for 48-72 hours at 37°C . Small indefinite quantity drops of methyl red indicator were placed in the incubated tubes. The consequences were ascertained subsequently.

Catalse test:

a small quantity of colony growth was transferred via the utilization of a loop on the surface of a dry-cleaned glass slide. A drop of 3% H2O2 was settled on the glass slide. The development of oxygen bubbles was determined.

Voges-Proskauer(VP) test:

The medium was allowed to equilateral to room temperature prior to inoculation. The medium was lightly inoculated exploiting organisms arrogated from an 18-24 hour clear culture. At 37 0C for 24 hours incubation was performed aerobically. 2 ml of the broth were aliquot-ed to a clean test tube. Point in time six drops of 5% of alpha-naphthol were added and blended well to aerate. Later 2 drops of 40% potassium hydroxide were added and intermingled well to oxygenate. a pink-red color at the surface within 30 min was observed. The tube was shaken vigorously during 30 minutes time period.

Molecular detection of ADI yielding bacteria:

Molecular characterization was carried out by DNA Northern hybridization and blotting technique. DNA was transferred and immobilized to nylon membranes, then complementary single-stranded probes were labeled non-radioactively. When hybridized to the filter, probes bounded to their complementary target sequences via hydrogen bonds. Unhybridized probes were then washed away

and the specifically-bound probes were detected by the color reaction.

Assessment of environmental and physiological agents influencing ADI production:

The determination of optimal environmental and physiological factors(PH, Temprature) affecting ADI production by some selected isolates was characterized during the isolation of ADI secreting bacterial strains on MAA and sub-culturing of the positive isolates on blood agar plates.

Characterization of bacterial arginine deiminase activators:

The determination of the optimal activators for the production and activity of the ADI enzyme from the positive cultured bacterial isolates was carried out on mineral arginine agar.

UV detection of production of Egyptian soil bacterial cultures containing arginine deiminase degrading enzyme: This was performed

exploiting direct Nesslerization assay for the estimation of optical density of metabolic nitrogenous containing compound(Ammonia) due to degrading ;-arginine present in media by bacterial ADI under PH7, Temp 37 0C, 0.05 mg/ml borate buffer and traces of Ni, Co and Mn metal ions as cofactors. All ingredients were bought from Algomhuria company for chemicals in Cairo, Egypt.19

Photo-metric determination of L-citrulline amino acid as a byproduct of L-arginine degradation by bacterial L-argenine deiminase: This was finished via utilizing diacetylmonoxime thiosemicarbazide performing assay for different concentrations of soil samples. All ingredients were obtained from Alnasr company for pharmaceutical chemicals in Abo Zabal, Qalyobia, Egypt. The intensity of the light was directly proportional to citrulline concentration .The amount of released citrulline amino acid was directly proportional to the enzyme activity.20

Salicylate method:

Arginine deiminase catalyzes the hydrolysis of arginine amino acid to citrulline amino acid and ammonium. The released ammonium was identified and assayed spectrophotometerically at UV wavelength 425 nm. The intensity of the light was directly proportional to ammonium concentration. The amount of released ammonium was directly proportional to the enzyme activity.

Construction of primer for expression of ADI by bacterial and fungal recombinant DNA hybridization:

The main steps of homologous primer(known sequence) design include: Recovery or getting the target gene (nucleotide sequence). Further, Restriction analysis of target gene using restriction Mapper program soft ware; Then, Finding of the reading frames utilizing Spin subprogram of the Staden package program; moreover, resolution of the nucleotide sequence of the forward primer on the parent DNA strand and the nucleotide sequence of the reverse primer on the complementary strand.

The fasta of the whole arginine deiminase genome from NCBI was copied and pasted in a separate word file. The coding sequence(CDS)of arginine deiminase enzyme from NCBI was copied as well and pasted in the word file likewise. Furthermore, PUC18 was chosen as the expression vector plasmid into which nucleotide sequence of ADI was inserted via restriction endunclease II enzymes(EcoR I and Hind III) besides ligase enzyme involvement. Suitable restriction endonuclease II enzymes for forward and reverse primers were designated according to webcutter website criteria. Criteria for choosing suitable restriction enzyme:

Enzyme should be available, present in multiple cloning site(MCS) of plasmid vector and obey orientation of ADI gene to be expressed.

After determining suitable restriction enzymes, the T annealing temperatures of forward and reverse primers for ADI gene expression were dictated exploiting PDRAW32 Microsoft application. Difference between annealing temperature of forward and reverse primers did not exceed 3 0C. This was optimal condition for expression of ADI. . Transformation of a proper expression host cell such as Saccharomyces cerevisiae BJ1824 or Eschrechia coli BL21(DES)polysS(which was characterized by expression of extracellular proteins) with the prepared recombinant DNA(gene of interest+ expression vector) was finished through restriction endonuclease enzymes(EcoR I and Hind III) in addition to ligase enzyme. The induction of transcription and translation was brought about with the aid of inducible IPTG addition. Selection of the right clone was achieved too. The partial extraction of extracellular arginine deiminase enzyme from the

superannuate of a centrifuge tube after centrifuge for 3 minutes was finished by crystallization with ammonium sulfate, followed by complete yield of the extracellular ADI enzyme of interest thru metal ligand affinity chromatography. The recombinant plasmid was designated and propagated first in Escherichia coli Top 10, then transformed into E.coli BL21(DES)polysS or Saccharomyces cerevisiae BJ1824. For ADI production using galactose as an inducer, YNBG selective medium(0.67% yeast nitrogen base without amino acids supplemented with appropriate nutrients and 2% galactose) was used for the growth of yeast transformants at 30 C, followed by maintenance in YPG-rich media(2% bacteriopeptone,1% yeast extract and 2% galactose).19 LB agar and broth were utilized for routine bacterial culture and incubation lasted for 24 hours at 37 0C. The antibiotics were added to the media according to the references recommendations.21

Clarification and the purification of recombinant ADI:

ADI was an extracellular protein that was separated from the supernatant of the centrifuge tube by precipitation by Ammonium sulfate(52 ml of 4.1 M saturated solution of ammonium sulfate were added to each 100 ml of the supernatant) after centrifugation for 3 minutes at 4000 rpm, then purified by ion exchange resin chromatography. The amounts and sizes of recombinant proteins were further analyzed via the Western blot technique:

Recombinant proteins were separated by agarose gel electrophoresis according to their sizes followed by denaturation and transfer to nitrocellulose membrane where they were hybridized into radio-labeled probes.

Estimation of ADI cellular position:

Thru Nesslerization and salicylate acid techniques, the incubated MAA media were centrifuged at 3000 rpm; point in time the supenatent and lysate were assayed.

Estimation of antioxidant actions of ADI:

This was performed through Nitric oxide scavenging activity assay.

In brief, at 25oC for 3 hours in front of a viewable poly-chromatic light-colored source (tungsten lamp 25 Watt) 6ml of the mixture of reaction incorporating Sodium nitroprusside(SNP) 6mM in phosphate buffered saline pH 7.4 with ADI extract was incubated. Hence radical of NO rendered interacted with oxygen to yield nitrite ion (NO_2^-) assessed at thirty minutes intervals by combining 2 ml of intermixture of incubation with a balanced quantity of reagent of Griess(sulphanilamide 2% in phosphoric acid 6% and Nnaphthylethylenediamine dihydrochloride0.2%). At 540 nm the chromophore optical density was determined. In existence of ADI extract the nitrite yielded was computed by victimizing a standard curve founded on notable sodium nitrite solutions concentrations.22

Assessment of secretion of antibodies to ADI:

The quantity of IgG anti-ADI antibodies in mouse serum were

Sealed with efficient ELISA operator.

The kinetic parameters Km and Vmax determination:

The parameters of kinetic, Michaelis–Menten constant (Km) and maximum velocity (Vmax) of purified ADI were ascertained with suited concentrations of L-arginine (2–11 mM) as the substrate. Data was connected to the nonlinear exponential stage union curve of regression. GraphPad Prism 5 software was utilized during this process. The outcome of L-asparaginase was computed aside measurement of the hydrolysis rate of L-arginine below standard laboratory circumstances victimizing the equation of Michaelis-Menten.

The deactivation rate constant (KD) and half-life time (t1/2)Estimation:

The thermal inactivation constant (KD) and thermal inactivation half-life (t1/2) of the purified ADI enzyme secreted by Bacillus cereus were determined by running software of Graph-pad Prism number five .

Molecular weight determination:

Observation of the purity and mass of the refined ADI enzyme were done aside using Western blot technique consisting of 0.2 %SDS according to Laemmli perception with a 10 in percent detaching acrylamide gel (pH 8.8) and a 5% stacking gel. pH was 6.8. Gel staining was performed with R-250 blue brilliant coomassie recorded by a staining step with a mixture of methanol-acetic acid and water in ratio 5:2:6. Molecular Weight of ADI was observed by victimizing a marker of standard molecular mass protein in the compass of 20-130 kDa. Consequently, the molecular weight of ADI was identified followed by confirmation via a mass spectrometer.

Physicochemical factors characterization of of the refined ADI:

The activity of the purified ADI was characterized in the range of pH 4-12 with the L-arginine substrate liquefied in different Tris-HCl pH 9, glycine Na-OH buffer at pH 8- 9. besides at a temperature range of 10-70°C in 0.07 buffer of M Tris-HCl below the test conditions, incubating the test mixture was finished; The assessment of the temperature effect on ADI state was performed. Besides, incubation of the refined enzyme ; in the presence of various substrate concentrations 2–9 mM Broad concentrations of substrate were discovered for activeness of ADI . To assess the consequence of time of incubation on ADI action, the mixture of chemical reaction was incubated for assorted times 0, 10, 20, 30, 40, 50, 60 and 80 minutes.

Experimental animals:

From pharmacology and toxicology department of pharmacy faculty, University of Cairo of Cairo, Egypt male rabbit animal models weighing about 2kg and mice weighing 40-50 gm were accepted and employed to display and attempt the cytotoxic consequences of ADI medication cultivated from Bacillus cereus. Mice were housed in a conditioned atmosphere at temperature of 25 ± 1 °C and 55 ± 5% relative humidity with orderly 12 h dark/ 12 h light cycles and autonomous accession to standard laboratory food and water. All involvements were conducted Below the downright regulations and guidelines for laboratory animals sanctioned thru Ethical Committee of Pharmacy Faculty,University of Cairo, Egypt.

Induction of tumorigenicity in mice models of xenografts:

Below pathogen free circumstances 6-8 weeks-old male mice weighing 45-50 gm were retained rumored to establishment counseling. For setting up tumors of xenografts implantation of mice flanks subcutaneously with each aliquot of about 1.0 × 105 cancer cell lines(hepatic, lung, pancreatic, colorectal, renal, heart and melanoma cancer cell lines) suspended in 225 μL of PBS comprising 31% of Growth Factor Ablated Matrigel (obtained from Dickinson of Becton, USA) was achieved . The mice were haphazardly sorted into 3 groups with 5 mice in each group two weeks later. Either placebo(PBS), Doxorubicin standard anticancer drug 10 U/mice or ADI 5 U/animal model alone, in 100 μL of PBS were given every four days intraperitoneally to mice. Every 4 days, the volume of tumor (V) mm3 was measured exploiting the mathematical statement: $V = d2 \times D \times 0.3$ where d was latitudinal diameter; D represented longitudinal diameter). The tumors were excised and weighed after the mice were sacrificed 28 days subsequently.

Determination of selectivity index of ADI:

an antineoplastic agent selectivity index was evaluated using the equation: SI=IC50 of test enzyme in natural cell line/IC50 of test enzyme in cancer cell line. IC50 represents the concentration of test enzyme needed to kill 50% of cells. It was deliberated with exploitation of software of Graph Pad Prism version five.

The formulation of ADI in a proper dosage form:

In our study, different pharmaceutical dosage forms and routes of administration were attempted to determine the optimal dosage form and route of administration of bacterial ADI enzyme as an anticancer agent against auxotrophic cancers for L-arginine:

Injectable products were prepared in presence of isotonic aqueous solutions which have PH close to that of blood and body tissues

(PH 7.4). Thiamphenicol antimicrobial agent was added to injections prepared in containers. Aqueous solutions were given through intramuscular injection, and the release of ADI was controlled by increasing vehicle viscosity by using carboxymethylcellulose(CMC). Ethylene glycol was added afterward for prolonging the duration of action to once daily dose administration instead of multiple-dose injections.

Tablets of micro-particles of ADI 10 mcg/g were prepared by wet granulation method. Magnesium aluminum silicate 3% was added as an excipient. It was a binder, glidant, and disintegrant. Starch 17% w/w as diluent. Magnesium stearate 1% w/w was added as a lubricant agent. The evaluation of different drug delivery systems was done through rabbit animal models induced by hepatic carcinoma.

In vitro cell viability assay of ADI as anticancer agent:

JHH4 hepatic carcinoma cell line was used for assessment of the physiologic, pharmacologic, and toxicological effects of the enzyme on the hepatic cancer cells.

