SCREENING AND PRODUCTION OF MICROBIAL L-ARGINASE ENZYME AS ANTICANCER AGENT FROM DIFFERENT SOIL ENVIRONMENTS IN EGYPT

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ABSTRACT:

Background: *Hydrolysis of L-arginine to L-ornithine and urea is catalyzed by the crucial L-arginase enzyme. L-arginase nowadays is necessary for medicine due to its significance as an anticancer agent against auxotrophic cancers to arginine. It exists and is widely distributed in plants, fungi, chordates, and non chordates, however, there are few available reports for L-arginase from bacteria. Auxotrophic cancer cells for L-arginine can not synthesize their L-arginine as a sole metabolic source of carbon and nitrogen, while normal cells can synthesize their L-arginine for normal growth. Deprivation of auxotrophic cancer cells from L-arginine causes their killing.*

The aim of the study: *Our study was aimed at isolation, screening, and molecular detection of L-arginase expressing bacteria from different soil environments in Egypt and characterization of the enzyme of activity as an anticancer mediator against auxotrophic cancer cells for arginine.*

Materials and methods: *50Grassland soil samples were collected from different locations in Egypt. The activity of L-arginase was confirmed by rapid plate assay screening using mineral arginine agar (MAA) media and estimating the optical density of Urea level spectrophotometrically. DNA Northen blotting hybridization technique, Gram stain, and biochemical reactions were used to identify the predominant soil bacterial isolates producing L-arginase. Tissue culturing techniques were applied for screening of anticancer activity of bacterial L-arginase against auxotrophic cancers for arginine.*

Results: *Only the bacterial isolates which were able to utilize L-arginine as the sole metabolic source for carbon and nitrogen were grown on MAA media. Molecular detection and the biochemical reactions revealed that* ***Bacillus subtilis, strain W23 (74%)****, and* ***Bacillus anthracis, strain 34F2 (26%)*** *were the predominant bacterial isolates producing L-arginase as an anticancer mediator for auxotrophic cancers for arginine.*

Bacterial L-arginase showed excellent anticancer activity against auxotrophic cancers for L-arginine in presence of Co, Ni, and Mn metal ions as co-factors. The molecular weight of bacterial L-arginase was found to be 39KDa as determined by the mass spectrometer.

IC50 of bacterial L-arginase was 2.31 U/ml against hepatic cancer cell line,2.19 U/ml against melanoma cancer cell line and 4.51 U/ml against colorectal cancer cell line,4.64 U/ml against lung adenocarcinoma cell line (Calu-3) and 4.28 U/ml against cardiac

cancer line(HL1). The Km and Vmax values of L-arginase were 8.63 mmol/l and 7.41 µmol /min.

Conclusion: *Our study was a promising approach study due to discovering a new bacterial L-arginase enzyme as an anticancer agent from different soil environments in Egypt.*

Keywords: *L-arginase; bacterial; molecular detection; cancer.*

INTRODUCTION

Difference between benign and malignant tumors: Malignant tumors are neoplasm (cancer), while benign tumors are not cancer[(1)]. Benign tumors do not spread to other tissues and Locally being grown. they may represent a danger if they badly affect vital organs like the brain[(2)]. Malignant tumors can invade and spread to other tissues. This is called metastasis[(3)]. Cancer metastasis is crucial as it aids in determining the staging and the treatment[(4)]. Metastatic neoplasms are secondary neoplasms as their origin is from primary cancer[(5)].

I. Auxotrophic cancer cells are the leading cause of death in humans worldwide. approximately 70% of cancer types are auxotrophic for L-arginine as a sole metabolic source of carbon and nitrogen[(6)]. **Auxotrophic cancer cells deficient in arginine biosynthesis:** Auxotrophic cancer cells such as hepatic, lung, heart, colorectal, and melanoma cancer cells cannot synthesize arginine amino acid which is essential for their survival and growth[(7)]. Normal cells can synthesize it. Deprivation of these cancer cells from arginine by bacterial arginase is of great value[(8)]. Auxotrophic cancers for L-arginine cannot biosynthesize arginine due to a lack of expression of argininosuccinate synthetase-1[(9)]. **The physiologic importance of arginine for cell biology in cancer cells:** Arginine is necessary for the biosynthesis of proline, glutamate amino acids which are essential for their growth, polyamine production which prevents their apoptosis, and production of nitric oxide by nitric oxide synthase which is a principal vasodilator [(10)]. L-arginine directly activates mTOR expression in auxotrophic cancer cells thus causing carcinogenesis as it is a nutrient-sensing kinase [(11)]. Mitochondrial activities of auxotrophic cancer cells are regulated by L-arginine levels inside these cells[(12)]. **Degradation of arginine amino acid:** It is degraded either by arginase enzyme in the arginine urease cycle with the production of urea and L-ornithine[(13)]. Or by arginine deiminase enzyme (dihydrolase enzyme) in the dihydrolase pathway[(14)]. Lack 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)]. In this present study, we searched for new treatments for auxotrophic cancers for arginine such as bacterial L-arginase enzyme. The source of enzyme was from bacteria due to higher productivity, yield and purity of the bacterial enzymes than other sources such as fungi and mammals. We aimed at collecting grassland soil samples from different environments in Egypt; boost isolation of L-arginase producing bacteria on selective media followed by determination of physiologic and environmental factors poignant the growth of some selected bacterial isolates producing L-arginase and characterization of L-arginase production and activity by some selected bacterial isolates.

