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Cytotoxic effect of the purified inulinase from locally Isolate *Staphylococcus aureus* on Hep-2 cancer cell line *in vitro*

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Abstract
Inulinase is an enzyme catalyzing the hydrolysis of inulin, a plant reserve polysaccharide, into fructoses and fructo- oligosaccharides which are widely used in pharmaceutical and food industry. Here we reported inulinase production from *Staphylococcus* as there is not found any report on inulinase production from *Staphylococcus*. Eight(32%) isolates of *Staphylococcus aureus* were isolated from different agricultural rhizosphere soil samples and screened for higher inulinase production, *Staphylococcus aureus*S_3 gave higher inhibition zone around the colony in comparison with other isolates. Inulinase was purified to homogeneity with ammonium sulfate at 70% saturation followed by DEAE-Cellulose A-50 ion exchange chromatography and sephadex G-100 gel filtration chromatography with a recovery yield of 21% and 31.63 fold of purification. The results revealed the purified inulinase has a clear cytotoxic activity on cancer cell (Hep-2)with high significances on this tumor cell line during the three incubation periods, suggesting that the cytotoxic effect of this enzyme is a dose and time dependent, The higher level of inhibition(66%) was obtained at a higher concentration 300µg/ml after 72hour of exposure, this result increased the benefit from using the inulinase enzyme as anticancer drug.
Keywords: inulinase, *Staphylococcus aureus*, Hep-2 cancer cell line

Introduction

The rhizosphere is considered a hot spot of bacterial diversity that harbours bacterial flora whose diversity and adapted to the root presence, and in particular to favour plant growth. This is in turn beneficial to the whole rhizosphere microbiota through the highly nutritive and energetically rhizodepositions[1]. Rhizosphere is an important source of various microorganisms that have benefit role in survival and plant growth such as *Bacillus, Enterobacter, Acinetobacter, Alcalogenes, Arthrobacter, Flavobacterium, Rhizobium, Erwinia, Azospirillum, Burkholderia*, *Enterobacter, Pseudomonas*, *Staphylococcus, Rhizobium* and *Serratia*[2,3]. They promote plant growth via acidolytic solubilization of minerals, chelation of iron, production of enzymes to mineralize N, S or P from organic compounds to provide plants corresponding inorganic ions, nitrogen fixation, phytohormone production and exoploymer synthesis [1].

Staphylococcus are pathogens of man and other mammals. *S aureus* produces a variety of polysaccharides and extracellular proteins, some of which are correlated with their virulence. Virulence results from the combined effect of many factors expressed during infection[4]. *Staphylococcus aureus* is the most important pathogen related to food poisoning. The primary habitat of *Staphylococcus aureus* is the mucous membranes of the human nasopharynx and animal skin. *Staphylococcus aureus* is also present in soil, water, dust and air sources [5]. The broad use of antibiotics in the treatment of bacterial infections has led to diffuse of resistant bacteria. *Staphylococcus aureus* is an important pathogen both in nosocomial and healthcare associated infections. This bacterium has successful strategies for resisting the action of used antibiotics[6]. Methicillin resistant *Staphylococcus aureus* is now widely spreading in the community setting, thus the development of new drugs or alternative therapies is urgently necessary.

Inulinase (2,1-b-D-fructano -hydrolases EC 3.2.1.7) hydrolyses the inulin, a reserve carbohydrate in the roots and tubers of composite plants such as Jerusalem artichoke, Chicory, Sunflower and Dahlia[7], into inulooligosaccharides and pure fructose. The inulooligosaccharides have wide applications in various types of foods like confectionery, fruit preparations, milk desserts, yogurt and fresh cheese, baked goods, chocolate, ice cream, and sauces[8]. The fructose is considered an important alternative for fructose syrups production [9]. This sugar is used in many food and beverage industries, in addition to sweeteners and reveals several benefits in comparison with sucrose, since it less cariogenic, highly soluble and hygroscopic and so that it less tends to crystals formation, has low calorie content and does not cause arterioscelerosis. fructose also may be used by diabetic patients and mask the bitter taste of saccharm[10]. The produced inulooligosacchrides are considered a prebiotic agents because their acting as growth factors for improvements the microbial flora in the intestine[11], relief of constipation, decrease of total cholesterol and lipid in the serum and promotion of animal growth[12].