LIM1215 human colorectal cancer cell line was used for assessment of the physiologic,

pharmacologic, and toxicological effects of the enzyme on the colorectal cancer cells.

UPMM3 melanoma cancer cell line was used for assessment of the physiologic, pharmacologic, and toxicological effects of the enzyme on the melanoma cancer cells.

Calu-3 lung adenocarcinoma cell line was used for assessment of the physiologic, pharmacologic, and toxicological effects of the enzyme on the lung cancer cells.

HL1 cardiac cancer cell line was used for assessment of the physiologic, pharmacologic, and toxicological effects of the enzyme on the cardiac cancer cells.

PANC-1 pancreatic cancer cell line was used for assessment of the physiologic, pharmacologic, and toxicological effects of the enzyme on the pancreatic cancer cells.

RCC4 renal cancer cell line was used for assessment of the physiologic, pharmacologic, and toxicological effects of the enzyme on the kidney cancer cells.

The Vero cell line was used for the assessment of the physiologic, pharmacologic, and toxicological effects of the enzyme on normal mammalian cells. All these cell lines were purchased from an accegen biotic company. All these lines of cells of various kinds were preserved in 1% penicillin/streptomycin and 10% heat-inactivated fetal bovine serum in a humidified incubator(5% CO2, 37°C).

MTT((dimethylthiazol-2-yl)diphenyl tetrazonium)method was used for in vitro cell viability assay of ADI:

To culture and measure cell viability, wells of 96-well microplates were seeded with 10-50 µl of cell suspension at a density of 5000 cells per well. Plates were incubated for 24 hours at 37°C in a CO2 incubator and then exposed to different concentrations of ADI. 10 µL of 10% MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for cell viability assay was added to each well and incubated for 3 hours. 100 µl of DMSO (dimethylsulfoxide) was then added to the wells and the absorbance at 570 nm was read on a BioTek ELIZA reader. Cell viability was calculated as the percentage of healthy cells in the samples rather than controls (not treated with ADI). IC50 values were determined as the concentration that inhibited cell proliferation by 50%.

Assessment of antiproliferative actions of ADI via BrdU incorporation assay:

Genomic DNA synthesis and cell proliferation rates were assessed using the 50-bromo-20-deoxyuridine (BrdU) colorimetric ELISA kit (Roche, Germany). Different cell lines were seeded with appropriate concentrations of yielded ADI in 96-well culture plates at a density of 5000 cells per well for 48 hours and incubated with BrdU labeling solution for 8 hours at 37 °C (Liboska et al. 2012). Cells were then fixed and DNA denatured with FixDenat solution. Fixed cells were incubated with peroxidase-conjugated anti-BrdU antibody and exposed to the substrate tetramethylbenzidine. Finally the plate was read at 370 nm in a microplate reader. DNA synthesis per cell was calculated by dividing total DNA synthesis by the percentage of viable cells.

Caspase-3 activity assay for evaluation of programmed auxotrophic cancer cell death activity of ADI:

A caspase-3 assay kit was used to detect the activity of caspases, which play a central role in cell apoptosis. A colorimetric assay of caspase-3 activity was performed according to the manufacturer's protocol (Sigma-Aldrich Chemie GmbH). ADI-treated cell lines were lysed and 10 micrograms of supernatant was added to 85 microliters of assay buffer and 10 microliters of caspase-3 substrate, acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DVD-pNA). , 96 wells for 4 h at 37 °C. Multiple changes in caspase-3 activity were assessed by measuring p-nitroanilide (p-NA) levels. This compound was released by the enzymatic activity of caspase-3 by calculating the absorbance of p-NA at 405 nm.

Statistical analysis

All candidates were conducted in triplets. Their presentation was by means and standard deviation.One way analysis of variance (p value≤.05) was used as means for performing statistical analysis and also, statistical analysis based on excel-spreadsheet-software.

Results

Physiochemical parameters influencing kinetic activity of ADI:

Reaction pH exhibited a substantial characteristic in the activity of most ADI. The purified ADI was active over a wide pH range from 7.7 to 11 with a value of 49.458 U/ml at pH 9. The

enzymatic effect decreased at higher pH. Even at pH 11,ADI retained 71.29% of its activity and at pH 6 retained 61.87%. The effect of ADI from Bacillus cereus was gradually enhanced with explosive incubation times up to 50 minutes (ADI effect 69.245 U/ml). The molecular mass was 48 KDa as ascertained by a mass spectrometer. Vmax of 8.78 µmol/min and a Km value of 8.75 mmol/L was shown by ADI. The heat inactivation half-life time (t1/2) was 69.13 min at 65°C(Kd 0.039 min-1); while it was 73.05 min at 56°C(Kd 0.061 min-1). 52-64 % tumor growth inhibition was observed in mice treated with test ADI during randomized human clinical trials phases1/2 ; while 74-80% tumor growth suppression was revealed during animal testing(preclinical trials). The pure ADI presented a 6.576 purification fold and a total action of 791.108 with a particularized activeness of 70.044 U/mg protein. Less detectable IgG antibodies to ADI were noticed utilizing the ELISA technique. ADI displayed a peak action at pH 9, 37 °C temperature, time of incubation approximately 50 min and optimal concentration of substrate 10 mM.

Antioxidant activity of ADI:

It displayed worthless antioxidant consequences and tiny a scavenging activeness against cancer cells as compared to vitamin C as a standard antioxidant drug.

Distribution of ADI producing bacteria in grassland soil samples collected from different areas:

The study started by screening 50 soil samples for ADI producing bacteria. The grass land soil samples were from different areas in Egypt:

Menyat Sheben Elkanater, Qalyobia (n=1)

Eltal Bani Tamim, Sheben Elkanater, Qalyobia(n=2)

Monshaat Elkeram,Sheben Elkanater, Qalyobia(n=3)

Nawa,Sheben Elkanater, Qalyobia (n=4)

Kafr Sheben Elkanater, Qalyobia (n=5)

Elsahafa,Mashtol Elsok, Sharqia (n=6)

El manyer, Mashtol Elsok, Sharqia (n=7)

El manasra, Mashtol Elsok, Sharqia (n=8)

Brash, Mashtol Elsok, Sharqia (n=9)

Kafr Brash, Mashtol Elsok, Sharqia (n=10)

Eldir, Tokh,Qlayobia (n=11)

Arab Elhaswa, Tokh,Qlayobia (n=12)

Dandana, Tokh, Qlayobia (n=13)

Elmanzala, Tokh,Qlayobia (n=14)

Moshtohr, Tokh,Qlayobia (n=15)

Senhera, Qaha, Qalyobia(n=16)

Salamant, Belbis, Sharqia(n=17)

Menyat Salamant, Belbis, Sharqia(n=18)

Tal Rozon, Belbis, Sharqia(n=19)

Elsalam, Belbis, Sharqia(n=20)

Elzawamel, Belbis, Sharqia(n=21)

Awlad Seif, Belbis, Sharqia(n=22)

Burdin, Elzagazig, Sharqia(n=23)

Alaslogy, Elzagazig, Sharqia(n=24)

Elzahraa,Elzagazig, Sharqia(n=25)

Alnakarya, Elzagazig, Sharqia(n=26)

Alreyad, Elzagazig, Sharqia(n=27)

Alzanklon, Elzagazig, Sharqia(n=28)

Alsaada, Elzagazig, Sharqia(n=29)

Alelwya, Elzagazig, Sharqia(n=30)

Aboyasin, Abokabir, Sharqia(n=31)

Alahraz, Abokabir, Sharqia(n=32)

Alrahmanya,Abokabir,Sharqia(n=33)

Alkramos, Abokabir, Sharqia(n=34)

Manshyet Radwan, Abokabir, Sharqia(n=35)

Algamalya, Alhusynia, Sharqia(n=36)

Alhegazya, Alhusynia, Sharqia(n=37)

Alnasrya, Alhusynia, Sharqia(n=38)

Bahr Albakr, Alhusynia, Sharqia(n=39)

Sanhagr, Sharqia(n=40)

Nay, Qalyob, Qalyobia(n=41)

Kafr Ramada, Qalyob, Qalyobia(n=42)

Tanan,Qalyob, Qalyobia(n=43)

Meet Halfa, Qalyob, Qalyobia(n=44)

Alsad, Qalyob, Qalyobia(n=45)

Balaqs, Qalyob, Qalyobia(n=46)

Halaba, Qalyob, Qalyobia(n=47)

Meet Nama, Shubra Alkhima, qalyobia(n=48)

Kafr Hamza, Khanka, Qalyobia(n=49)

Arab Alelykat, Khanka, Qalyobia(n=50)

Morphololology, the biochemical tests and subculture of the isolates on sheep blood agar:

The positive bacterial isolates(1, 3, 7, 9, 11, 14, 16, 17, 19, 22, 23, 28, 31, 33, 35, 38, 39, 42, 46, 47, 49, 2, 5, 6, 15, 18, 21, 25, 26, 29, 37, 40, 41, 43, 45, 48, 50) on the mineral argenine agar were observed under a light microscope gram staining. They exhibited a charecterstic morphology of gram

positive, large rods, flagellated, motile.The colonies were large, irregular, yellowish white in colour and raised wrinkly colonies. They exhibited beta-hemolysis. They also, exhibited characteristic biochemical reactions of Bacillus cereus.

Determination of enviromental and physiologic factors affecting the growth of ADI producing bacteria:

The optimal conditions for the growth of ADI producing bacteria are PH 7.3 at temperature 37 0C.

Characterization of bacterial ADI production:

Activators of ADI enzyme production:

0.5 g/L KCL, 0.5 g/L MgSO4, 1.0 g/L KH2PO4, 0.1 g/L FeSO4, 0.1 g/L ZnSO4. The optimal PH and temperature for bacterial enzyme production were PH 7.3 at temperature 37 0C.

UV detection of production of Egyptian soil bacterial cultures containing ADI:

This was achieved threw using direct nesslerization test for the estimation of optical density of metabolic nitrogenous containing compound(Ammonia) due to degrading arginine present in media by bacterial arginine degrading enzymes(ADI) under PH7, Temp 37 0C, 0.05 g/L borate buffer and traces of Ni(0.02 mg/L), Co(0.04 mg/L) and Mn(0.03 mg/L) metal ions as co-factors.

The optimal conditions for bacterial arginine degrading enzymes production at different concentrations from 10-1 to 10-6 of the Egyptian soil containing bacteria producing arginine degrading enzymes using UV spectrophotometer were PH7.3 at temperature 37 0C and traces of activators Co, Ni, Mn.

Isolation and the screening of arginine deiminase producing bacteria:

A total of 37 bacterial isolates were isolated from different soil samples and were utilized for various screening studies. The current study involved the screening of isolated bacteria on minimal arginine agar media with the arginine as the sole metabolic source of nitrogen and carbon. Only the organisms that were able to utilize nitrogen and carbon could grow.

Identification and screening of arginine deiminase degrading enzyme:

The collected soil samples were further analyzed by nesslerization test. This method was applied for the determination of the ammonia concentration as a product of enzymatic degradation by arginine deiminase degrading enzyme. The concentration of the liberated ammonia by test samples demonstrated that arginine degrading enzymes were present in collected samples. Among the collected samples, samples from which were further identified as Bacillus cereus species produced the highest amount of arginine deiminase degrading enzyme.

The new primer for expression of arginine deiminase:

Forward primer for expression : Direction of sequence is from 5to 3

GGCAAAGTGAAGCCAACCAG

T. annealing=Tm-5=59.97-5=54.97 0C

Reverse primer for expression : Direction of sequence is from 5 to 3

TGGGGCCCTCGAGTTACATA

T.annealing =Tm-5=60.03-5=55.03 0C. The puc 18 plasmid was used as the the expression vector. The two restriction enzymes that were used and chosen according to webcutter website were EcoR I and Hind III.

The purity and yield of the produced test ADI enzyme:

The arginine deiminase produced from the new primer of expression in our study was characterized by high yield and purity.

Determination of activity of arginine deiminase enzyme by direct nesslerization and salicylate tests:

These tests revealed that the test arginine deiminase catalyzed the conversion of arginine amino acid into L-citrulline amino acid and ammonia. The liberated ammonia concentration was determined spectrophotometerically. The concentration and activity of arginine deiminase was directly proportional to the liberated ammonia concentration due to the degradation of L-arginine by ADI.

Determination of activity of arginine deiminase enzyme by the photo-metric measurement of L-citrulline concentration:

This test revealed that the test arginine deiminase catalyzed the conversion of arginine amino acid into L-citrulline amino acid and ammonia. The liberated citrulline amino acid concentration was determined spectrophotometerically. The concentration and activity of arginine deiminase was directly proportional to the liberated citrulline concentration due to the degradation of L-arginine by ADI.

In vitro cell viability assay:

This assay showed higher efficacy of the test arginine deiminase as anticancer agent against auxorophic cancer cell lines such as hepatic carcinoma cells, melanoma cells, lung cancer, heart cancer,pancreatic cancer,renal cancer and colorectal cancer cells prepared by tissue culture techniques. This enzyme showed no activity at acidic PH. It showed higher activity at alkaline PH but not exceeding PH 10 with the highest activity at PH 9. In presence of cobalt as co-factor at neutral PH 7.3, arginine deiminase was as effective as alkaline conditions.