MATERIAL AND METHODS:

Ethical statement:

In the attendant study, 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 Weatherall report with approval number P-18-4-2020. All efforts were performed to ablate the number of humans and animals utilized and their suffering during study.

Table 1. It represents 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	Artikel
Refrigerator 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

All chemicals and biochemical materials were purchased from Algomhoria pharmaceutical company in Cairo, Egypt. 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 cereus* strain AH173.

Collection of Soil Samples: Samples were collected randomly from acidic soils and soils beside wheat mills from different governorates in Egypt, then stored in sterile polythene bags at 4ºC until further use.

The study protocol was Collecting grassland soil samples from different environments; Isolation of L-arginase producing bacteria on selective media; Determination physiologic and environmental factors affecting the growth of some selected bacterial isolates producing L-arginase; characterization of L-arginase production and activity by some selected bacterial isolates. **Type of Study:** Screening experimental study. **Place and date of the study:** This study was done faculty of pharmacy, Cairo university, Egypt between April 2020 and June 2021. **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 animals; can be induced by hepatic carcinoma; blood ammonia levels can be easily estimated; obese animals**. Exclusion criteria were:**

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

Methods:

Isolation and Screening of L-arginase Producing Bacteria:

Soil samples were serially diluted, spread on the nutrient agar plates, and incubated at 37ºC for 24h. Bacterial colony morphology was studied, and examined for microscopic and biochemical characteristics. Isolates were screened for L-arginase activity by streaking on mineral arginine agar media (MAA) media constituting: KCl 0.7%, MgSO4 0.3%, KH2PO4 1.5%, FeSO4 0.2%, ZnSO4 0.3%, L-arginine 2.0%, and fluconazole (24 µg/ml) . The inoculated

plates were incubated for 24 h at 37ºC. Only the bacterial isolates expressing L-arginase which were able to utilize L-arginine as a sole metabolic source for carbon and nitrogen were able to grow on MAA media.

Determination of bacterial L-arginase Activity by diacetyl monoxime test in presence of Co, Mn, and Ni metal ions as co-factors for the enzyme:

L-arginase activity was determined based on the amount of urea released in the reaction. L-arginase enzyme production and activity were analyzed based on the spectrophotometric measurement of the liberated urea concentration by the diacetyl monoxime method at 520 nm wavelength which resulted from the hydrolysis of L-arginine by this enzyme. In this test, urea reacts with diacetyl monoxime in presence of sulfuric, phosphoric acids, and ferric chloride forming a pink-colored complex. The absorbance (optical density) of this complex is determined colorimetrically by a UV spectrophotometer. The intensity of light absorbed (absorbance=optical density) was directly proportional to the concentration of the liberated urea. The more the absorbance the more the biological activity of the L-arginase enzyme.

Assessment of L-arginase activity exploiting Nesslerization test:

The exhibition and activity were analyzed founded on the spectrophotometric mensuration of the emancipated ammonia concentration by the Nessler's performing at 425 nm wavelength. The higher the optical density the higher the biological activity of the L-arginase enzyme. L-arginine was utilized as the sole carbon and nitrogen metabolic source for growth of bacterial L-arginase from different soil samples serial dilutions on MAA media.

Identification and Characterization of Predominant Bacterial Isolates Producing L-arginase:

The potent isolates were identified based on morphological and biochemical characteristics. Further molecular characterization was carried out by DNA Northern hybridization and blotting technique at Kassab microbiology and immunology research laboratory in Qalyobia, Egypt. 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.

In vitro cell viability assay:

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.

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.

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

To culture and measure cell viability,96-well microplate wells were seeded with 10–50µl cell suspension at a density of 5000 cells per well. The plates were incubated for 24 h at 37°C inside a CO2 incubator and then exposed to different concentrations of the produced L-arginase. For cell viability assay, 10µl of 10% MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)

was added to each well and incubated for 3 h. Then 100µl of DMSO (dimethyl sulfoxide) was added to the wells and the optical absorbance was measured at 570 nm on a BioTek ELIZA reader. The cell viability was calculated as a percentage of healthy cell count in the samples instead of the control (not treated with L-arginase). IC50values were determined as the concentration resulting in 50% inhibition in the growth of the cells.

Assessment of antiproliferative actions of L-arginase via BrdU incorporation assay:

Genomic DNA synthesis and cell proliferation rates were assessed using a colorimetric 50-Bromo-20-deoxyuridine (BrdU) ELISA kit (Roche, Germany). Different cell lines were plated at a density of 5000 cells per well in a 96-well culture plate with the appropriate concentration of produced L-arginase for 48 h and then were incubated with the BrdU labeling solution at 37°C for 8 h (Liboskaet al. 2012). Afterward, the cells were fixed and DNA was denatured using the Fix Denat solution. Fixed cells were incubated with peroxidase-conjugated anti-BrdU antibody and then exposed to substrate tetramethylbenzidine. Lastly, plates were read at 370 nm by a microplate reader. DNA synthesis per cell was calculated by dividing the total DNA synthesis by the percent of viable cells.

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

A caspase-3 assay kit was used for detecting the activity of caspases that plays a central role in cell apoptosis. The colorimetric caspase-3 activity test was employed according to the manufacturer's protocols (Sigma-Aldrich Chemie Gmbh). L-arginase treated cell lines were lysed, 10 micrograms of the supernatant were incubated with 85microliter of assay buffer and 10microliter of caspase-3 substrate, acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DVD-pNA), in a96-well for 4 h at 37°C. Fold changes in caspase-3 activity were evaluated by measuring the concentrations of p-ni-troanilide (p-NA). This compound was released due to the enzymatic activity of caspase-3 by calculating the absorbance values of p-NA at 405 nm.

