Cytotoxic Potential of Purified Arginine Deiminase from *Streptococcus* spp

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Abstract

The results that presented in the study were based on collecting one hundred and ten clinical samples of oral cavity, burns and wounds. Primary and secondary diagnositic showed that 14 out of these isolates belonged to *Streptococcus* spp.. The ability of these isolates in arginine deiminase enzyme production was screened and the results showed that these isolates were arginine deiminase producers with variable degrees. It has been found that one of these isolates which were *Streptococcus mitis* S5 had the highest specific activity (0.184 U/mg protein), therefore it was chosen for further study. Results of the optimum conditions for arginine deaminase production reveal that the maximum arginine deaminase production was achieved after supplementation of the production medium (pH 7.5) with 1% maltose as carbon source, 1.2% tryptone as nitrogen source, 30mM as arginine concentration and incubated at 37ºC for 18h. Under these conditions, ADI purified in three steps including; precipitation with 70% saturated ammonium sulphate, dialysis then ion exchange chromatography using DEAE- cellulose column and the last step was gel filtration chromatography throughout Sephadex G150 column. Specific activity of the purified enzyme was increased up to 18 U/mg with 5 folds of purification and 70.8 % enzyme recovery.

***Key words**: Streptococcus spp., arginine deaminase, DEAE- cellulose, chromotography.*

Introduction

Enzymes are nature's sustainable catalysts. They are biocompatible, biodegradable and are derived from renewable resources. Almost all chemical reactions in a biological cell need enzymes in order to occur at rates sufficient for life [(1)]. One of these important enzymes is arginine deiminase (ADI) that produced from *Streptococcus* spp. Genus *Streptococcus*, represent group of spheroidal bacteria belonging to the family Streptococcaceae. The genus *Streptococcus* is Gram-positive bacteria that exert a strong impact on the health of humans and animals. ADI catalyzes a conversion of arginine to ornithine, ammonium, and carbon dioxide, while generating ATP from ADP and phosphate[(2)]**.** Arginine belongs to the semi-essential amino acids for humans. It is irreplaceable for synthesis of proteins, urea, polyamines, agmatine and amino acids like glutamate and proline. The enzymes implicated in the three steps of the pathway are arginine deiminase (ADI, EC 3.5.3.6), ornithine transcarbamylase (OTC, EC 2.1.3.3), and carbamate kinase (CK, EC 2.7.2.2) [(3)]. ADI pathway is a multi-enzyme pathway encoded by *arc* operons genes named *arcA*, *arcB*, and *arc*, which hydrolyze arginine to ornithine, with the byproducts of ammonia, CO_2 and ATP[(4)]. ADI pathway is widely distributed among bacteria and archaea, where it is often a major means of energy production. Many reports have dealt with the occurrence of arginine deiminase in prokaryotes and eukaryotes. Cancer is a major burden of disease worldwide [(5)]. Each year, tens of millions of people are diagnosed with cancer and more than half of the patients eventually die from it. Among the available treatments, nutrient depletion therapy is equally effective. Most of cancer cells require unceasing supply of l-arginine for their growth and survival; they use these amino acids as a source of nitrogen for the synthesis of their cell components [(6)]. Since, the last three decades, ADI enzyme has been widely used in target- specific chemotherapy to treat different types of cancer and

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hepatocellular carcinomas (HCC) and melanomas were one of these cancer types. Clinicians and researchers have also harnessed ADI against other arginine auxotrophic tumors such as pancreatic cancer, prostate cancer, leukemia, colon cancer and breast cancer [7].

Materials and methods

· **Samples collection**

A total of 110 specimens were collected from Medical City Hospital in Baghdad during the period from 29/9/2019 to 30/12/2019. These specimens involved oral cavity, burns and wounds. Swab specimens were aseptically transferred under cooling conditions to the laboratory for analysis.