The determination of optimum and suitable dosage form and route of administration of ADI:

The route of administration of arginine deiminase was by intramuscular or subcutaneous injection. It had a short half life and needed to be given several times daily. In our study we could overcome this problem by extending its duration of action by addition of polyethylene glycol.

Characterization of the production and biological activity of bacterial ADI produced by recombinant DNA technology:

ADI was produced as extracellular protein and purified from the superannuate of a centrifuge tube by ion exchange resin chromatography after precipitation by ammonium sulfate. Recombinant ADI showed high yield, productivity, stability, specificity, and few side effects as an anticancer mediator against auxotrophic cancers for L-arginine.

The determination of the cytotoxic activity of bacterial ADI against human cancer cell lines:

The purified bacterial ADI enzyme produced by recombinant DNA technology was assessed on 7 cancer cell lines for its in vitro anticancer effect via the standard MTT assay. Bacterial ADI

enzyme displayed varying degrees of inhibitory activity against the tested human cancer cell lines(JHH4 hepatic carcinoma cell line, LIM1215 human colorectal cancer cell line, UPMM3 melanoma cancer cell line, Calu-3 lung adenocarcinoma cell line, HL1 cardiac cancer cell line, PANC-1 pancreatic cancer cell line and RCC4 renal cancer cell line) and the normal Vero cell line. ADI at concentration 10 U/ml inhibited the cell viability of the cancer cell lines after 24 hrs of incubation by 78.9%(JHH4), 74.3%(LIM1215), 67.2%(UPMM3) and 70.3%(Calu-3) and 68.3%(HL1) respectively, 75.49% (PANC-1), 64.61%(RCC4); while inhibited the cell viability of the normal Vero cell line by 51.4%.

IC50 of ADI against hepatic cancer cell line(JHH4) was 2.31 U/ml, 4.51 U/ml against colorectal cancer cell line(IM1215), 2.19U/ml against melanoma cancer cell line(UPMM3), 4.64 U/ml against lung adenocarcinoma cell line (Calu-3), 3.07 U/ml against PANC-1 pancreatic cancer cell line, 3.81U/ml against RCC4 renal cancer cell line and 4.28 U/ml against cardiac cancer line(HL1); while 8.7U/ml against the normal cell line.

Standard doxorubicin as an anticancer agent inhibited the viability of cancer cell lines by 89.3%(JHH4), 91.1%(LIM1215), 92.7%(UPMM3), 90.1%(Calu-3), and 88.2%(HL1) respectively, while inhibited the cell viability of normal Vero cell line by 92.3%. Ic50 of standard doxorubicin against hepatic cancer cell line(JHH4) was 5.2 U/ml, 4.19 U/ml against colorectal cancer cell line(IM1215), 4.47U/ml against melanoma cancer cell line(UPMM3),4.52 U/ml against lung adenocarcinoma cell line(Calu-3), 3.04 U/ml against PANC-1 pancreatic cancer cell line, 3.22 U/ml against RCC4 renal cancer cell lineand 4.82 U/ml against cardiac cancer line(HL1), while 8.66 U/ml against the normal Vero cell line.

Selectivity index of bacterial ADI against cancer cell lines:

It was 3.81 against (JHH4) hepatic carcinoma cell line, 3.33 against ((IM1215) colorectal cancer cell line, 3.52 against (UPMM3)melanoma cancer cell line, 3.12 against (Calu-3) lung adenocarcinoma cancer cell line, 3.27 against PANC-1 pancreatic cancer cell line, 3.14 against RCC4 renal cancer cell line and 3.74 against (HL1) cardiac cancer cell line.

BrdU incorporation in vitro:

To explore if the ADI treatment has an inhibitory effect on DNA synthesis in auxotrophic cancer cell lines, a colorimetric BrdU proliferation assay was applied. A dose-dependent reduction in proliferation of auxotrophic cancer cells was observed after ADI treatment for 48 h. 39 and 84% for JHH4 ,11 and 54% for IM1215,41 and 89% for UPMM3, 23 and 67% for Calu-3, 37 and 76% for PANC-1, 29 and 84% for RCC4 and 29 and 76% for HL1 reduction in cell proliferation was observed by ADI at 0.3, 0.5, 0.8, 0.4,0.7,0.6 and 0.9 IU/ml respectively.

Caspase-3 activity assay:

Caspase-3 apoptosis index in the ADI treated JHH4, IM1215, UPMM3, Calu-3, and HL1 cells were increased in a dose-dependent manner. The results provide support for the fact that the produced ADI induces apoptotic cell death by increasing the enzymatic activity of the caspase-3. ADI at 0.5 IU/ml concentration induces the activity of caspase-3 up to 14, 13, 15, 18, 11, 17, 16 and 19% in JHH4, IM1215, UPMM3, Calu-3, PANC-1, RCC4 and HL1 cells respectively. Also, at 0.9 IU/ml concentration this induction was increased up to 23, 19, 31, 28, 22, 25 and 35% in JHH4, IM1215, UPMM3, Calu-3, PANC-1, RCC4 and HL1 cells respectively.

ADI augments in vivo inhibition of tumorigenesis auxotrophic cancer cells:

Auxotrophic cancer cells for L-arginine were subcutaneouly ingrained into naked mice to yield a xenograft. The mice were processed with ADI (2 U/mouse), doxorubicin(2 U/mouse) and PBS (placebo 2 U/mouse) when the xenograft tumors were full-grown to about 50 mm3. The neoplastic tumors From days 25–30 markedly vitiated during the period of management in all groups except the control group. Tumor growth was regressed with ADI management.

Table 1. Ingredients of Metallic Arginine Agar:.

Ingredient	**Unit of measurement**
Potassium chloride	0.5 mg
Magnesium sulfate	0.5 mg
$KH2PO_4$	1.0 g
Ferrous sulfate	0.1 g
Zinc sulfate	0.1 g
L.arginine	1.0 g
Agar	2%

Table 2. list of ingredients of blood agar

Ingredient	**Unit of measurement**
peptone	10.0 g/L
tryptose	10.0 g/L
Sodium chloride	5.0 g/L
Agar	15.0g/L
Distilled water	960 ml

Table 3. List of instruments:

Instrument	**Model and manufacturer**
Autoclaves	**Tomy, japan**
Aerobic incubator	**Sanyo, Japan**
Digital balance	**Mettler Toledo, Switzerland**
Oven	**Binder, Germany**

Deep freezer -80 ^{0}C	**Artiko**
Refrigator 5	**whirpool**
PH meter electrode	**Mettler-toledo,UK**
Deep freezer -20	**whirlpool**
Gyratory shaker	**Corning gyratory shaker,Japan**
190-1100nm Ultraviolet visible spectrophotometer	**UV1600PC, China**
Light(optical) microscope	**Amscope 120X-1200X, China**

Table 4. Prevalence of ADI producing bacteria among grassland samples:

Grassland soil sample(n)	Positive isolate	Negative isolate
n1	+	
n2	+	
n3	+	
n4		-
n5	+	
n6	+	
n7	+	
n8		-
n9	+	
n10		-
n11	+	
n12		-
n13		-
n14	+	
n15	+	
n16	+	
n17	+	
n18	+	
n19	+	
n20		-
n21	+	
n22	+	
n23	+	
n24		-
n25	+	
n26	+	
n27		-
n28	+	
n29	+	
n30		-

n31	+	
n32		-
n33	+	
n34		-
n35	+	
n36		-
n37	+	
n38	+	
n39	+	
n40	+	
n41	+	
n42	+	
n43	+	
n44		-
n45	+	
n46	+	
n47	+	
n48	+	
n49	+	
n50	+	
Total	**37**	**13**

Table 5. It shows biochemical reactions

Biochemical test	**Result**
Catalase test	positive
Lecithinase test	positive
Methyl red	negative
Indole test	positive
Voges proskauer	positive

Table 6. It shows Nesslerization assay:

Concentration of soil bacterial culture containing ADI(serial dilutions from 10^{-1} to 10^{-6})	Optical density of ammonia at 425 nm by UV spectrophotmeter
0.00	0.00
10^{-1}	0.438
10^{-2}	0.376
10^{-3}	0.307
10^{-4}	0.226
10^{-5}	0.151
10^{-6}	0.078

Table 7. Distribution of cellular positions of ADI:

Proportion	**Percent(%)**
Extracellular	67
Surface bound	23
Cytosolic	10

Table 8. It shows a computation of bacterial arginine deiminase action via the modified acetyl monoxime thiosemicarbazide technique:

Concentration Of soil bacterial culture containing arginine deiminase enzyme(serial dilutions from 10^{-1} to 10^{-6} mg/l)	The absorbance of liberated L-citrulline at 520 nm by UV spectrophotometer
0.00	0.00
1/10	0.410
1/100	0.382
1/1000	0.341
1/10000	0.283
1/100000	0.177
1/1000000	0.076

Table 9. It shows a computation of bacterial arginine deiminase enzyme action via the salicylate technique:

Concentration Of soil bacterial culture containing arginine deiminase enzyme (serial dilutions from 10^{-1} to 10^{-6} mg/l)	The absorbance of liberated ammonia at 425 nm by UV spectrophotometer
0.00	0.00
1/10	0.421
1/100	0.386
1/1000	0.372
1/10000	0.297
1/100000	0.188
1/1000000	0.082

Table 10. It shows the action of bacterial arginine deiminase enzyme as a function of the reaction PH:

PH	Arginine deiminase enzyme action
3.5	10
4.5	20
5.5	30
6.5	40
7.5	45
8.5	50
9.5	47
10.5	39

Table 11. It shows the influence of various incubation times on arginine deiminase enzyme activity:

Incubation time(min)	Arginine deiminase enzyme action(U/ml)
10	30
20	45
30	50
40	55
50	75
60	71
70	64
80	59

Table 12. It shows the Michaelis-Menten plot for arginine deiminase enzyme:

Substrate concentration(mM)	Rate of reaction(µM/min)
1	40
2	80
3	90
4	100
5	110
6	117
7	118
8	120
9	122
10	124

Table 13. It shows the Influence of substrate concentration on the enzyme activity of arginine deiminase enzyme:

Substrate concentration(mM)	Arginine deiminase enzyme activity (U/ml)
1	42
2	78
3	91
4	102
5	115
6	117
7	118
8	119
9	120
10	122

Table 14. It shows the effect of the temperature on arginine deiminase enzyme activity:

Temperature ^{0}C	Arginine deiminase enzyme activity U/ml
20	70
25	75
30	89.5
35	90
40	89
45	80
50	79
55	70
60	64
65	60

Table 15. It demonstrates the scavenging(antioxidant) activity of vitamin C via nitric oxide scavenging assay:

Concentration of sample(mcg/l)	Percentage of inhibition(%)
100	20
200	30
300	52
400	68
500	79

Table 16. It shows tumerogenicity detection of vehicle influence:

Tumor volume(mm^3)	Time(days)
100	5
200	10
300	15
400	20
500	25
600	30
700	35
800	40

Table 17. It shows tumerogenicity detection of ADI influence:

Tumor volume(mm^3)	Time(days)
400	5
350	10
300	15
250	20
250	25
200	30
150	35
100	40

Table 18. It displays the formulation of ADI as a sterile solution at PH 7:

Ingredient	concentration
ADI	15 mg
PEG20	45 mg
Mono-basic sodium phosphate	USP, 1.5 mg +5%
Di-basic sodium phosphate	USP, 3 mg + 5%
Sodium chloride	USP, 7.5 mg +5%
Water for injection	Query size to 1 ml

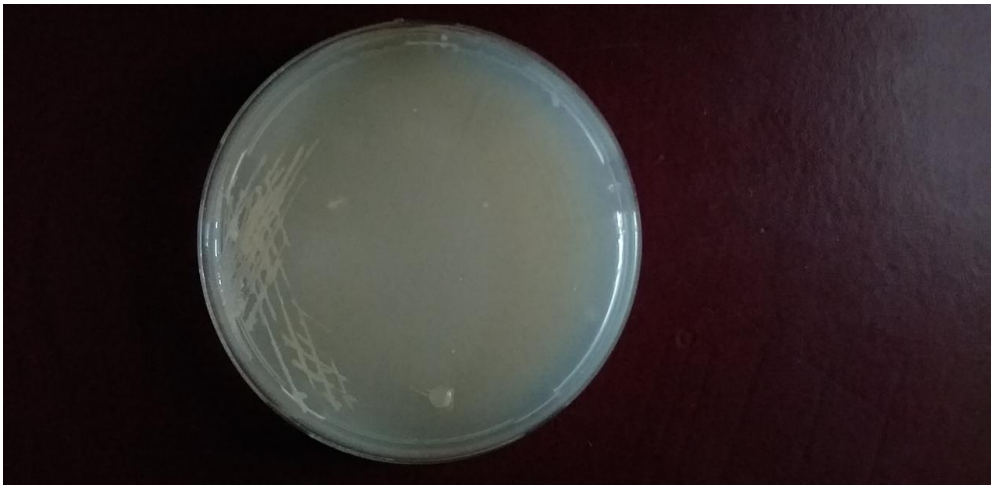

Figure 1. It shows positive Bacillus cereus bacterial isolates concealing ADI on MAA.