Microorganisms are an important source for commercial production of inulinases. Microbial inulinases are an important class of industrial enzymes, which are usually inducible and extracellular[11].Earlier studies focused on inulinase production from various microorganisms such as yeasts, fungi and bacteria[9,11].Among the bacteria, *Bacillus* spp., *Pseudomonas* spp. and *Streptomyces* sp. have been reported as high-yielding inulinase producers[13]. The bacteria *Staphylococcus aureus* has been rarely used for this purpose. To our knowledge

there is not any report about inulinase production by this bacteria. Therefore, the aim of this study was to screen inulinase production by *Staphylococcus aureus*, in addition to purification and investigation the cytotoxic effects of inulinase on viability of cancer cell line(Hep-2).

Materials and Methods

Samples collection

Twenty-five agricultural rhizosphere soil samples were collected from different locations by using pre-sterilized sample bottles and sterile spatula from garden in Al-Mustansiriya university. One gram of each of the samples was suspended in 10 ml of sterile distilled water and shake vigorously for 10 min. later, 0.1 of the resulting liquid was spread on the surface of blood agar and MacConkeys agar, then incubated at 30°C for 18-24 hour using L- shaped glass rod[14].

Bacteriological analysis

The bacterial isolates were observed for morphological characters and identified by using the tests guided by Berge's Manual of Systemic Bacteriology. Suspicious isolates of *Staphylococcus aureus* were identified by characteristic colonies (Round, smooth, white, creamy colonies with beta-hemolysis), Gram staining pattern and standard biochemical reaction like catalase test(+),oxidase test(-), coagulase test(+), citrate utilization test(+), DNase test(-), histamine assimilation test(-), glucose oxidation test, gelatin liquefaction test(-), urease activity(-), unability to motile, cocci in clusters and ability to grow on manitol salt agar[15,16]. Also the selective medium(Baird-Parker Agar) was used for the isolation of *Staphylococcus aureus* and prepared according to the method that described by[17]. Further, the *Staphylococcus aureus* isolate was confirmed by using Vitek 2 system by using Vitek GPI card (bio Merieux, France) according to the manufacturer's instructions.

Primary screening for inulinase producers.

All the bacterial isolates were inoculated in to the inulin agar plates containing 2g/L of inulin, 10g/L of yeast extract , 20 g/L of $MgSo_4.7H_2O$, 2g/L of KCl, 10% of NaCl, 20g/L of agar. Inulin was used as the sole source of carbon in this medium, thus, bacteria growth after 24 hrs of incubation at 30℃ shows this presence of inulinase activity, then the diameters of clear zones around the colonies were measured [18].

Secondary screening for inulinase producers

The selected bacteria isolates were transferred into 10 ml of a medium contained (g/L): inulin 20, yeast extract 20, $(NH_4)_2H_2PO_4$ 5, $NH_4.H_2PO_4$ 2, $MnCl.4H_2O$ 0.5, KCl 0.5, $MgSO_4.7H_2O$ 0.5, $FeSO_4.7H_2O$ 0.01, and pH was adjusted to 7.0. The bacteria isolates were incubated on the rotary shaker at 100 rpm at 30℃ for 24 hour. After removal of cells by centrifugation at 10000xg for 20 min. the enzyme activity, protein content and specific activity were assayed for resulted supernatant[19].

Assay of inulinase

Endoinulinase activity was assayed by incubating 2 ml enzyme solution with 2% (w/v) inulin prepared in 10 mM citrate-phosphate buffer pH 7.0 at 35℃ for 60 min. After incubation, the reaction tubes were kept in a boiling water bath for 10 min. to stop the enzyme reaction and then cooled to room temperature. The reaction mixture was assayed for reducing sugar as fructose by DNS method as described by[18] by reading the absorbance at 575 nm. The calibration curve was prepared with fructose solutions of known concentration and blanks were run simultaneously with enzyme and substrate solutions. One unit of inulinase activity was defined as the amount of enzyme, which produced 1µ mole of fructose under the assay conditions.

Protein determination estimation

Protein concentration was determined by the method of [20] and using bovine serum albumin as a standard.