· **Isolation of *Streptococcus* spp.**

According to [8], swabs were taken carefully from the sites of infection and placed in tubes containing transferred medium to maintain the swab wet during transferring to laboratory. Each specimen was inoculated on *Streptococcus* isolation mitis salivarius which is selective agar media. All plates were incubated aerobically in incubator at 37ºC for 24 hours.

· **Identification of *Streptococcus* spp.[9]**

1.Cultural examination

Morphological characteristics of colonies were studied on mitis salivarius agar and blood agar. Color, size and edge of colonies were observed after 24 hours of incubation at 37˚C.

2.Microscopical examination

A single colony of each isolate was fixed on a clean slide to study gram stain, under light microscope

3.Identification of suspected bacteria by VITEK 2 system

The VITEK 2 is an automated microbiology system utilizing

growth-based technology and used for bacterial identification. The suspected isolates obtained after biochemical tests were subjected to VITEK 2 tests.

· Purification of arginine deiminase

1.Precipitation of enzyme by ammonium sulfate

Solid ammonium sulfate was gradually added in saturation ratios (30%, 50%, 70% and 90%) to 100 ml of crude enzyme at 4 Cº.The component was mixed gently for 45 min. Then it was centrifuged at 6,000 rpm for 20 min., the supernatant was discared and the precipitate was dissolved in a suitable volume of potassium phosphate buffer.

2.Dialysis

The enzyme solution was dialyzed after precipitation with ammonium sulfate against distilled water for 24 h under cooling conditions (4Cº) with stirring and changing the D.W for four times.

3.Purification by ion exchange chromatography

A DEAE-cellulose column (1.5×20 cm) was prepared according to Whitaker and Bernard, (1972) by dissolving 20 g of resin in 1L of distilled water. Then beads were left to settle down and washed several times with D.W until getting clear appearance.

4.Purification by gel filtration chromatography

Sephadex G-150 (2 × 30 cm) column was prepared as recommended by Pharmacia Fine Chemicals Company. Aquantity of sephadex G-150 was suspended in 0.05 M phosphate buffer pH8, heated at 90ºC for 5 hours to complete swelling of the beads, degassed and packed in a glass column, the column was equilibrated with the same buffer buffer.

· Cytotoxic Effect of arginine deiminase on HepG2 cancer cell line using MTT assay

In vitro method was performed to investigate the effect of arginine deiminase on HepG2 cancer cell line. The procedure of [10] was followed by using different concentrations (6.25, 12.5, 25, 50, 100 and 200 µg/mL) of crude and purified arginine deiminase enzyme and was performed using MTT ready to use kit. An ELISA reader was used for measurement at 575 nm wave length. The data of optical density was subjected to statistical analysis in order to evaluate the concentration of compounds required to cause 50 % reduction in cell viability for each cell line.

· **HCS assay**

Five independent parameters including Viable cell count(VCC), Total nuclear intensity(NI), cell membrane permeability(CMP), mitochondrial membrane potential(MMP) and cytochrome C which refers to the cell health. Different concentrations (6.25, 12.5, 25, 50, 100 and 200 µg/ml) of the purified arginine deiminase of *Streptococcus* were tested on HepG2 cell line. The assay was carried on at Biotechnology research center. This assay was carried out according to [10] and evaluated by Array Scan HCS Reader.

2.2.8 Statistical analysis

One mode examination of variance ANOVA (Duncan) was made to test whether group alteration was important or not, statistical significance was defined as $p \leq 0.05$. Data were expressed as mean± standard error and statistical significances were carried out using Graph Pad Prism version 6 (Graph Pad Software Inc., La Jolla).

Results and Discussion

· Cultural microscopic characteristics and some biochemical characterization

From hundred and ten clinical specimens that collected from different types of infections only 14 of them were able to grow on blood agar and the colonies appeared white in color with hemolysis surrounding the colonies, and culturing on mitis salivarius agar appear pale blue colonies. bacterial isolates were appeared positive in gram staining and the cells mainly nonmotile that occur in pairs or chains. All isolates were negative for catalase, grew under aerobic conditions and positive for methyl red.