Figure 2. It demonstrates subculture of the positive bacterial isolates of Bacillus cereus secreting ADI(taken from positive bacterial bacillus cereus isolates on MAA) on the nutrient agar plate.

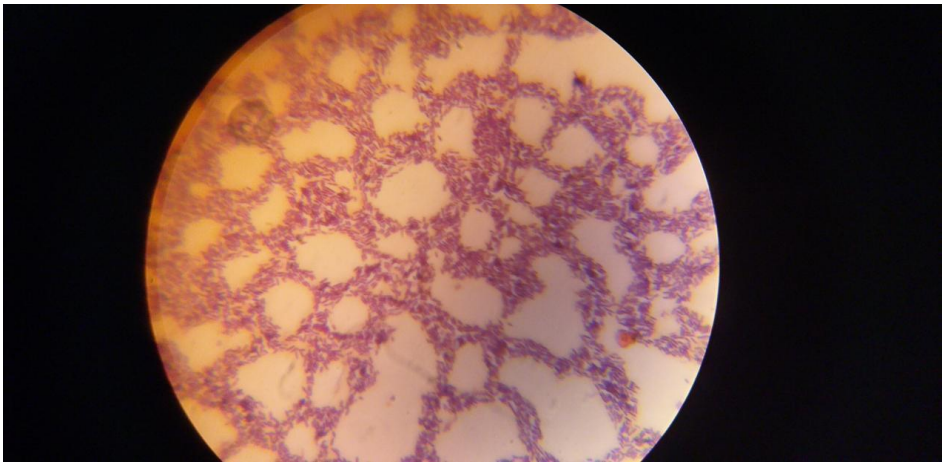

Figure 3. It displays Gram positive Bacillus cereus secreting ADI

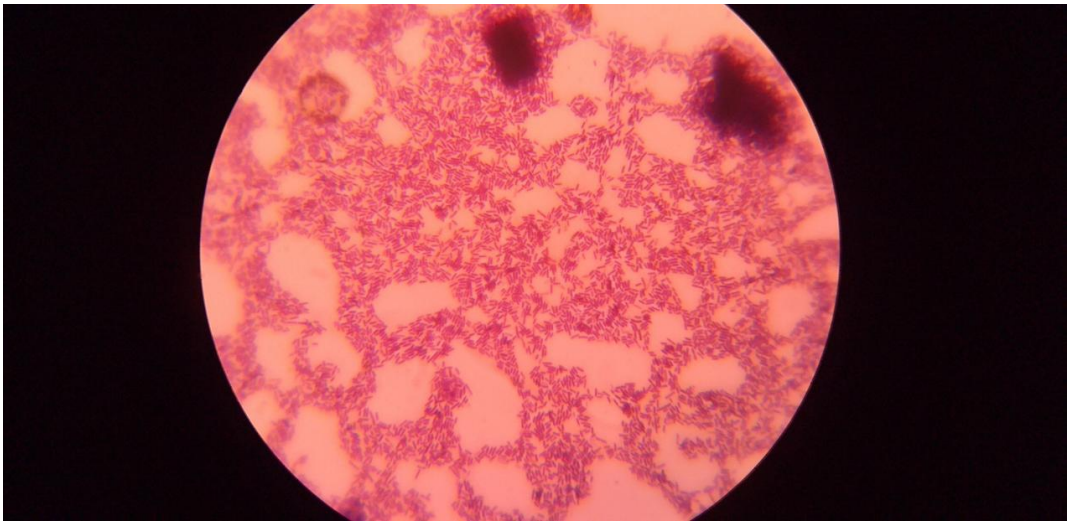

Figure 4. It shows Gram-positive bacilli producing ADI enzyme as the anticancer agent from the soil environment in Egypt.

Figure 5. It shows positive catalase test with postive bacterial Bacillus cereus isolates yielding ADI on MAA.

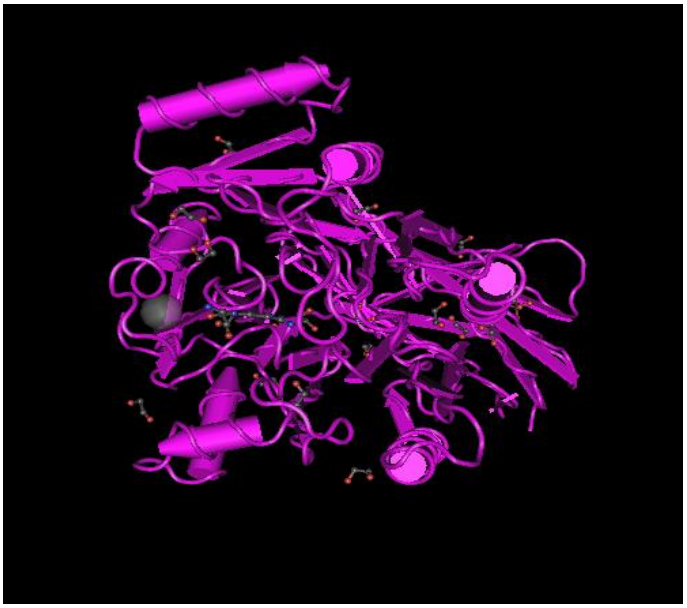

Figure 6. 3D structure of Arginine deiminase. It was composed of 418 amino acids.

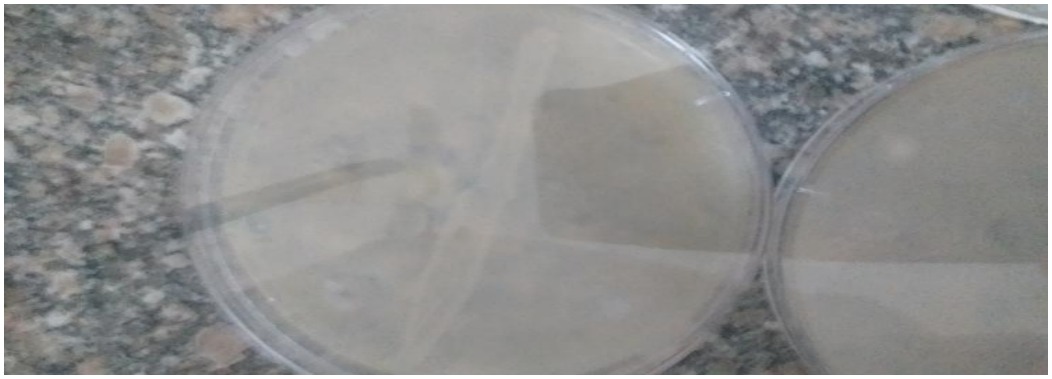

Figure 7. Biochemical reaction presents positive lecithinase test with positive bacterial Bacillus cereus isolates on MAA.

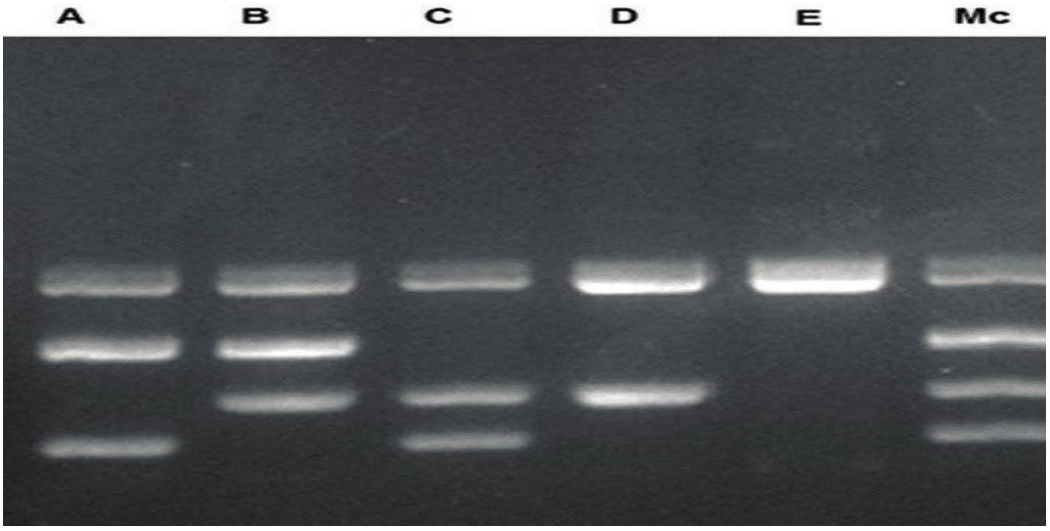

Figure 8. It shows different amounts and sizes of recombinant proteins of arginine deiminase measured via the Western blot technique. The maximum yield of ADI was 75-80 mg/l. The purity of recombinant arginine deiminase was approximately 86%.

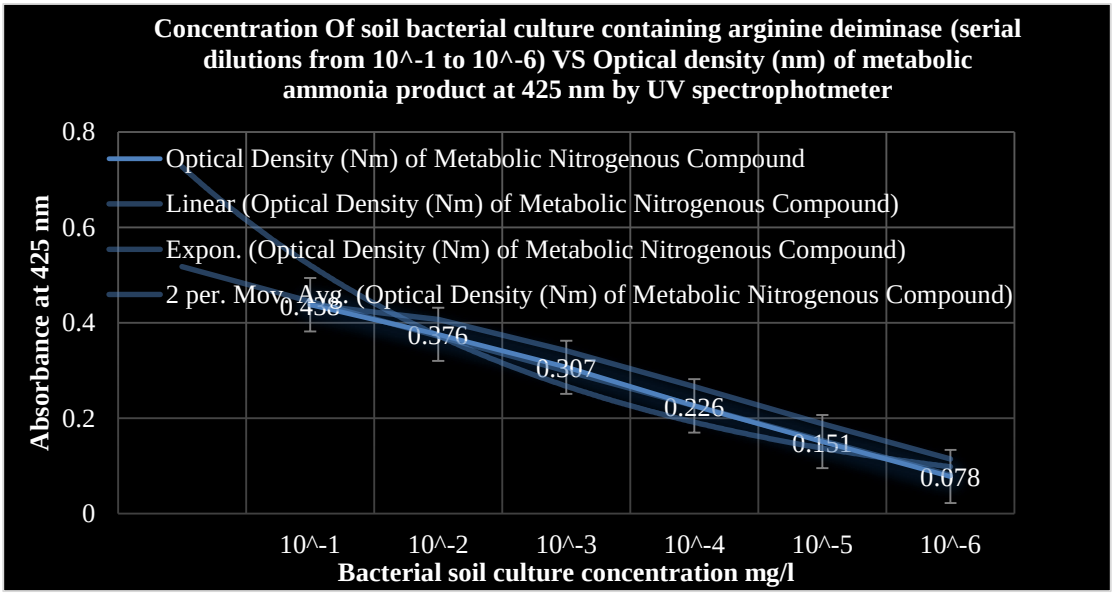

Figure 9. It represents a Nesslerization screening assay of different concentrations of soil bacterial isolates producing ADI.

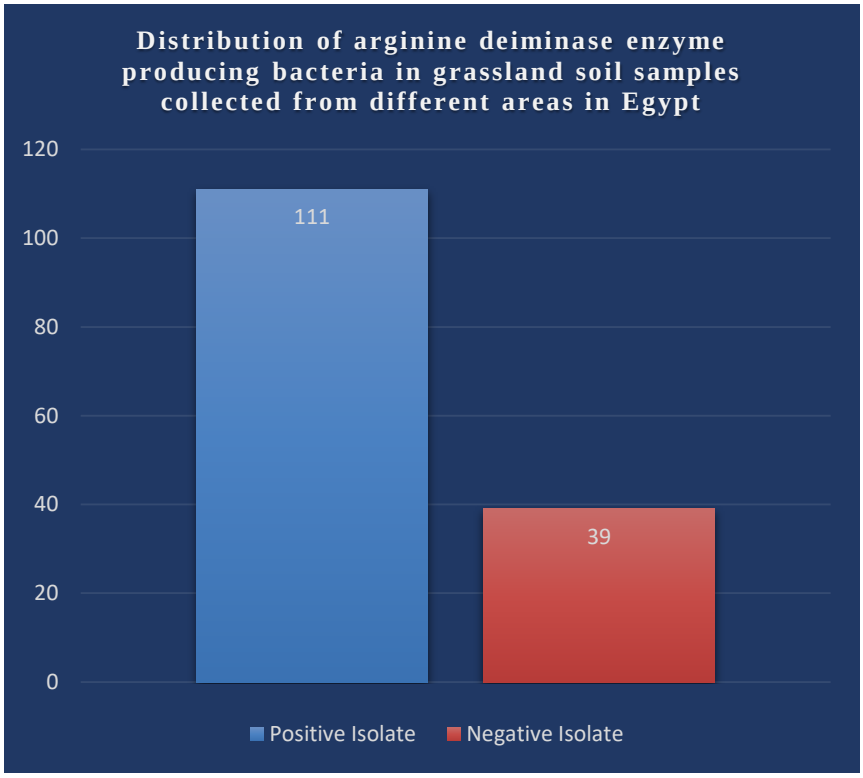

Figure 10. It represents a distribution of arginine deiminase enzyme producing bacteria.