The design of a new primer by bioinformatics for expression of bacterial L-arginase enzyme from *Bacillus subtilis*:

Forward primer for expression:

The direction of the sequence is from 5^- to 3^-

GGGTTGACTGACTGGAGAGC

T. annealing=Tm-5=60.04-5=55.04 ^{0}C

Reverse primer for expression: The direction of the sequence is from 5^- to 3^-

TGCATTTCCTGGCCAGATGTA

T.annealing =Tm-5=59.72-5=54.72 ^{0}C

Production of L.arginase by recombinant DNA technology:

The synthesis of L-arginase by recombinant DNA technology using *Saccharomyces cerevisiae BJ1824* as expression host. C-terminal was 6x histidine, promoter AUG1, inducer was methanol and PYES2-DEST52 was the expression system vector. Genes of L-arginase were cloned using PCR and then sub-cloned into PYES2-

DEST52 using Hind III and EcoRI restriction endonucleases II for the digestion of the plasmid, followed by ligation by ligase enzyme. The recombinant plasmid was designated and propagated first in *Escherichia coli* Top 10, then transformed into *Saccharomyces cerevisiae BJ1824.* For L-arginase 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).

Clarification and the purification of recombinant L-arginase:

The enzyme of interest 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 are hybridized into radio-labeled probes.

The determination of optimal dosage form and route of administration of the enzyme of interest:

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 L-arginase 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 L-arginase 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 L-arginase 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.

Figure 1. Bacterial positive isolates on MAA.

Figure 2. Positive isolates of the subculture of bacterial *Bacillus* sp (taken from positive bacterial *bacillus* sp isolates on MAA) on the nutrient agar plate.

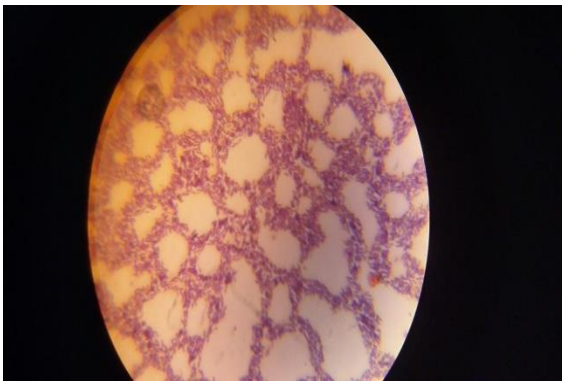


Figure 3. It shows Gram-positive bacilli producing L-arginase enzyme as the anticancer agent from the soil environment in Egypt.

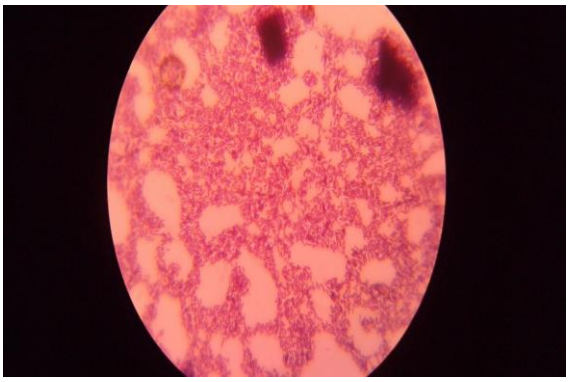


Figure 4. It shows Gram-positive bacilli producing L-arginase enzyme as the anticancer agent from the soil environment in Egypt.

Figure 5. Biochemical reaction demonstrates positive catalase test with positive bacterial *Bacillus Spp* isolates on MAA.

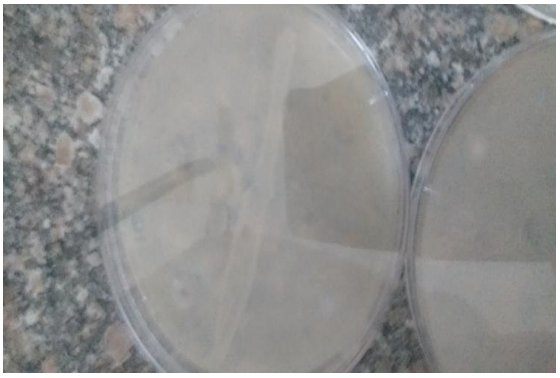


Figure 6. Biochemical reaction presents positive lecithinase test with positive bacterial *Bacillus Spp* isolates on MAA.

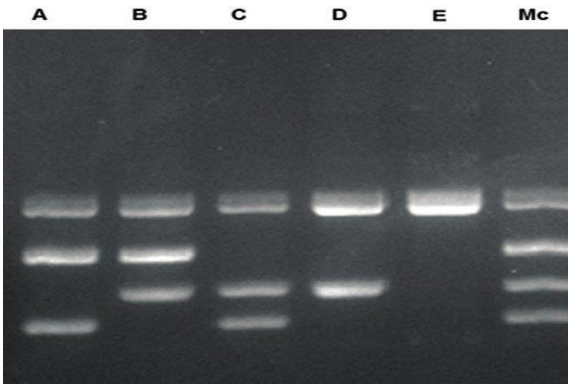


Figure 7. It shows different amounts and sizes of recombinant proteins of L-arginase measured via the Western blot technique. The maximum yield of L-arginase was 70-75 mg/l.The purity of recombinant L-arginase was approximately 85%.