· **Identification of *Streptococcus* by VITEK system**

After isolation and confirmation for some tests of bacterial isolate, the 14 isolates were subjected to identification by VITEK 2 system. GP (gram positive) cards were used in VITEK 2 system.

· **Screening the ability of *Streptococcus mitis* in arginine deiminase production**

For the detection of the efficient bacterial isolates in arginine

deaminase production, spectrophotometrically a method were performed using quantitative screening by measuring the formation of L citrulline from arginine. all isolates had the ability to produce the enzyme with a specific activity ranging between 0.055-0.184 U/mg protein. Among these isolates *Streptococcus mitis* S5 was the most efficient in arginine deiminase production because the specific activity of arginine deiminase in crude filtrate of this isolate was 0.184 U/mg.

· **Purification of arginine deiminase**

1. Ammonium sulfate precipitation and dialysis

High enzyme specific activity (4.8 U/mg protein) was obtained up on precipitation of arginine deiminase at 70% ammonium sulfate saturation ratio. At low ion concentrations, the solubility of proteins increases with increasing salt concentration, an effect termed salting in. As the salt concentration is further increased, the solubility of the protein begins to decrease. At a sufficiently high ionic strength, the protein will precipitate out of the solution, an effect termed salting out.

2. Ion exchange chromatography

The precipitated enzyme concentrated by ammonium sulfate was

partially purified using DEAE-cellulose ion exchange chromatography

column. Three protein peaks obtained in the washing step, and two protein peaks obtained after elution by the gradient concentrations of sodium chloride. All these protein peaks were assayed to detect arginine deaminase activity. Results showed that eluted proteins (Fractions 45 to 50) contained most of arginine deaminase activity, and the enzyme specific activity was measured to be 1.3U\mg protein with 34.28 % overall yield and 5.71 purification fold.

3. Gel filtration chromatography

Sephadex G-150 represent the final step in the purification of arginine deiminase produced by the local isolate *Streptococcus mitis* S5. After purification by ion exchange step, fractions representing arginine deiminase activity were collected, and applied to Sephadex G-150 previously equilibrated with 0.05M potassium

phosphate buffer pH 7. Sephadex G-150 has separation limits ranging between (1-150 KDa) allowing larger capacity of separation with a high purification degree.