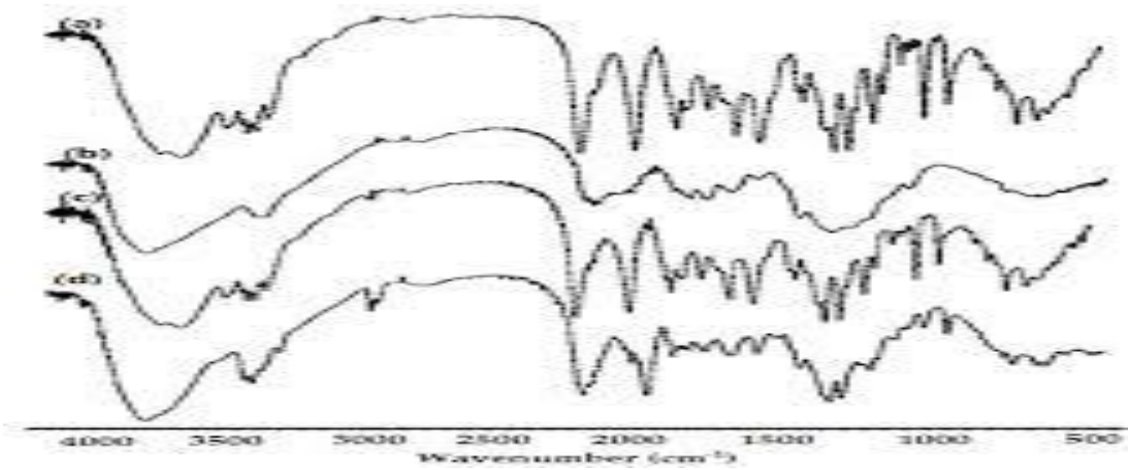

Figure 11. FTIR spectroscopy shows no drug-drug interaction or incompatibility between arginine deiminase enzyme and excipients.

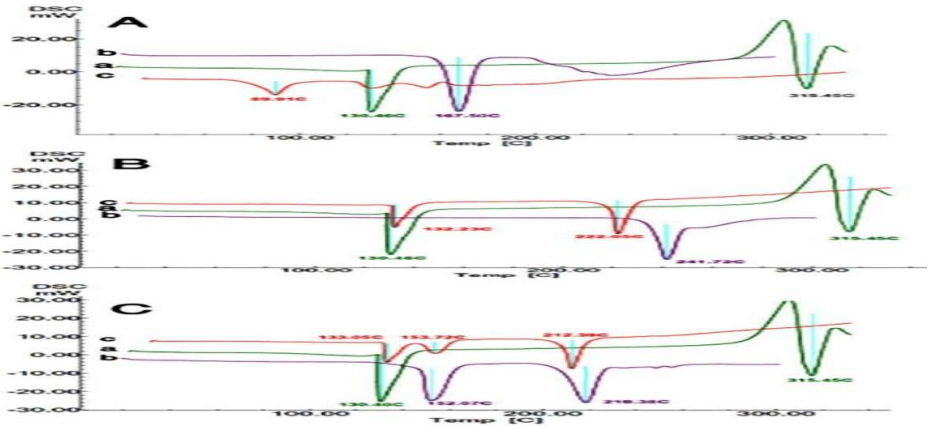

Figure 12. DSC study shows no drug-drug interaction or incompatibility between arginine deiminase enzyme and excipients.

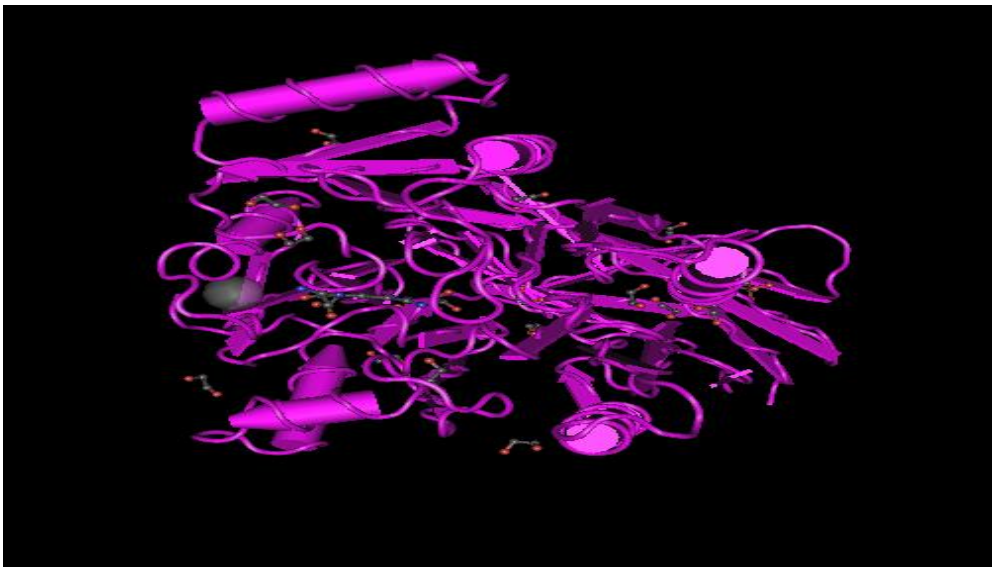

Figure 13. 3D structure of Arginine deiminase. It is composed of 418 amino acids.

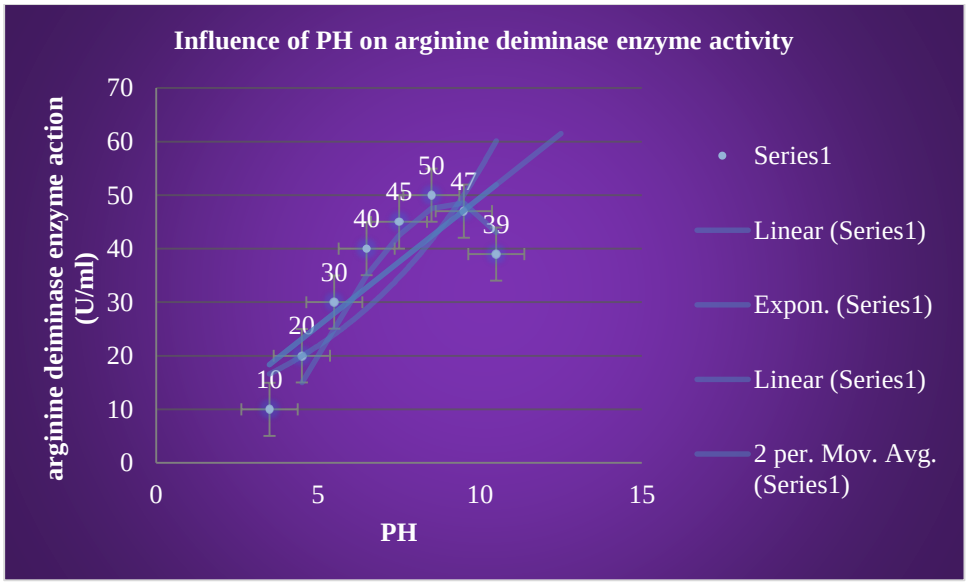

Figure 14. It shows the action of bacterial arginine deiminase enzyme as a function of the pH of the reaction

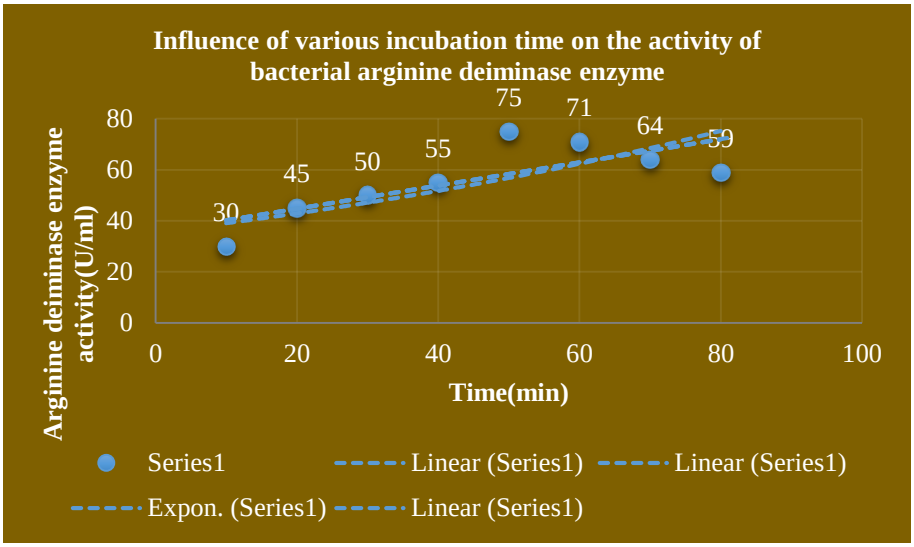

Figure 15. It shows the influence of various incubation times on arginine deiminase enzyme activity.

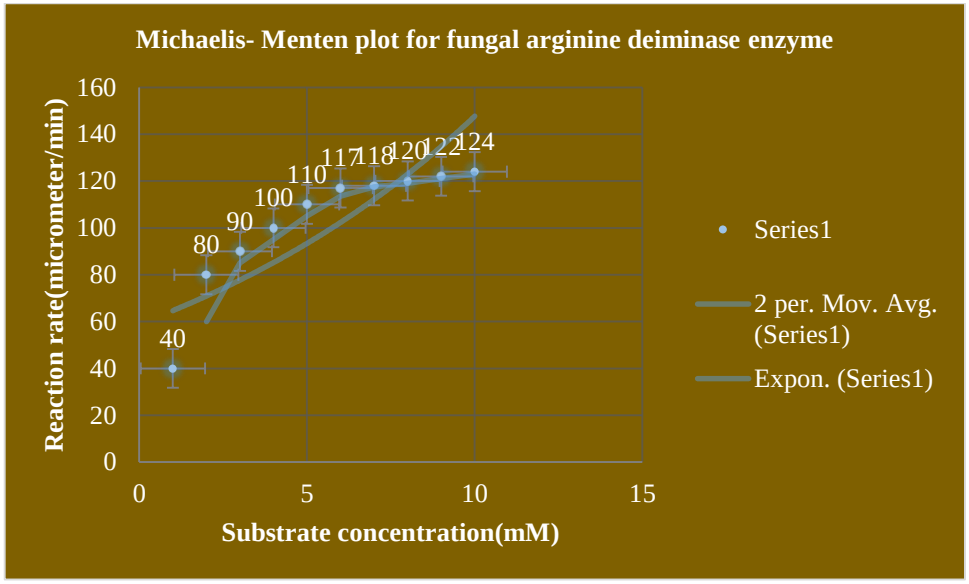

Figure 16. It displays the Michaelis-Menten plot for arginine deiminase enzyme

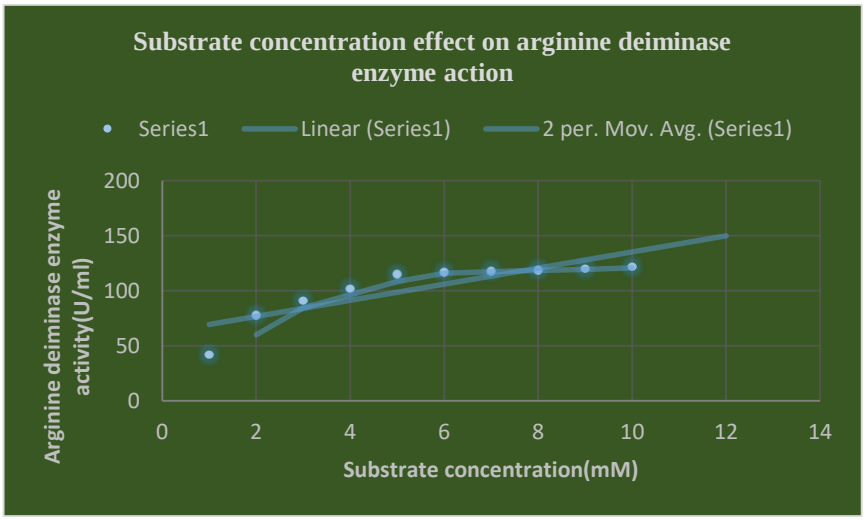

Figure 17. It shows the Influence of substrate concentration on the enzyme activity of arginine deiminase enzyme

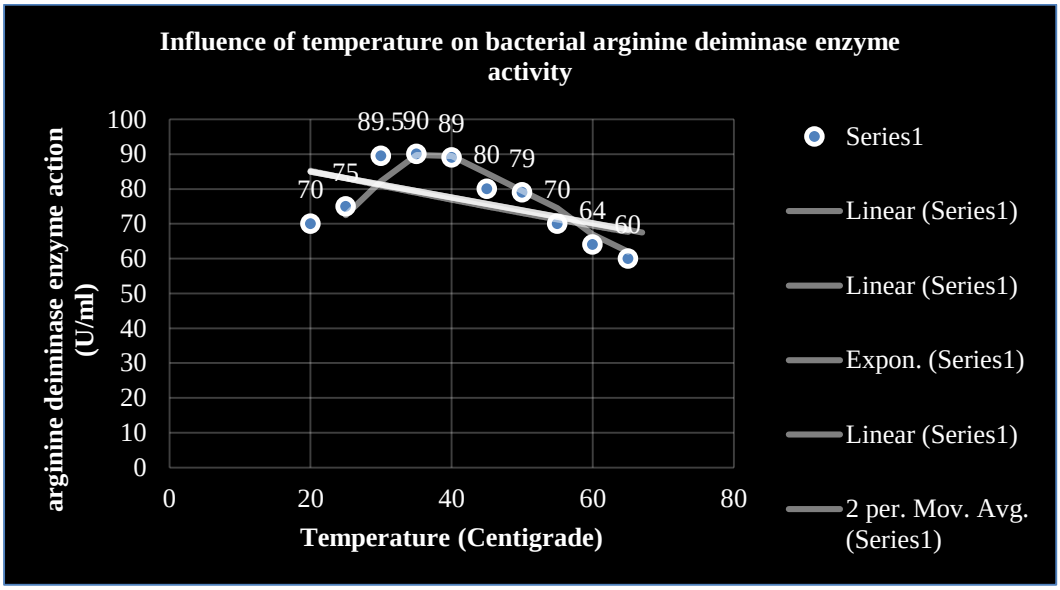

Figure 18. It represents the effects of temperature on bacterial arginine deiminase enzyme activity.