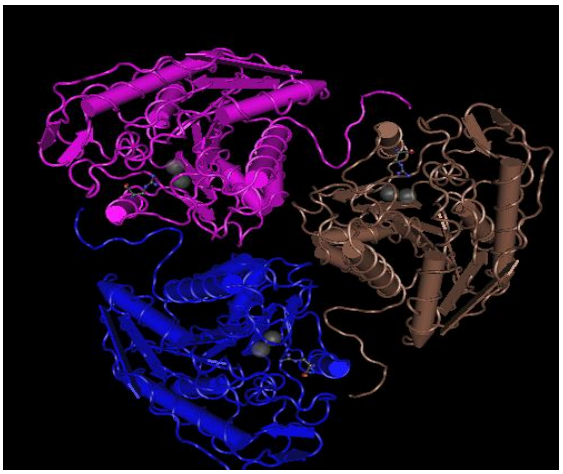
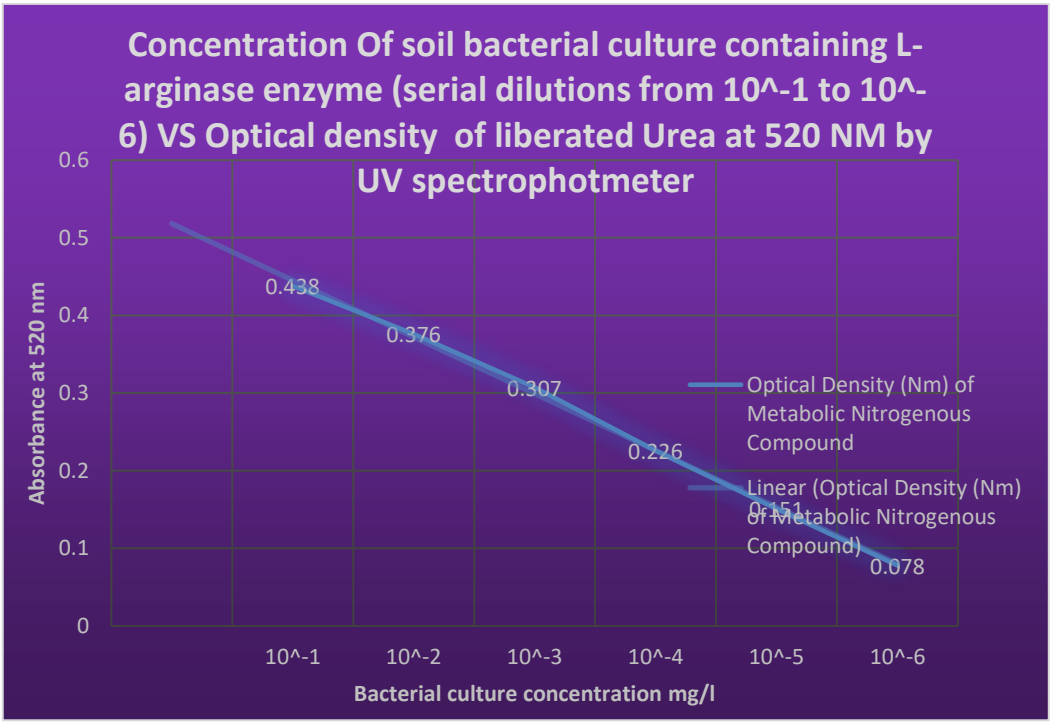


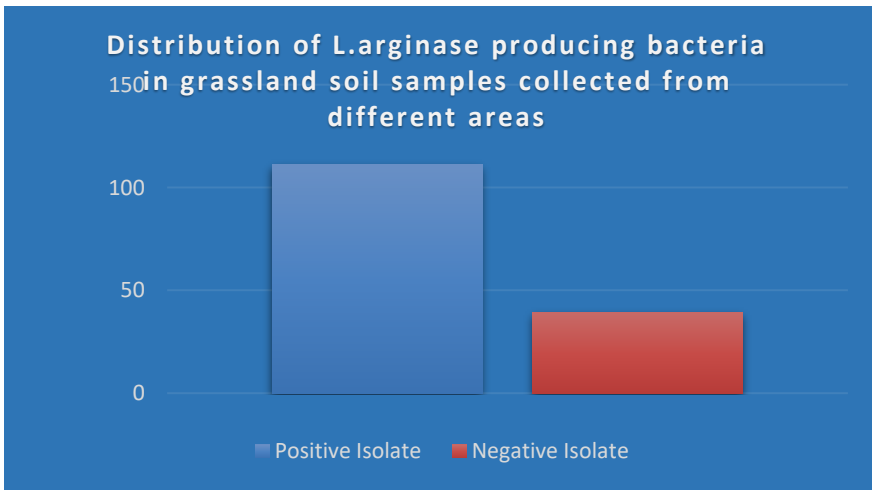
Figure 8. It represents 3D structure of L-arginase enzyme. **L-arginase comprises 323 aminoacids structure.**

Statistical analysis:

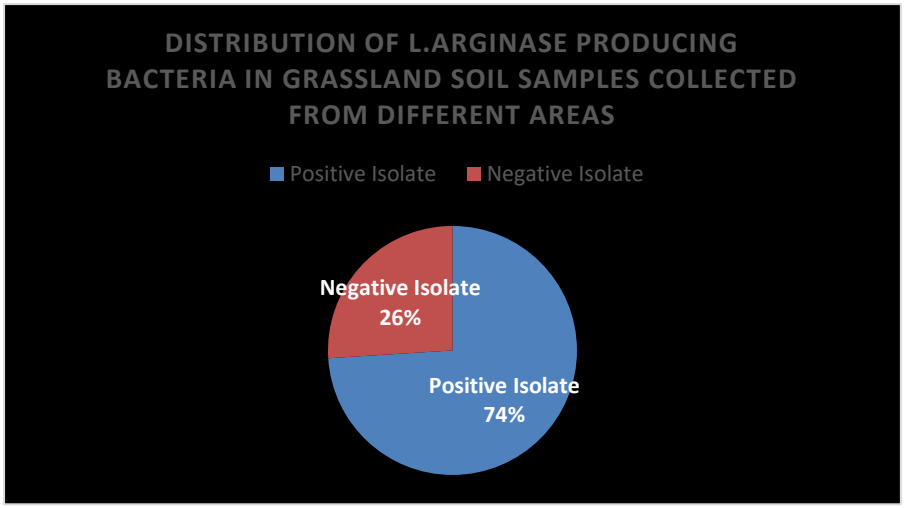
All cultures were conducted in triplets. Their presentation was by means and standard deviation. One-way analysis of variance (p value≤.05) was used as means for performing statistical analysis and also, statistical analysis based on excel-spreadsheet-software.



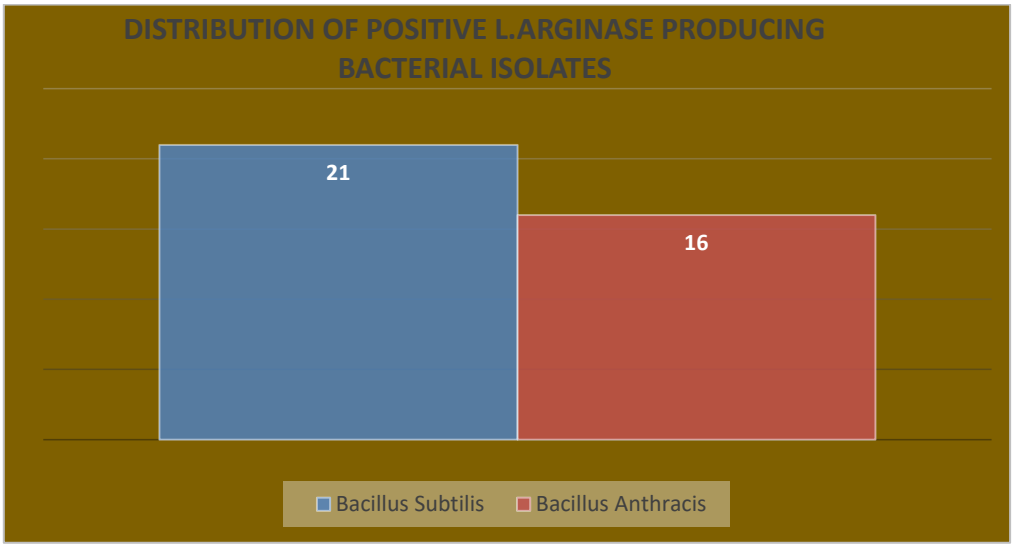
Graph 1. Concentration Of soil bacterial culture containing L-arginase enzyme (serial dilutions from 10^-1 to 10^-6) VS Optical density of liberated Urea at 520 NM by UV spectrophotmeter



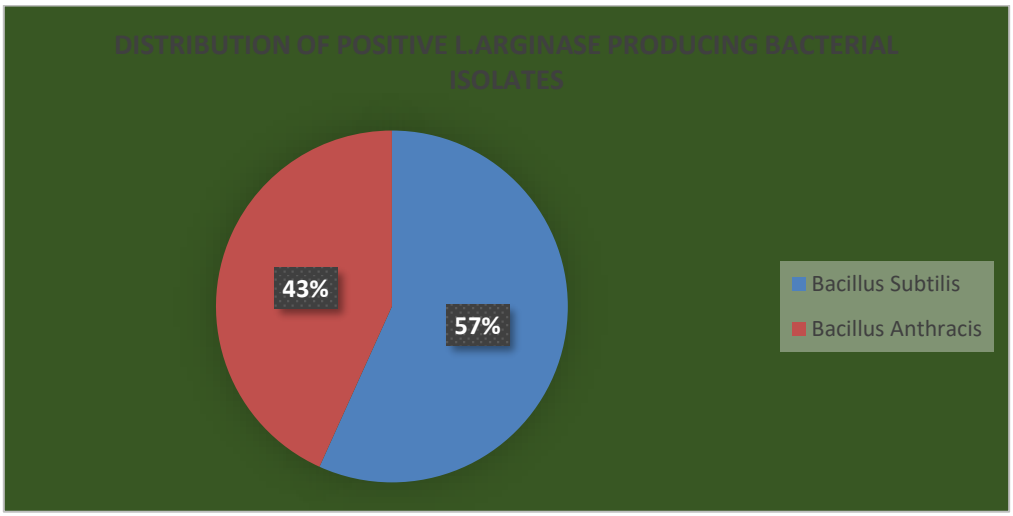
Graph 2. Distribution of L.arginase producing bacteria in grassland soil samples collected from different areas