Purification of inulinase

Culture supernatant that was obtained by centrifugation (8,000 × *g* for 30 min, 4°C) of selected isolate after incubation at 30ºC in inulin broth medium for 24 hour, subjected to fractionation by ammonium sulphate precepitation at 30-80% saturations. The sample was left overnight at 4℃ and the precipitate was collected by centrifugation at 1000 rpm for 15 min and dissolved in 20 mM potassium phosphate buffer, pH 7.0) then dialyzed overnight against the same buffer. The dialyzed protein was loaded on a DEAE-Cellulose A-50 column(2cm diameterx25cm long) and eluted with gradient of 0.1-0.5 M NaCl prepared in the same buffer at a flow rate of 0.6ml/min. The active fractions that showing the highest inulinase activity were pooled and applied to sephadex G-100 column (2x70 cm) that was pre-equilibrated with the above buffer. The column was eluted with same buffer at flow rate 0.5 ml/min. Protein concentration at 280 nm and inulinase were estimated and the active fractions were pooled for further studies.

Cell line preparation and cytotoxicity assay

The cytotoxic effect was tested for purified inulinase by using four concentrations (37.5,75, 150 and 300µg/ml) on growth of cancer cell line Hep-2 (Human larynx epidermoid carcinoma) and Rat embryo fibroblast (REF) which were obtained by the center Biotechnology research center of Al-Nahraine University. The cells were grown in RPMI-1640 medium containing 10% fetal calf serum to form confluent monolayer. The attached cells washed firstly with PBS and harvested from the flask by treatment with trypsin-versine solution. The cells were

counted by trypan-blue (about 95% viability), then cell suspension used for determination of cytotoxicity, 200µl of cell suspension were seeded in 96-well microtiter plate at density 1×10^5cells/ml and incubated in CO2 incubator at 37ºC until the separation of the cells from the ground flask.

In cytotxicity assay, 200µl of different concentration of inulinase (37.5, 75, 150 and 300µg/ml) were prepared in serum free media (SFM) and added to cells, then incubated at 37ºC for 24,48 and 72 hour.Three replicate wells were used for each concentration and negative control wells were treated with SFM. At the end of exposure periods, the cells were stained by 100µl of crystal violet solution and incubated at 37 ºC for 30 min. The optical density of each well was read by micro-ELISA reader at 492nm.The percentage of inhibition was calculated according to [21].

Inhibition rate%(IR)= ((Optical density of control - Optical density of test)/optical density of control) × 100

Statistical analysis

The experiments data were analyzed using statistical software SPSS version 16, significant differences between control and sample means were assessed using student´s T-test and P values ≤ 0.05 were considered significant.

Results and Discussion

Isolation of *Staphylococcus aureus*

Twenty- five agricultural rhizosphere soil samples were collected from rhizosphere area that has the dimensions(150 miles long and 60 miles wide). out of thirteen(52%) *Staphylococcus* spp., eight(32%)(figure-1a). *Staphylococcus aureus* was grew in the selective medium as shown in figure(1b). Baird-Parker Agar is a moderately selective and differential medium for the isolation and enumeration of *Staphylococcus aureus* in foods, in addition to environmental and clinical specimens. *Staphylococcus aureus* can grow in this medium with dark gray to black, shiny, convex colonies with entire margins and clear zones with or without an opaque zone around the colonies[17]. *Staphylococcus* may consider a very important agents in the aerobic mineralization of organic materials in nature because of their occurrence in soil and their nutritional properties[3]. Most *Staphylococcus aureus* isolates can diffuse in water and soil because of their do not need to growth factors, since they can grow in a mineral medium containing acetate or any organic material as carbon and energy source[5]. *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* are the common found in all the soil samples such as road side soil, industrial soil and Garden soil[3].