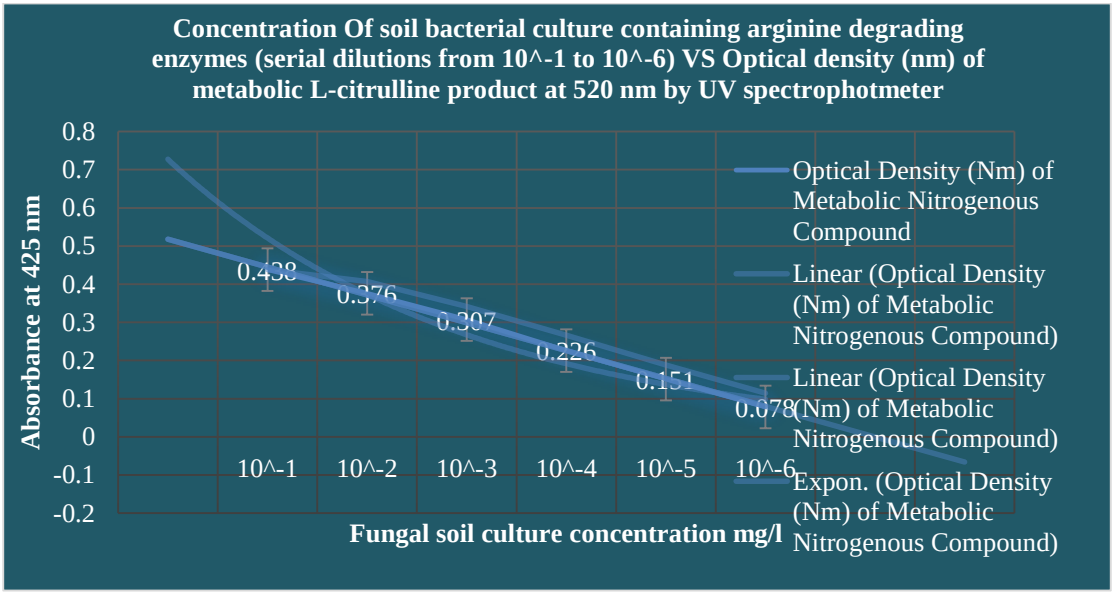

Figure 19. It represents a modified diacetylmonoxime thiosemicarbazide screening assay of different concentrations of soil bacterial arginine deiminase enzyme.

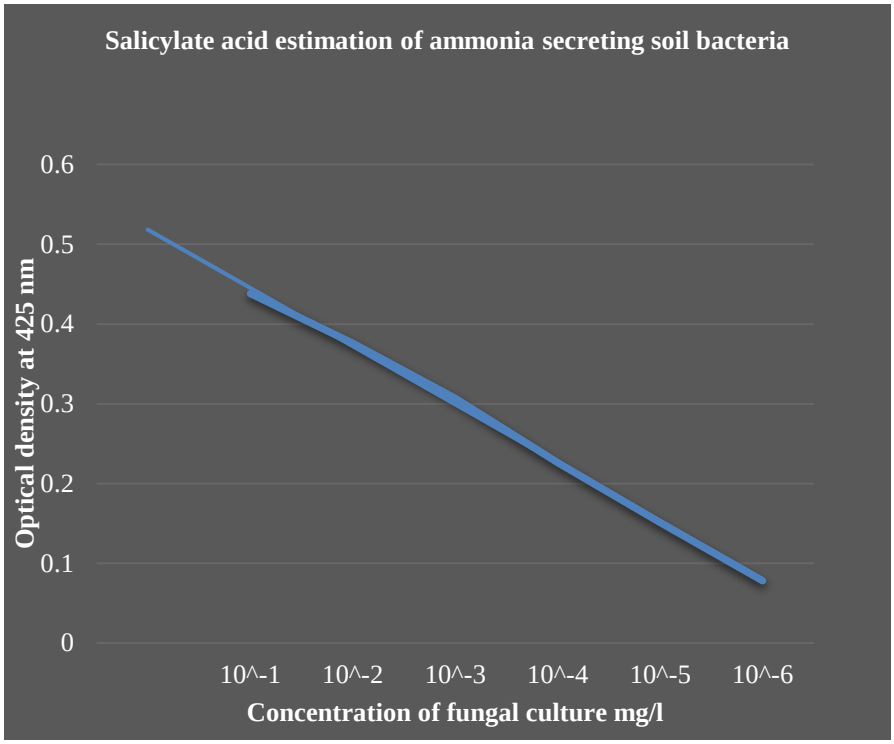

Figure 20. Liberated ammonia at 425 nm by different Concentrations Of soil bacterial culture containing arginine deiminase enzyme.

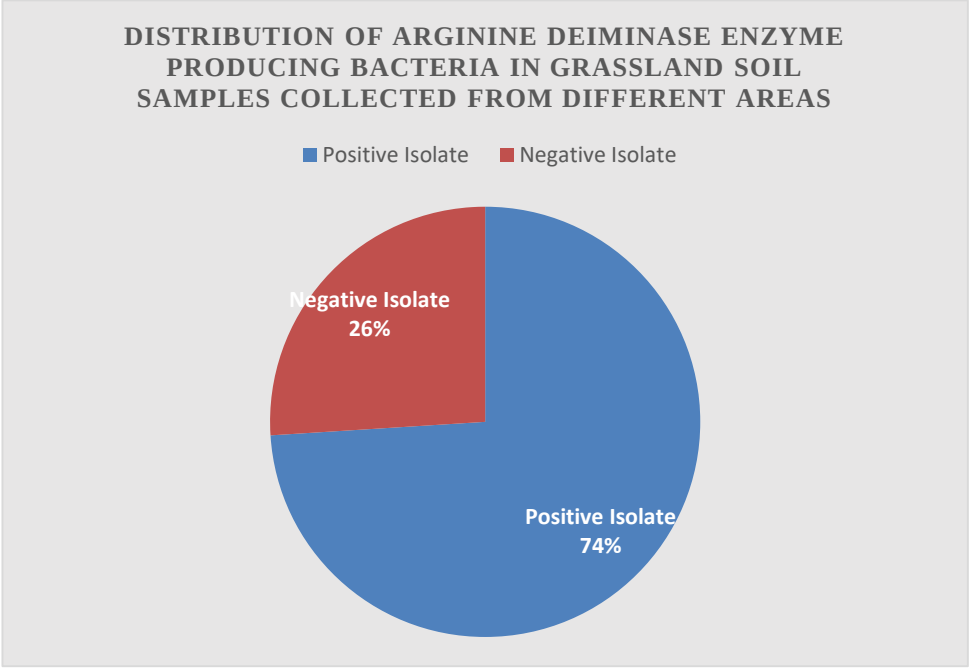

Figure 21. Distribution of arginine deiminase enzyme producing bacteria in grassland soil samples collected from different areas.

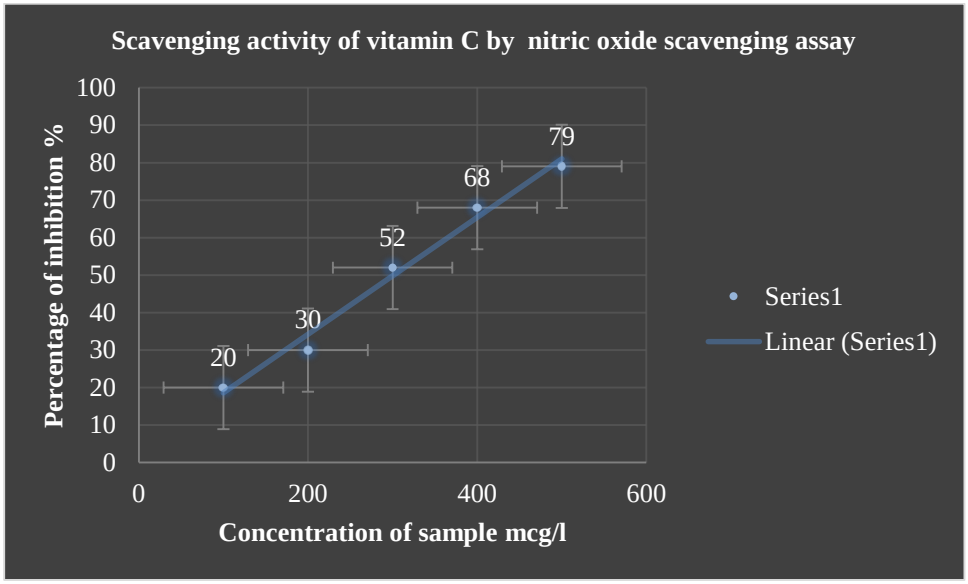

Figure 22. It represents the scavenging activity(antioxidant activity) of standard vitamin C through nitric oxide scavenging assay.

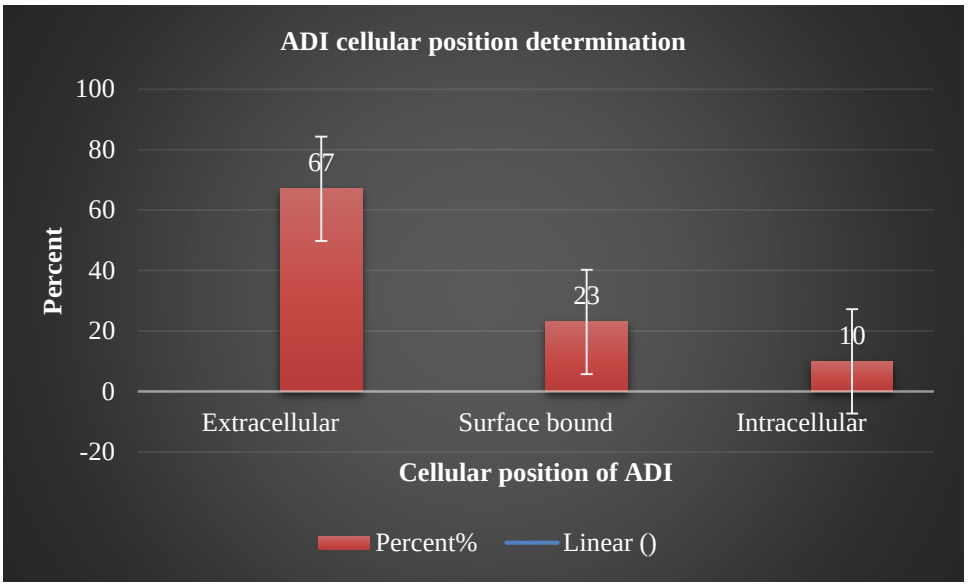

Figure 23. It displays the cellular position detection of ADI through Nesslerization assay.