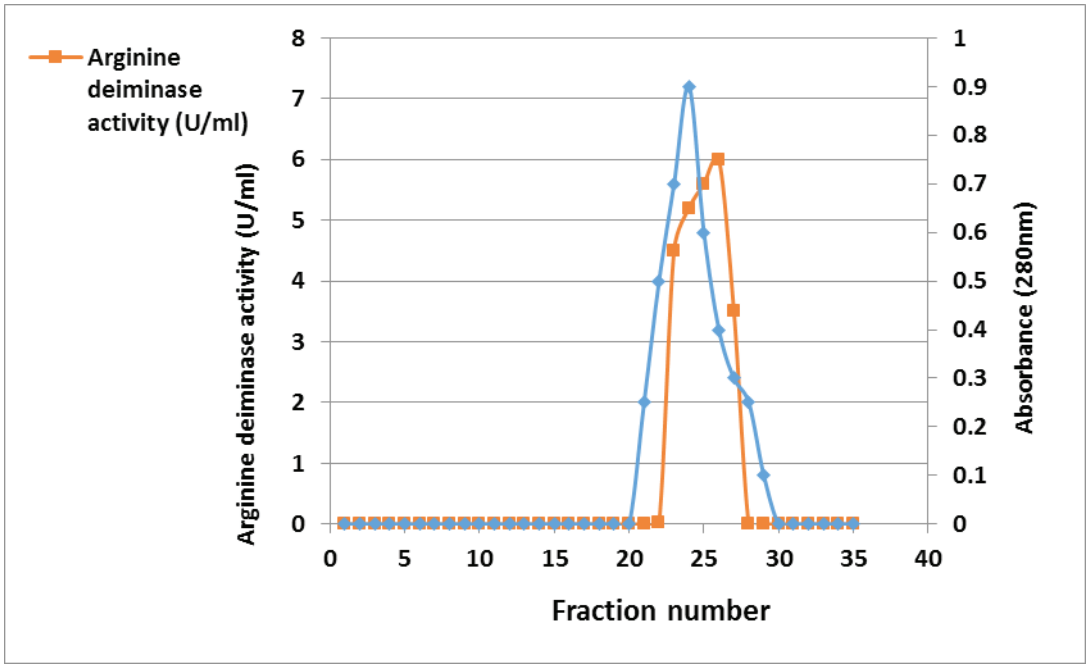

Figure (1): Gel filtration chromatography of arginine deiminase produced by *Streptococcus* S5 using Sephadex G-150 column (1.5cmx35cm) equilibrated with 0.05M sodium phosphate buffer pH 7 at flow rate at 30 ml/h.

Scientific researchers found that the specific activity of ADI enzyme purified from *Lactococcus lactis* spp. 140.3 U/mg using the sequential Q-Sepharose anion exchange and Sephacryl S-200 gel filtration column chromatography [11].

Table (1): Purification steps for arginine deiminase produced by *Streptococcus* S5

Purification step	Volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (folds)	Yield (%)
Crude enzyme	50	3.2	0.9	3.5	160	1	100
Ammonium sulphate precipitation 70%	35	3.9	0.8	4.8	136.5	1.3	85.3
Dialysis	25	4.9	0.7	7	122.5	2	76.5
DEAE-cellulose	18	6.3	0.35	18	113.4	5	70.8
Sephadex G150	15	6	0.22	27.2	90	7.79	56

· **Cytotoxic effect of crude and purified arginine deiminase In Vitro using MTT assay**

The test of 3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was accomplished to assess the cytotoxic effect of arginine deiminase produced by *Streptococcus mitis* on liver cancer cell line (HepG2). MTT Assay was made to calculate the cell viability and inhibition rate on the tumor cell line by using different concentrations of ADI enzume. The percentage viability of treated cells was calculated in a comparison with normal cell line WRL-68 [12]. The cytotoxic effect of arginine deiminase detected in concentration ranged from 6.2-200 µg/ml on HepG2. As shown in (Table 2) there were a decrease in cell viability in a dose-dependent pattern. The cell viability is reduced by increasing the concentration of arginine deiminase enzyme. The decreasing in HepG2 cell viability (%) was noted by 200µg/ml (46.18±6.60) while the highest HepG2 cell viability at 12.5 µg/ml reached to (95.25±1.20).(Figure 2) showed that crude arginine deiminase exhibited significantly the most potent cytotoxic activity with IC50 value of 148.4 µg/ml. however an IC50 of 212.2 µg/ml was obtained from the effect of arginine deiminase on WRI-68 normal cell line. The purified arginine deiminase exhibited significantly the most potent cytotoxic activity with IC50 value of 83.49 µg/ml. however an IC50 of 137.6 µg/ml was obtained from the effect of arginine deiminase on WRI-68 normal cell line (Figure 3) with cell viability ranged from (94.59±0.52 to 74.62±4.39) from 6.25 to 200 µg/ml. Certain cancers may be auxotrophic for a particular amino acid, and amino acid deprivation is one method to treat these tumors. Arginine deprivation is a novel approach to target tumors which lack argininosuccinate synthetase (ASS) expression. ASS is a key enzyme which converts citrulline to arginine [13].