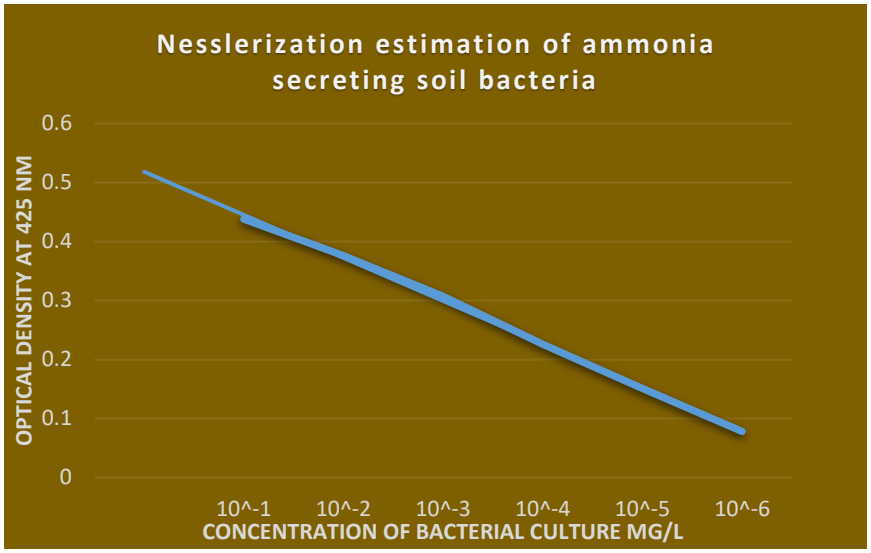
Graph 3. Distribution of L.arginase producing bacteria in grassland soil samples collected from different areas.



Graph 4. Distribution of positive L.arginase producing bacterial isolates.



Graph 5. Distribution of positive L-arginase secreting bacterial isolates.



Graph 6. Liberated ammonia at 425 nm by different Concentrations Of soil bacterial culture containing L-arginase enzyme.

RESULTS:

Distribution of L.arginase producing bacteria in grassland soil samples collected from different areas:

The study started by screening 50 soil samples for L-arginase-producing bacteria. The grassland soil samples were from different areas in Egypt:

1-Menyat Sheben Elkanater, Qalyobia (n=1)

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

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

4-Nawa,Sheben Elkanater,Qalyobia (n=4)

5-Kafr Sheben Elkanater,Qalyobia (n=5)

6-Elsahafa,Mashtol Elsok,Sharqia (n=6)

7-El more many, Mashtol Elsok, Sharqia (n=7)

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

9-Brash,Mashtol Elsok,Sharqia (n=9)

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

11-Eldir,Tokh,Qlayobia (n=11)

12-Arab Elhaswa,Tokh,Qlayobia (n=12)

13-Dandana,Tokh,Qlayobia (n=13)

14-Elmanzala, Tokh, Qlayobia (n=14)

15-Motor, Tokh, Qlayobia (n=15)

16-Senhera, Qaha, Qalyobia (n=16)

17- Salamant, Belbis, Sharqia (n=17)

18-Menyat Salamant, Belbis,Sharqia (n=18)

19-Tal Rozon,Belbis, Sharqia (n=19)

20-Elsalam, Belbis, Sharqia (n=20)

21-Elzawamel ,Belbis, Sharqia (n=21)

22-Awlad Seif, Belbis, Sharqia (n=22)

23-Burdin, Elzagazig, Sharqia (n=23)

24-Alaslogy, Elzagazig, Sharqia (n=24)

25-Elzahraa, Elzagazig,Sharqia (n=25)

26-Alnakarya, Elzagazig, Sharqia (n=26)

27-Alreyad, Elzagazig, Sharqia (n=27)

28-Alzanklon,Elzagazig,Sharqia (n=28)

29-Alsaada,Elzagazig,Sharqia (n=29)

30-Alelwya,Elzagazig,Sharqia (n=30)

31-Aboyasin,Abokabir,Sharqia (n=31)

32-Alahraz,Abokabir,Sharqia (n=32)

33-Alrahmanya, Abokabir, Sharqia (n=33)

34-Alkramos, Abokabir, Sharqia (n=34)

35-Manshyet Radwan, Abokabir, Sharqia (n=35)

36-Algamalya, Alhusynia, Sharqia(n=36)

37-Alhegazya,Alhusynia,Sharqia(n=37)

38-Alnasrya,Alhusynia,Sharqia(n=38)

39-Bahr Albakr, Alhusynia, Sharqia (n=39)

40-Sanhagr,Sharqia (n=40)

41-Nay,Qalyob,Qalyobia (n=41)

42-Kafr Ramada, Qalyob, Qalyobia (n=42)

43-Tanan,Qalyob, Qalyobia (n=43)

44-Meet Halfa,Qalyob, Qalyobia (n=44)

45-Alsad,Qalyob,Qalyobia(n=45)

46-Balaqs, Qalyob, Qalyobia (n=46)

47-Halaba,Qalyob,Qalyobia(n=47)

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

49-Kafr Hamza ,Khanka, Qalyobia (n=49)

50-Arab Alelykat ,Khanka, Qalyobia (n=50)

Table 2. Prevalence of L-arginase-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

Morphology, 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) on the mineral arginine agar were observed under a light microscope gram staining. They exhibited a characteristic 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:**

Table 3. It shows characteristic biochemical reactions:

Biochemical reaction	Result
Catalase test	positive
Lecithinase test	negative
Methyl red test	negative
Indole test	negative
Voges Proskauer test	positive

The positive bacterial isolates (2, 5,6, 15, 18, 21, 25, 26, 29, 37, 40, 41, 43, 45, 48, 50)

on the mineral, arginine agar was observed under a light microscope gram staining. They exhibited a characteristic morphology of gram-positive, large rods, and non-motile. The colonies were large, irregular, and greyish-white in color. They exhibited no hemolysis on blood agar. They also exhibited **characteristic biochemical reactions**:

Table 4. It shows characteristic biochemical reactions:

Biochemical test	Result
Catalase test	positive
Lecithinase test	positive
Methyl red	negative
Indole test	positive
Voges Proskauer	positive

Determination of environmental and physiologic factors affecting the growth of L.arginase producing bacteria:

The optimal conditions for the growth of L.arginase-producing bacteria are PH 7.3 at a temperature of 37 C.