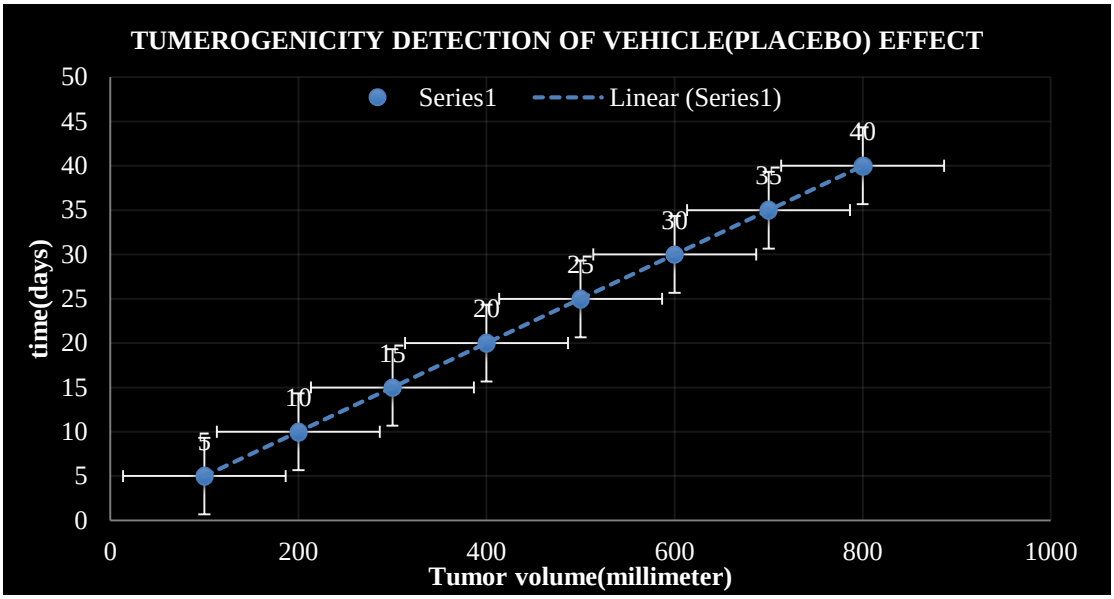

Figure 24. It shows tumerogenicity detection of vehicle influence.

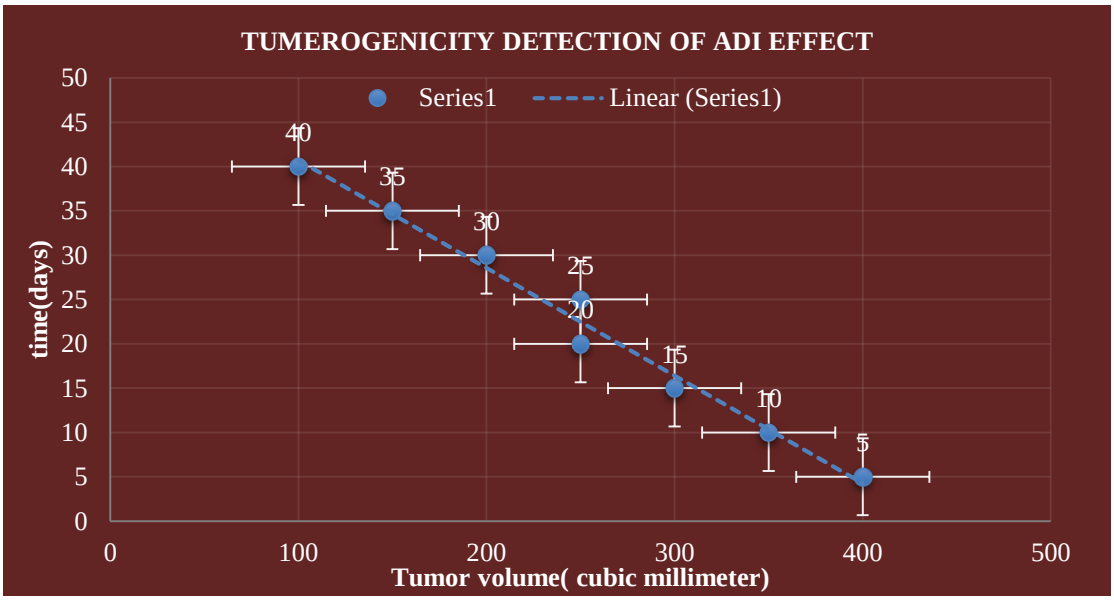

Figure 25. It shows tumerogenicity detection of ADI influence.

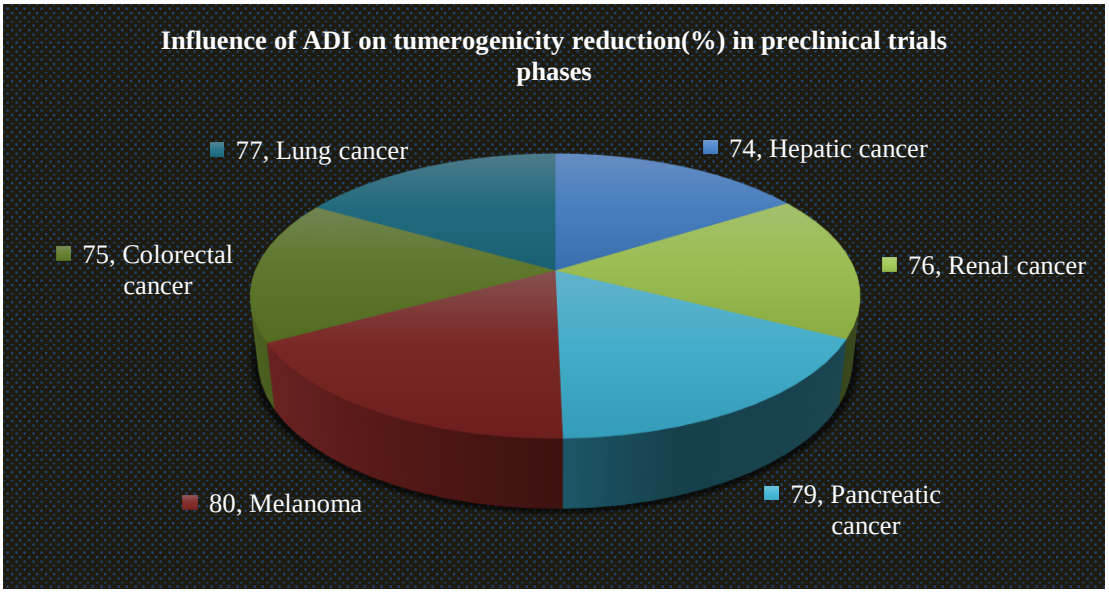

Figure 26. It represents the percentage of the decrements in tumor size in preclinical trials trials phases by ADI.

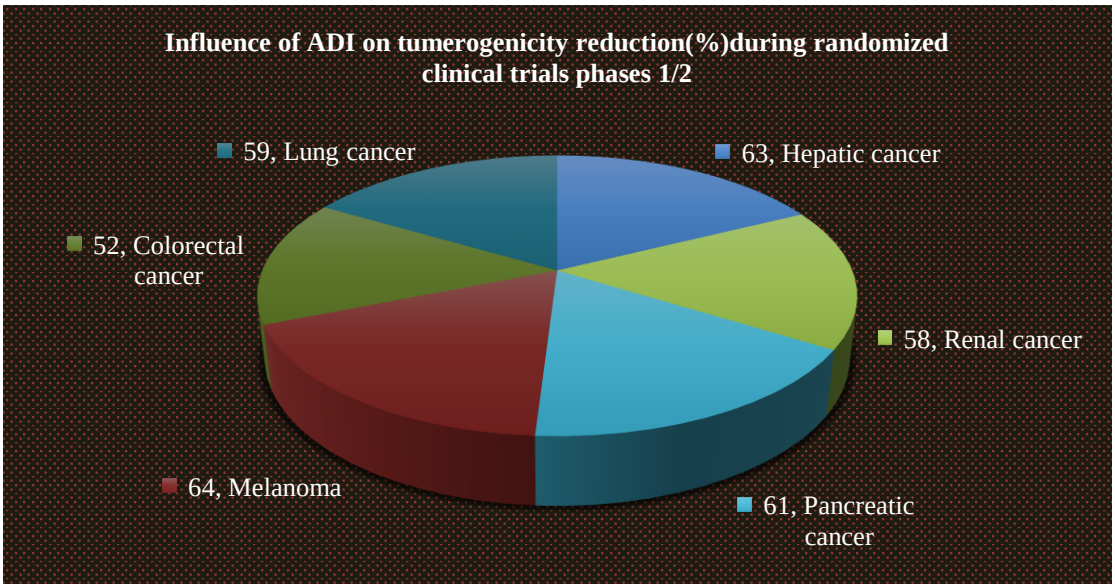

Figure 27. It represents the percentage of the decrement in tumor size in randomized clinical trials phases 1/2 by ADI.

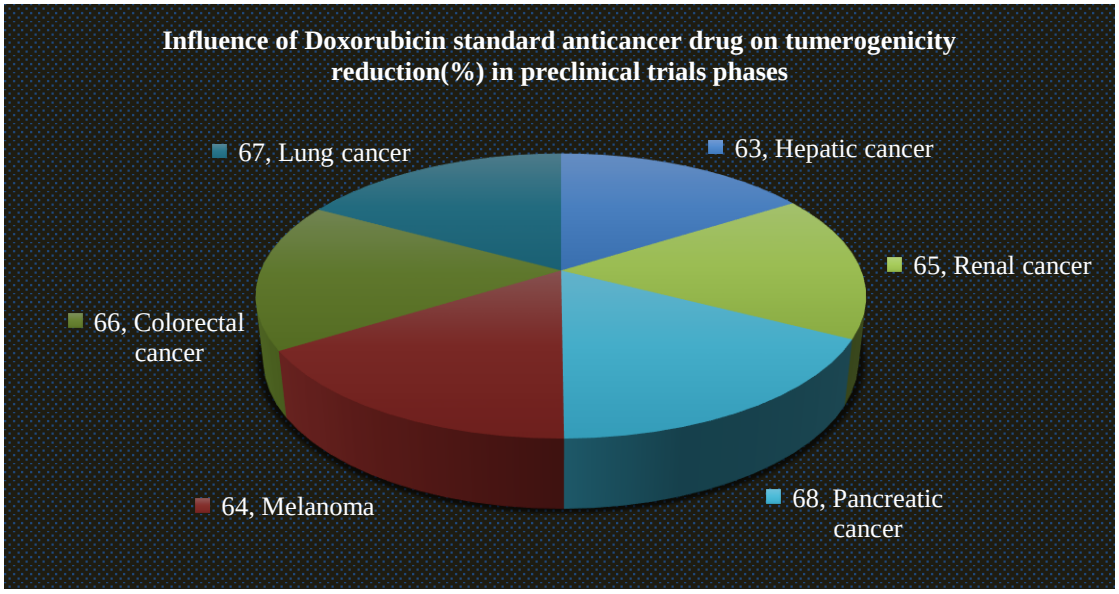

Figure 28. It represents the percentage of the decrement in tumor size by standard doxorubicin anticancer drug in preclinical trials phases.

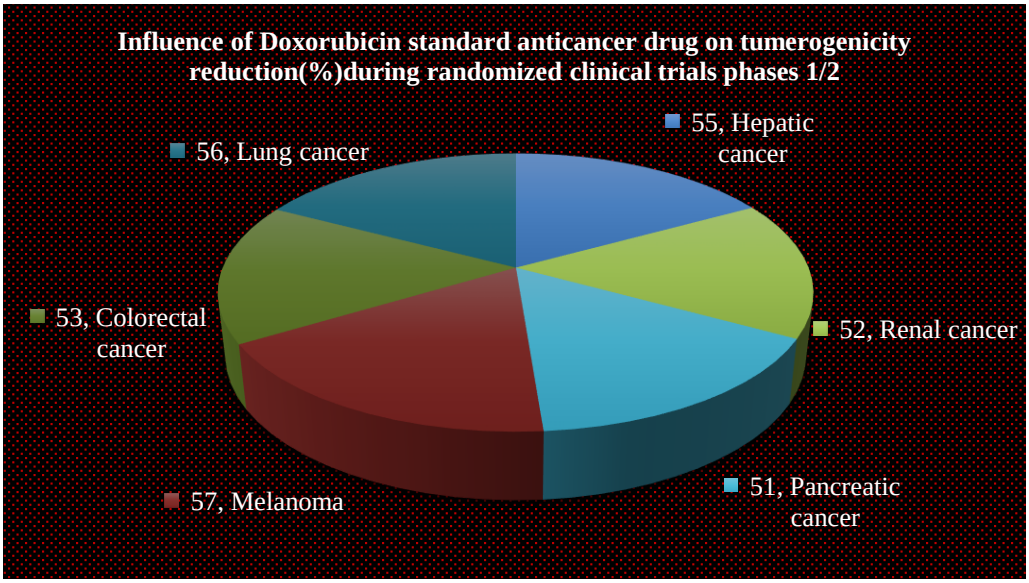

Figure 29. It represents the percentage of the decrement in tumor size via standard doxorubicin anticancer drug in randomized clinical trials phases 1/2.