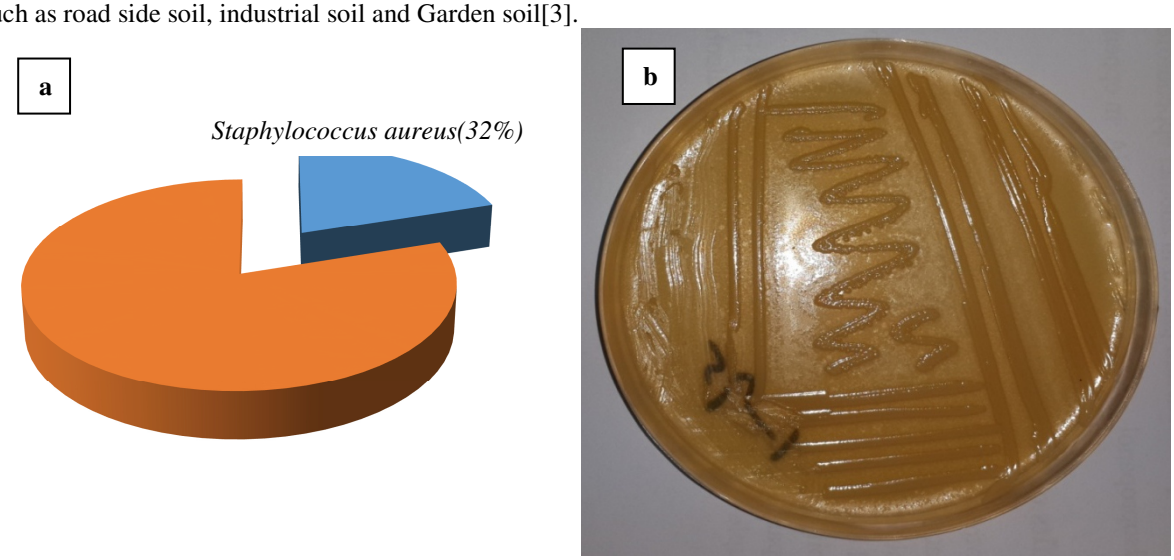

Figure-1: a) Percentage of *Staphylococcus aureus* from different soil sources.
b) Baird-Parker Agar medium for isolation of *Staphylococcus aureus*

Screening of inulinase producers

Among eight *Staphylococcus aureus* isolates initially subjected to rapid screening for extracellular inulinase production using inulin agar plates, five isolates were found to be positive for inulinase activity by their growth on this medium. To select the best inulinase producer, all five of these positive isolates were subjected to secondary screening in liquid culture and it was found that *Staphylococcus aureus* S_3 produced the highest level of inulinase with activity (165.8)U/ml (table-1). In this study, this isolate was selected for further studies. Inulinase production by *Pseudomonas aeruginosa*, *Xanthomonas oryzae*, *Lactobacillus casei* and *Achromobacter* sp. was started from 8th hour and the maximum enzyme activity was seen during the late logarithmic phase at 22nd hour of fermentation(22), in addition, *Flavobacterium multivorum* produced cell bound inulinase at the end of the growth phase (23). *Bacillus* spp. were considered as active producers of extracellular inulinase, since they produced 30-42 U/ml inulinase activity by growing on the sucrose as

substrate(24). when 50g inulin per liter was used as substrate, *Pseudomonas* sp. gave maximum activity of endo-inulinase(25). In a study done by(26) reported that inulin was the best carbon source for inulinase production by *Streptomyces* sp. since the activity reached to 0.55 U/ml with pure inulin and 0.85 U/ml with the inulin extract at 1% (w/v).

Table-1: Diameter of inhibition zone and inulinase activity for *Staphylococcus aureus* isolates

Isolate no.	Inulinase activity (U/ ml)	Protein conc. (mg/ml)	Diameter of inhibition zone (cm)
***Staphylococcus aureus* S_1**	158.6	115	1.3
***Staphylococcus aureus* S_2**	132.1	99	1.5
***Staphylococcus aureus* S_3**	165.8	112.6	2.4
***Staphylococcus aureus* S_4**	144.9	101	2.1
***Staphylococcus aureus* S_5**	149.4	108	1. 8

Purification of inulinase

Indoinulinase was obtained as an extracellular enzyme in the culture broth of *Staphylococcus aureus* S_3. Table (2) summarizes results of endoinulinase purification. The precipitation step was carried out by precipitation of protein from the cell-free supernatant by using ammonium sulphate at 70% saturation in this step the specific activity reached to 7.01 U/mg protein. The most commonly used salt for this procedure is $(NH_4)_2SO_4$ due to its high solubility, lack of buffering capacity, minimal cost, and the low density of the resulting solution relative to other salts, which aids in centrifugation separation(27). Ammonium sulfate precipitation followed by dialysis step to remove this salt from the sample and led to concentrate the product. The dialyzed sample was first loaded on a DEAE-Cellulose A-50 column. The elution with 0.1 to 0.5M NaCl gradient led to observe three peaks of proteins and the inulinase activity was located in the first protein peak(figure-2). In this step inulinase was purified 16.46 fold with a yield of 25.1%. The collected fractions was applied to sephadex G-100 column. The eluted fractions showed one protein peak that contained the inulinase activity as shown in figure(3) and the yield of inulinase was 21% with 31.63fold of purification and specific activity 46.5U/mg. For large scale production of inulooligosaccharides from inulin-containing agricultural crops, the crude endoinulinase preparation is directly useful(28). The inulinase was purified from *Arthrobacter globiformis* S64-1 with 442-fold of purification by DEAE-Toyopearl chromatographies[29].In a study done by[30] found that inulinase produced by *Aspergillus niveus* was purified by using ammonium sulphate and DEAE- cellulose chromatography with a yield of 27.26%. In contrast,[26] purified inulinase from *Streptomyces* sp. by using ammonium sulphate followed by four steps of column chromatography.