Table (2): Cytotoxicity effect of arginine deiminase on HepG2 and WRL-68 cells after 24 hours incubation at 37ºC

Arginine deiminase concentrations µg/ml	Viable cell count of HepG2 cell line Mean± S.D.	Viable cell count of WRL-68 cell line Mean± S.D.
200	46.18±6.60	74.62±4.39
100	66.22±1.24	79.74±1.50
50	75.30±3.45	86.84±3.17
25	84.60±2.64	94.40±3.37
12.5	95.25±1.20	93.44±1.90
6.25	94.40±0.98	94.59±0.52

Hepatocellular carcinoma was increased in incidence worldwide, liver transplantation or complete surgical resection remains the only known cure for this disease. For patients who have unresectable disease, treatment options may involve chemoembolisation or radiofrequency ablation of the liver; yet, the prognosis is dismal for most patients because drug therapy has not been demonstrated to improve survival. Therefore there is a huge need for more effective therapy for cancers such as melanoma and hepatocellular carcinoma. ADI depletion is a novel targeted therapy that appears to have antitumor activity in melanoma and hepatocellular carcinoma, tumors that are auxotrophic for arginine. The importance of arginine in the cell because its participation in multiple pathways that affected main cellular functions such as nitric oxide production,

creatine production and polyamine synthesis. In tumor cells, arginine influences their growth/proliferation [14] and diet restriction has been shown to inhibit metastatic tumor growth. ADI supposed to be a particularly valuable source of effective anti-proliferative and cytotoxic agents against different cell lines. Thereby, they were proficient to cause metabolic stress and inhibit HepG2 cells growth by inducing apoptosis and necrotic phenotypes [15].

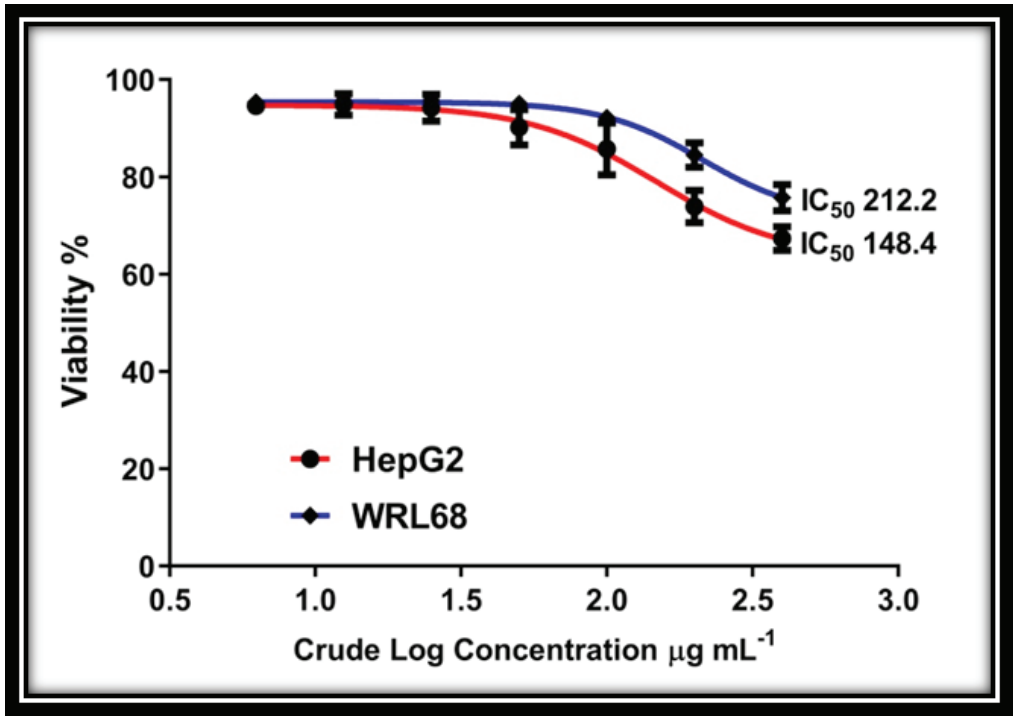

Figure (2): Cytotoxic effect of crude arginine deiminase on HepG2 and WRL-68 cells after 24 hours incubation at 37 ºC.