Discussion

Grandness of ADI:

Arginine debasing enzymes are indispensable for the management of auxotrophic cancers for L- arginine such as hepatic carcinoma, melanoma, lung cancer, heart cancer,pancreatic,kidney and colorectal cancers. Auxotrophic cancers have not the power to synthesize arginine but natural cells can synthesize it, thus auxotrophic cancers depend on receiving the arginine amino acid from the external environment. Arginine is essential for auxotrophic cancers because they can not synthesize it, but is not basal for normal cells because they can synthesize it. Arginine degrading enzymes include arginine deiminase, L-arginase enzyme and arginine decarboxylase enzyme. Our study was concerned with isolation and screening of bacterial producing ADI enzyme as anticancer agent for management of auxotrophic cancers from contrastive soil environments in Egypt. Also, it was concerned with resolution of environmental and physiological factors touching the growth of some ADI producing bacterial isolates. Characterization of ADI enzyme activity was also enclosed in our study. In our study

some bacterial isolates were investigated for production of ADI on mineral arginine agar selective media(MAA). These bacterial isolates which displayed positive growth on MAA used the arginine as the sole metabolic origin of carbon and nitrogen for their maturation. Only the bacterial producing ADI isolates demonstrated onto-genesis on MAA. The optimal environmental and physiological elements affecting growth of these positive isolates were PH 7.3 at temperature 37 0C. The morphological and the biochemical reactions revealed that Bacillus cereus and Bacillus anthracis were the major positive bacterial producing ADI isolates of soil samples collected from assorted soil environments. Nesslers test was utilized for discovery and screening of the presence of bacterial arginine degrading enzymes via their ability to bring forth ammonia from debasing the arginine in various dilutions of soil samples. Gram positive Bacillus cereus and anthracis bestowed the high quantities of bacterial arginine degrading enzymes. This was confirmed by biochemical reactions and positive isolation on mineral arginine agar media. The optimum conditions for characterization of ADI production were with the following activators KCL, ZnSO4, FeSO4, K2HPO4, MgSO4 at PH 7.3 at temperature 37 0C.

Isolation and the screening of ADI producing bacteria:

A total of 37 bacterial isolates were isolated from different soil samples and used for various screening studies.

The current study involved the screening of isolated bacteria on MAA media with arginine as the sole metabolic source of nitrogen and carbon. Only the organisms that were able to utilize nitrogen and carbon were grown on MAA media.

Identification and the characterization of the most potent bacteria:

The morphological characteristics of 37 isolates which showed high ADI activity indicated that the organism belonged to the genus Bacillus. The biochemical characterization by biochemical tests revealed that it has about 82% similarity with Bacillus cereus and about 18% similarity with Bacillus anthracis. Comparing our results with another study conducted in India(Isolation, screening, and characterization of ADI producing soil bacteria, July 2019), it was found that the prevalent ADI producing bacteria were Bacillus cereus and Bacillus anthracis in our results, while It was Pseudomonas aeuroginosa in Indian study.

Molecular detection and characterization by DNA hybridization and Northern blotting technique revealed that the most potent bacterial isolates were Bacillus cereus strain AH173 , strain W23(82%), and Bacillus anthracis, strain 34F2(18%).

Identification and the characterization of the most potent bacteria:

The morphological characteristics of 37 isolates which showed high arginine deiminase activity indicated that the organism belonged to the genus Bacillus.The biochemical characterization by biochemical reactions revealed that it was similar to Bacillus cereus.Comparing our results with another study conducted in Iran(Isolation, screening and characterization of arginine deiminase producing soil bacteria), it was found that the prevalent producing bacteria were Bacillus cereus group in our results, while It was Pseudomonas aeuroginsae species in the other study.

Environmental and physiological factors affecting the growth of producing bacteria:

The optimal conditions for the growth of ADI were PH7.3 at a temperature of 37 0C.

Characterization of bacterial ADI production:

The activators of the enzyme production :0.5 g/l KCL,0.5 g/l MGSO4,1.0 g/l FESO4,0.1 g/l ZnSO4.

The design of a new primer for expression of bacterial ADI enzyme from Bacillus cereus:

In our study, we could design a new primer for the expression of bacterial ADI. It increased the purity, productivity, and yield of recombinant ADI as an anticancer agent against auxotrophic cancers for L-arginine.

The determination of the cytotoxic activity of bacterial ADI against human cancer cell lines:

In comparison with previous studies and standard doxorubicin as an anticancer agent against auxotrophic cancers for arginine, Our bacterial ADI was found to have strong antiproliferative effects and strong cytotoxic activity against hepatic carcinoma and melanoma, while it showed moderate cytotoxic activity and antiproliferative effects against colorectal cancer, lung adenocarcinoma, and heart cancer. Our bacterial ADI demonstrated weak cytotoxic activity and no antiproliferative effects against normal human cells suggesting that it is an ideal anticancer mediator against auxotrophic cancers for L-arginine with few side effects on the normal human cells.

The determination of optimal dosage form and route of administration of bacterial ADI:

In our study, the optimal dosage and route of administration of bacterial ADI enzyme were by intramuscular or subcutaneous injection. It had a short half-life (t1/2=3hours) and needed to be given

several times daily. In our study we could overcome this problem by extending its duration of action by addition of polyethylene glycol, therefore we recommend an intramuscular or subcutaneous injection of ADI once daily as an anticancer agent against auxotrophic cancers for L-arginine. The oral drug delivery system of ADI was not convenient due to the chemical instability of the enzyme in acidic environments in the stomach and degradation by proteolytic enzymes in the digestive tract such as pepsin and trypsin protease enzymes.

Selectivity index of bacterial ADI against cancer cell lines:

In comparison with previous studies and standard doxorubicin as an anticancer agent against auxotrophic cancers for arginine, Our bacterial ADI was found to have higher selectivity towards auxotrophic cancers for L-arginine such as hepatic, lung, colorectal, melanoma and heart cancers.

Mechanism of action:

Bacterial ADI in our study was found to have anticancer activity against auxotrophic cancers for L-arginine due to cancer cytostatic effect, caused apoptosis of the auxotrophic cancer cells for L-arginine and deprived the auxotrophic cancer cells of L-arginine which was the essential metabolic source for carbon and nitrogen for these cells. Malignant neoplasms of auxotrophic cancers for L-arginine with ablated expression of arginosuccinate synthetase (ASS) were affiliated with local invasion and much lymph node metastasis with high expression of survivin. Management of ASS-deficient auxotrophic cancer cells with ADI diminished their growth in a time and dose dependent way. Moreover, the antineoplastic consequences on auxotrophic cancers cells for L arginine were augmented by arginine deiminase via multiple mechanisms regarding up-regulation of the expression of caspase 9 and 3, inhibition of activation of the NF-κB survival pathway and induction of cell cycle arrest in the S phase. Moreover.23

Therapeutic indications:

In our study, ADI showed great efficacy against auxotrophic cancers for L-arginine and solid tumors which form a mass of the cells inside the organs of the human body but it was ineffective against liquid tumors which circulate all over the human body, have rapidly dividing cells and high replication rates such as myeloma, lymphoma, and leukemia.24

Conjugation of ADI with PEG20 diminished the immunogenicity. This was observed via ELISA immunogenicity categorization. As well, ADI extended the time period of action.25

Conclusion:

Gram-positive Bacillus cereus and Bacillus anthracis displayed high levels of bacterial ADI. This was addicted by biochemical reactions, molecular detection by DNA hybridization, blotting, and positive isolation on arginine mineral agar. The optimal circumstances for characterization of ADI manufacture were pH 7.3 and temperature 37 °C with the succeeding activators KCL, ZnSO4, FeSO4, K2HPO4, and MgSO4. Bacterial ADI was a promising as a co-chemotherapeutic agent for auxotrophic cancers of solid tumors ;consequently it is suggested to exhibit its large scale production.

Acknowledgement:

A patent 882/2021 authorized by ministry of scientific research in Egypt.

Conflict of interest:

There is no conflict of interest.

Fund:

This study was carried out in a research project number 46361/2020 funded by STDF.

Data availability:

Raw data were generated at faculty of pharmacy, Cairo university,Egypt. Derived data supporting the findings of this study are available from the corresponding author Dr. Mohammed Kassab up on request.

References

1- **Bardal Stan,Waechter Jason, Martin Douglas. 2020.**Applied pharmacology.V14: Chemotherapeutic drugs. Elsevier Edinburgh, London. 2020.

2- **Caroline S,Zeind Michael G. 2018.** Applied therapeutics, the clinical use of drugs.V11:Complications of End-Stage Liver Disease.Wolters Kluwer, London. 2018.

3- **Christin Riessa et al. 2018.** Arginine-Depleting Enzymes-An increasingly Recognized Treatment strategy for Therapy-Refractory Malignancies. Journal of cellular physiology and biochemistry;51:854–870. 2018.

4- D**ipro Cecily, Schwinghammer Terry, Dipro Joseph, Well Barbara. 2021**.pharmacotherapy handbook.V11:Hepatic carcinoma. McGraw Hill Education, New York. 2021.

5- **Fisher Bruce, Champe Pamela, Harvey Richard. 2021.** Lippincott illustrated reviews microbiology.V6:Virology. Wolters Kluwer, London. 2021.

6- **Golderg Stephen. 2020.** Clinical physiology is made ridiculously simple.V6: Physiology of the liver. Med Master, Miami, United States of America. 2020

7- **Levinson Warren. 2021.**Review of medical microbiology and immunology. V15: Immunology. McGraw Hill Education, New York. 2021

8- **Meeting Patricia J. 2019.** Physiology.V16: Gastrointestinal physiology.McGraw Hill Education, New York. 2019

9- **Narjes Ebrahimi et al. 2016.** Selective Isolation and Identification of Arginine Degrading Bacteria; the Optimized Arginine Deaminase Production by Enterobacter sp. Sign as a New Source of This Potentially Anti-Tumor Enzyme. Journal of applied pharmaceutical science. Volume 6(9). 2016

10- **Olson James. 2020.** Clinical pharmacology made ridiculously simple. V7: Anticancer drugs. MedMaster, Miami, United States of America. 2020

11- **Parveen Kumar. 2017.Kumar**, Clark's clinical medicine.V9: Oncology.Elsevier Edinburgh London. 2017.

12- **Parveen Nadaf et al. 2019.** Isolation, screeningand characterization of L-arginase producing soil bacteria. International journal of pharmaceutical sciences and research; 10(7):3440-44. 2019

13- **Trevor Anthony,Katzung Bertram,Kruidering-Hall Marieke. 2021.** Katzung Trevor pharmacology examination board review.V13: Anticancer drugs. McGraw Hill Education, New York. 2021.

14- **Swanson Larry N, Souney Paul F, Muntnick Alan H, Shargel Leon. 2019**. Comprehensive Pharmacy Review for NAPLEX.V10:Oncology.Wolters Kluwer, London. 2019

15- **Wilson Golder N. 2019**. Biochemistry and genetics. V8:Gastroenterology and nutrition. McGraw Hill Education, New York. 2019

16- **Houssam Al-Koussa et al. 2020**. Arginine deprivation: a potential therapeutic for cancer cell metastasis? A review. Cancer Cell International volume 20, Article number: 150. 2020.

17- **Ye Ni et al. 2008.** arginine deiminase, a potential anti-tumor drug. Journal of cancer lett; 2008 Mar 8;261(1): 1-11.

18- **Lynn Feun et al. 2011.** Pegylated arginine deiminase: a novel anticancer enzyme agent. Journal of Expert Opin Investig Drugs; 15(7):815-822.

19- **Ziyu Wang et al. 2021**. Aminoacid degrading enzymes and autography in cancer therapy. Journal of Front. Pharmacol., 11 January 2021; Sec. Pharmacology of anticancer drugs.

20- **Jiangbo Liu et al. 2014.** Arginine deiminase augments the chemosenstivity of arginosuccinate synthetase-deficient pancreatic cancer cells to gemcitabine via inhibition of NF-KB signaling. BMC Cancer journal 14, Article number: 686(2014).

21- **Anjana Sharma et al. 2017**. Preliminary evaluation of arginine deiminase activity of indigenous bacterial strains of suitable chemotherapeutic applications. Journal of biocatalysis and agricultural biotechnology. V 12, October 2017, Pages 66-77.

22- **Mahboubeh Zarei et al. 2019.** Arginine deiminase: Current understanding and applications. Journal of recent patents on biotechnologygy. V 13(2): 124-136.

23- **H Gong et al. 1999.** Arginine deiminase inhibits cell proliferation by arresting cell cycle and inducing apoptosis. Journal of Biochem Biophys Res Commun. 1999 Jul 22; 261(1): 10-4.

24- **Riess C. et al. 2018.** arginine depleting enzymes- an increasingly recognized treatment strategy for therapy- refractory malignancies journal of cellular physiology and biochemistry. 2018; 51: 854-870.

25- **Rakhi Dhankhar et al. 2021.** Invitro and in Silico analysis of recombinant arginine deiminase feom Pseudomonas furukawaii as a potential anticancer enzyme. Journal of research square. 2021; 43: 1051-1069.

Kassab, M. Identification and molecular characterization of arginine deiminase-producing bacteria in Egyptian soil from various soil environments. IJHS (Egypt) 2024; 2(2): 84-116.