Characterization of bacterial L.arginase production:

The optimal conditions for the production of L.arginase enzyme include:

a. Activators of the 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.

b. The optimal PH and temperature for bacterial enzyme production are PH 7.3 at a temperature of 37 C.

Estimation of biological activity of L-arginase enzyme:

Using direct diacetyl monoxime test for the estimation of optical density of the liberated metabolic nitrogenous containing compound(liberated Urea) due to degrading L-arginine present in media by bacterial L-arginase under PH7, Temp 37 C, .05 borate buffer and traces of Ni, Co, and Mn metal ions as cofactors.

Table 5. It shows an estimation of L-arginase activity by the direct diacetyl monoxime method:

Concentration Of soil bacterial culture containing L-arginase enzyme(serial dilutions from 10*-1 to 10*-6 mg/l)	The optical density of liberated metabolic nitrogenous compound(liberated urea) at 520 nm by UV spectrophotometer
0.00	0.00
1/10	0.438
1/100	0.376
1/1000	0.307
1/10000	0.226
1/100000	0.151
1/1000000	0.078

The optimal conditions for bacterial L-arginase production at different concentrations from 10^{-1} to 10^{-6} mg/l of the Egyptian soil containing bacteria producing L-arginase using UV spectrophotometer are PH7.3 at temperature 37 C and traces of activators CO, Ni, and Mn ions.

Nesslerization determination of L-arginase activity:

The biological activeness of bacterial L-arginase was directly proportional with the level of the liberated ammonia as indicated by increasing absorbance at 425 nm.

Table 6. It shows a computation of bacterial L-arginase action via the direct Nesslerization technique:

Concentration Of soil bacterial culture containing L-arginase 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	0.410
1/100	0.382
1/1000	0.341
1/10000	0.283
1/100000	0.177
1/1000000	0.076

In vitro cell viability assay:

This assay showed higher efficacy of the test L-arginase as an anticancer agent against auxotrophic cancer cell lines including hepatic carcinoma cells, melanoma cells, 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 8.7. In presence of Manganese ion as a cofactor at neutral PH 7.3, L-arginase was as effective as in alkaline conditions.

Determination of optimal dosage form and route of administration of bacterial L-arginase:

L-arginase showed a very short biological half-life (1 hour) when given intravenously.

The enzyme in injectable dosage form given intramuscularly or subcutaneously without polyethylene glycol had a short half-life (3 hours). The duration of action was extended to 18 hours after the addition of polyethylene glycol. Oral delivery systems showed poor efficacy due to chemical instability in an acidic environment and degradation by proteolytic enzymes in the gastrointestinal tract.

Characterization of the production and biological activity of bacterial L-arginase produced by recombinant DNA technology:

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

T**he determination of the cytotoxic activity of bacterial L-arginase against human cancer cell lines:**

The purified bacterial L-arginase enzyme produced by recombinant DNA technology was assessed on five cancer cell lines for its in vitro anticancer effect via the standard MTT assay. Bacterial L-arginase 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 and HL1 cardiac cancer cell line)** and the normal **Vero cell line.** L-arginase at concentration 10U/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, while inhibited the cell viability of the normal Vero cell line by 51.4%.

Ic50 of L-arginase 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) 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)and4.82 U/ml against cardiac cancer line(HL1), while 8.66 U/ml against the normal Vero cell line.

Selectivity index of bacterial L-arginase 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 and 3.74 against (HL1) cardiac cancer cell line.

BrdU incorporation in vitro:

To explore if the L-arginase 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 L-arginase 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, and 29 and 76% for HL1 reduction in cell proliferation was observed by L-arginase at 0.3,0.5,0.8,0.4 and 0.9 IU/ml respectively.

Caspase-3 activity assay:

Caspase-3 apoptosis index in the L-arginase 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 L-arginase induces apoptotic cell death by increasing the enzymatic activity of the

caspase-3.L-arginase at 0.5 IU/ml concentration induces the activity of caspase-3 up to 14,13,15,18,11and 19% in JHH4, IM1215, UPMM3, Calu-3, and HL1 cells respectively. Also, at 0.9 IU/ml concentration this induction was increased up to 23,19,31,28 and 35% inJHH4, IM1215, UPMM3, Calu-3 and HL1 cells respectively.

DISCUSSION

Isolation and the screening of L-arginase-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 screening of L-arginase:

The collected soil samples were further analyzed by a direct diacetyl monoxime test. This method has been applied for the determination of the Urea concentration as a product of enzymatic degradation by L-arginase. The concentration of the liberated Urea by test samples demonstrated that L-arginase was present in collected soil samples. Among the collected samples, samples from 1 to 37 which were further identified as *Bacillus* species produced the highest amount of L-arginase.

Identification and the characterization of the most potent bacteria:

The morphological characteristics of isolates from 1 to 37 which showed high L-arginase activity indicated that the organism belonged to the genus *Bacillus*. The biochemical characterization by biochemical tests revealed that it has about 74% similarity with *Bacillus subtilis* and about 26% similarity with *Bacillus anthracis*. Comparing our results with another study conducted in India (Isolation, screening, and characterization of L-arginase producing soil bacteria, July 2019), it was found that the prevalent L-arginase producing bacteria were *Bacillus subtilis* and *Bacillus anthracis* in our results, while It was *Pseudomonas* species in an Indian study.