Table(2): Steps of inulinase purification from *Staphylococcus aureus*

Purification step	Size(ml)	Inulinase activity (U/ml)	Protein conc.(mg/ml)	Specific activity (U/ mg)	Total activity	Purification fold	Yield (%)
Crude extract	150	165.8	112.6	1.47	24870	1	100
$(NH_4)_2SO_4$ precipitation	70	240.7	34.3	7.01	16849	4.76	67.7
DEAE-Cellulose A-50	20	312.8	12.9	24.2	6256	16.46	25.1
Sephadex G-100	15	349.3	7.5	46.5	5239	31.63	21.0

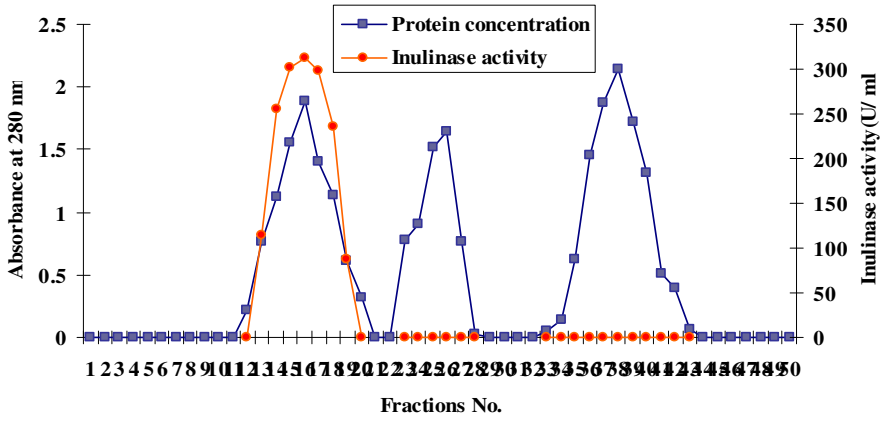

Figure (2): Purification of inulinase from *Staphylococcus aureus* using ion exchange on DEAE-Cellulose A-50 column.

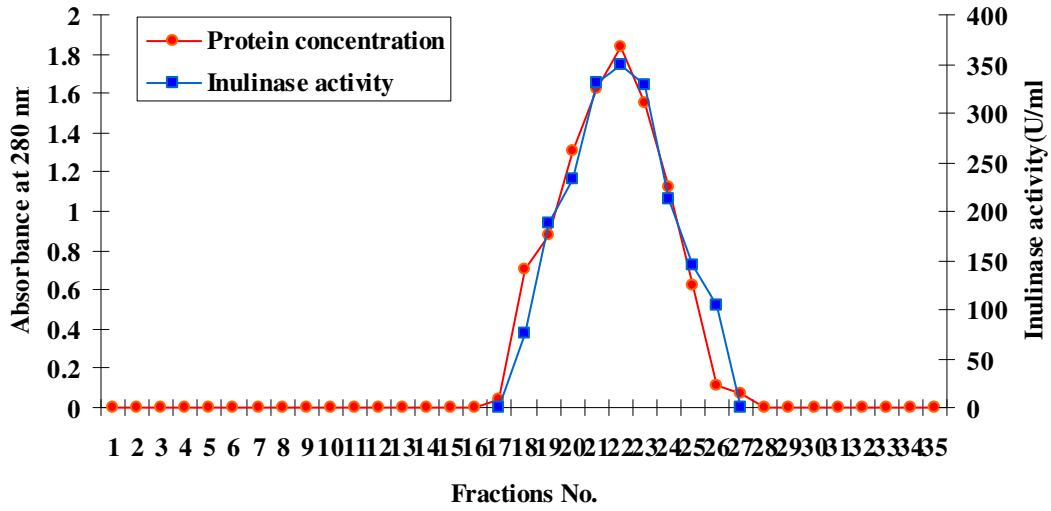

Figure (3): Purification of inulinase from *Staphylococcus aureus* using gel filtration on sephadex G-100 column.