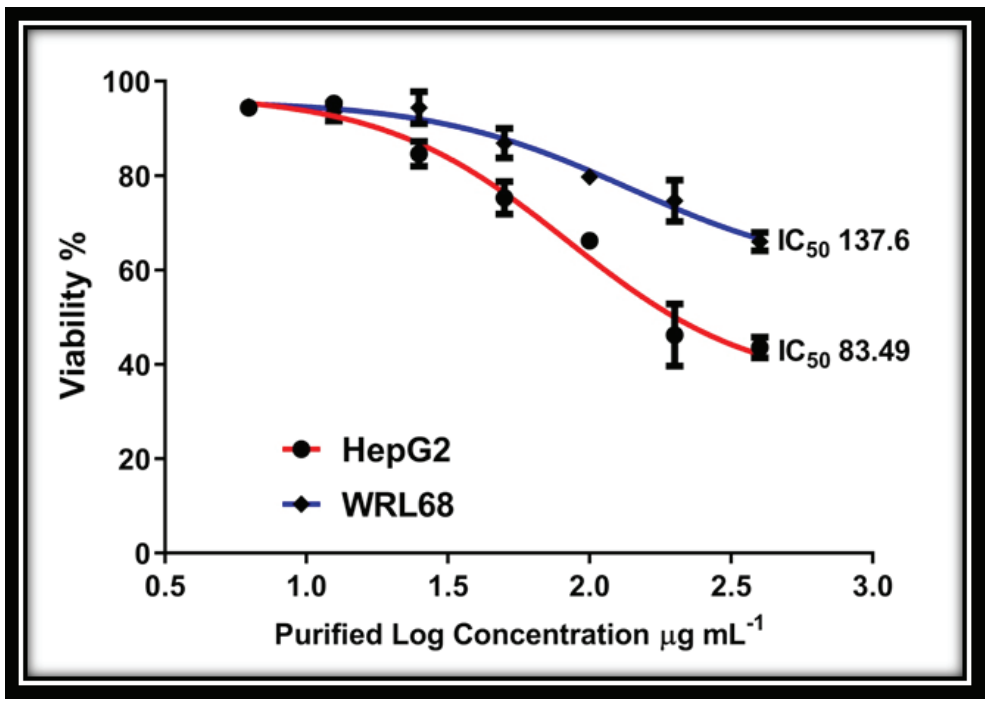

Figure (3): Cytotoxic effect of purified arginine deiminase on HepG2 and WRL-68 cells after 24 hours incubation at 37 ºC.

· **High Content Screening (HCS) of arginine deiminase purified from *Streptococcus mitis* on HepG2 cell line.**

High-Content Screening (HCS) is a cellular imaging-based approach that played a key role in detecting the toxicity and classification of compounds based on observed patterns of reversible and irreversible cellular injury. Also HCS provide multipara metric analysis of compound toxicity at the level of individual cells [16].

Six concentrations (200, 100, 50, 25, 12.5 and 6.25 µg/ml) of purified enzyme arginine deiminase were tested on HepG2 cell line to detect the changes in six cellular parameters (viable cell count VCC, nuclear intensity NI, cell membrane permeability CMP, mitochondrial membrane potential MMP and cytochrome C) after 24 hours of exposure.

Table (3) showed that 200 µg/ml has the highest significant effect on the all parameters when compared with control (untreated cell). 25 µg/ml, 12.5 µg/ml and 6.25 µg/ml Showed results close to those of the untreated cells which represent the negative control (-ve) with a very few significant differences.