Molecular detection and characterization by DNA hybridization and Northern blotting technique revealed that the most potent bacterial isolates were ***Bacillus subtilis, strain W23*** **(74%), and** ***Bacillus anthracis, strain 34F2*** **(26%)**

Environmental and physiological factors affecting the growth of L-arginase-producing bacteria:

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

Characterization of bacterial L-arginase 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 L-arginase enzyme from *Bacillus subtilis:*

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

The production and biological activity of bacterial L-arginase produced by recombinant DNA technology:

In our study**,** we could improve the production and the physicochemical characteristics of bacterial L-arginase enzyme produced by recombinant DNA technology in Saccharomyces cerevisiae expression host. In comparison with previous studies, our L-arginase showed

higher stability, specificity, productivity, yield, biological activity, and fewer side effects as an anticancer agent against auxotrophic cancers for L-arginine than previous bacterial L-arginase.

The determination of the cytotoxic activity of bacterial L-arginase against human cancer cell lines:

In comparison with previous studies and standard doxorubicin as an anticancer agent against auxotrophic cancers for arginine, Our bacterial L-arginase 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 L-arginase 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 L-arginase:

In our study, the optimal dosage and route of administration of bacterial L-arginase 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 L-arginase once daily as an anticancer agent against auxotrophic cancers for L-arginine. The oral drug delivery system of L-arginase 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 L-arginase against cancer cell lines:

In comparison with previous studies and standard doxorubicin as an anticancer agent against auxotrophic cancers for arginine, Our bacterial L-arginase 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 L-arginase 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.[16]

Therapeutic indications:

In our study, L-arginase 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.

Conclusion:

Gram-positive Bacillus subtilis and Bacillus anthracis displayed high levels of bacterial L-arginase. 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 L-arginase manufacture were pH 7.3 and temperature 37 °C with the succeeding activators KCL, ZnSO4, FeSO4, K2HPO4, and MgSO4. Bacterial L-arginase was a promising as a co-chemotherapeutic agent

for auxotrophic cancers of solid tumors; consequently it is suggested to exhibit its large scale production.

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

Conflict of interest:

There is no conflict of interest.

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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.

This study was a patent registered with number 882/2021 in the Egyptian ministry of scientific education.

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انتاج انزيم الال ارجنيز البكتيري من بيئات التربة المختلفة في مصر كعامل مضاد للسرطان

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الانزيمات المسئولة عن تكسير الحمض الاميني الارجنين ضرورية لعلاج السرطانات المعتمدة على حصولها على الارجنين من الوسط الخارجي لانها ليس لها القدرة على تصنيعها مثل سرطانات الكبد والقولون والمستقيم والورم الميلانيني الصبغي الخبيث بينما الخلايا العادية لها القدرة على تصنيعه.هذه الانزيمات تشمل الارجنين دي ايمنيز والارجنيز والارجنين ديكاربوكسيليز.دراستنا كانت معنية بعزل واستكشاف البكتريا المنتجة للارجنيز كعامل مضاد للسرطان من بيئات تربة مختلفة وايضا تعيين وتقرير العوامل البيئية والفسيولوجية المؤثرة على نمو بعض العزلات المنتجه للارجنيز وتحديد خصائص الانزيم .تم استعمال وسط خاص لزراعة البكتريا المنتجة لانزيم الارجنيز يسمى الوسط الارجنيني المعدني.البكتريا التي كان لها القدرة على انتاج انزيم الارجنيز واستعمال الارجنين كمصدر للكربون والنيتروجين لنموها هي التي نمت وعن طريق دراسة الشكل الظاهري لهذه العزلات واجراء الاختبارات الكيميائية الحيوية تبين انها بكتريا موجبة الجرام عصوية واغلبها من نوعي البكتريا سابتيليس وانثراكاس.

العوامل البيئية والفسيولوجية المؤثرة على نموالبكترياهي انها كانت من تربة الوسط بها حمضي وبجانب مطاحن الدقيق والبقوليات الغنية بالارجنين ودرجة حموضة الوسط الارجنيني المعدني كانت 7.3 عند 37 درجة مئوية في وجود الاوكسجين وتبين ان الاملاح المحفزة لانتاجه هي كبريتات الزنك والماغنسيوم والحديد وكلوريد البوتاسيوم واملاح بوتاسيوم الفوسفات الهيدروجينية. تم عمل اختبارداي اسيتيل مونوكسامين لقياس تركيز اليوريا الناتجة عن نشاط الارجنيز البكتيري واختبار نسلر المباشر لقياس نسبة النشادر للكشف عن البكتريا المنتجة للانزيم ونشاط الانزيم لتركيزات مختلفة من عينات التربة وتبين ان املاح المعادن المنشطة لفاعليته هي املاح كبريتات المنجنيز وثنائي كلورايد الكوبلت والنيكل عند درجة حموضة وسط معتدلة 7.3 و37 درجة مئوية في وجود الاوكسجين.

تم عمل اختبار لقاعلية الانزيم كعامل مضاد للسرطان وثبتت فاعليته وتم وتم انتاجه بواسطة تقنية الدي ان ايه المهجن مع تمكننا من انتاج حقن منه في حين لم يفلح انتاج اقراص منه.