Cell line preparation and cytotoxicity assay

The cytotoxicity activity of purified inulinase was carried out against tumor cell line (Hep-2) and normal cell line (REF)at different concentrations(37.5, 75, 150 and 300µg/ml). The results showed in figure (4) showed the significant differences ($P<0.05$) as the concentrations increased when the cancer cells (Hep-2 cell line) were treated with purified inulinase comparable with the control, since the inhibition percentage was increased from 10% to 40%, 17% to 52% and 23% to 66% at different concentrations ranged between 37.5 and 300µg/ml after 24,48h and 72h of exposure. The lowest cytotoxicity was found in 37.5 µg/ml concentration with 10% of cell growth inhibition after 24 hour of exposure and the highest inhibition percentage (cytotoxicity) reached 66% at high concentration (300µg / ml) of purified inulinase after 72 hour exposure time so that the inhibition rate was concentration dependent, as well as time dependent for Hep-2 cell line. This can be attributed to sensitivity of Hep-2 cell and may be due to the variation in the cytotoxic activity of lectin according to different time of exposure. The modification of cell surface might be due to the neoplastic transformation, change of glycoprotein in cell surface and cell adhesion molecules which might be correlated to the invasion of metastasis *in vivo*[31]. The activation of endogenous endonuclease that cleaves the nuclear DNA at random locations led to apoptosis[32], since an apoptosis is a major form of cell death and essential for normal development and for maintenance of homeostasis. The ability of purified inulinase to reduce or induce an apoptosis related with many several factors like enzyme concentration , mode of action of enzyme and cell type[33]. These effects may be due to the ability of inulinase to induce some enzymes such as cyclooxygenase (COX) that help the defense mechanisms in the body to detoxify carcinogens , reduction of oxidative stress, induction of the proapoptotic genes and reduction of antiapoptotic genes this lead to induce the programmed death of cell lines and arresting their proliferation[34].Another reason for cell death that the excessive consumption of glucose was neither used for synthesis nor oxidation, but rather secreted as lactate and the accumulation of lactate in the medium will affect the cell growth profile[35].

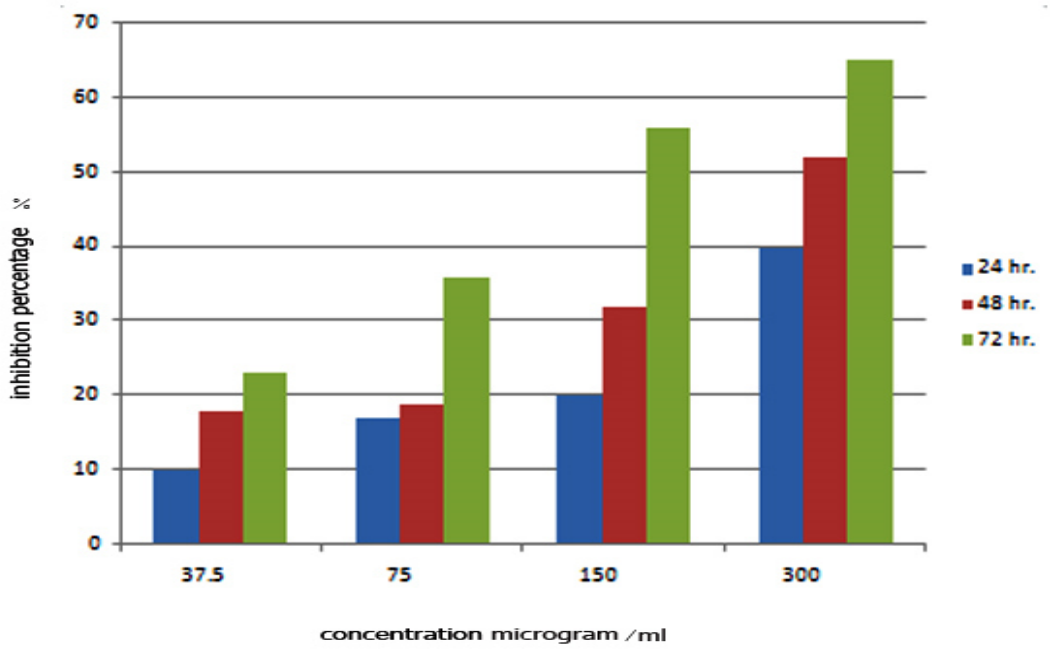

Figure (4): The cytotoxic effects of purified inulinase from *Staphylococcus aureus* on Hep-2 cell line *in vitro* during different exposure times

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