The results of viable cell count listed in table (3) showed that the purified arginine deiminase significantly affected on HepG2 cell line viability as it reduced to(853±103.9) of cells at 200µg/ml and (964±58.69) at 100 µg/ml when compared with control, while the viability for the other concentrations was 1013±23.33, 1232±45.25, 1208±9.899, 1214±24.75 (for 50µg/ml, 25µg/ml, 12.5µg/ml and 6.25µg/ml respectively) which did not showed significant differences from control. The reduction in the viability of HepG2 cell line correlated to the toxic effect of arginine deiminase. Previous research demonstrated that human HCC cell line HEPG2 was tested for its sensitivity to ADI inhibition. The results showed that when the ADI activity was reduced to 0.05 U/ml, the inhibition rates for HEPG2 was 60%. The result showed that HEPG2 cells are sensitive to the inhibition by ADI *in vitro*.

purified arginine deiminase shows significant increasing the nuclear intensity of HepG2 cell line. This increasing was dose dependent (937.0±39.60, 764.5±48.79 and 639.0±33.94 for 200µg/ml, 100µg/ml and 50µg/ml respectively). The highest percentage of increasing was 937.0±39.60 at 200µg/ml when compared with control. 25µg/ml, 12.5µg/ml and 6.25µg/ml did not show nuclear condensation, nuclear fragmentation, cell shrinkage, formation and aggregation of apoptotic bodies are important futures of apoptotic morphology of the cell [17]. The results stated that mitochondrial membrane of HepG2 cell was more permeable following treatment with the ADI at different concentration; this was led to death of cell either by apoptosis or necrosis. These events can cause the mitochondrial membrane to lose its potential with the subsequent release of cytochrome C.Also ADI induces DNA damage in cancer cells which is an indicator of apoptosis [18].

On the other hand mitochondrial membrane potential (MMP) listed in table (3) indicates that 200 µg/ml, 100 µg/ml and 50 µg/ml showed significantly decreased the MMP (182.5±34.65, 249.5±27.58 and 275.0±14.14 for 200µg/ml, 100µg/ml and 50µg/ml respectively). 200µg/ml affects significantly more than other concentrations when compared with control (451.0±18.38). Other concentrations (25µg/ml, 12.5µg/ml and 6.25µg/ml) did not show any significant differences from control.

MPP measurement was dependant on the mean intensity of MMP dye penetrating the mitochondria and the highest effect upon mitochondria was the less fluorescent intensity. Apoptosis is often dysregulated during cancer development and it is very significant to trigger proper apoptosis which occurs via the mitochondrial outer membrane permeabilization (MOMP) and resulting in caspase activation and protein substrate cleavage (MOMP pathway) [19].

It has been reported that changes in cell membrane permeability are often associated with a toxic or apoptotic responses, and the loss of cell membrane integrity is a common phenotypic feature of marked cytotoxicity [20].

Cytochrome C releasing results indicated that cytochrome C releasing rise significantly with the increasing of concentration when compared with control and the mean of increasing were 548.0±16.97, 458.5±30.41, 410.5±74.25 and 398.5±26.16 for 200µg/ml and 100µg/ml, 50µg/ml and 25µg/ml and respectively. While 12.5 µg/ml and 6.25 µg/ml showed non significant differences when compared with control 232.5±4.950.

Table(3): Cytotoxicity effect of purified arginine deiminase of *Streptococcus* on multi cellular parameters after 24 h. of incubation at 37° C.

HCS Parameters (Mean ± S.D.)					
Concentration µg/ml	**Viable cell count**	**Nuclear intensity**	**MMP**	**Cell membrane permeability**	**Cytochrome C Releasing**
Unteated	1353±270.1	470.0±15.56	451.0±18.38	81.50±9.192	232.5±4.950
200	853±103.9	937.0±39.60	182.5±34.65	103.0±2.828	548.0±16.97
100	964±58.69	764.5±48.79	249.5±27.58	79.00±5.657	458.5±30.41
50	1013±23.33	639.0±33.94	275.0±14.14	75.00±1.414	410.5±74.25
25	1232±45.25	521.0±5.657	323.5±28.99	74.00±4.243	398.5±26.16
12.5	1208±9.899	528.5±17.68	379.0±79.20	78.50±2.121	319.5±12.02
6.25	1214±24.75	518.5±17.68	404.0±86.27	77.00±9.899	253.0±45.25

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: None

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