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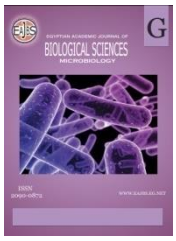

Study Cytotoxicity and Genotoxicity of Hemolysin Using Human Lymphocyte

Aqeel Hazim Salieh, Batol Imran Dheeb and Asmaa Easa Mahmood
Department of pathological analysis, college of applaied science, Samaraa university
*E. Mail:**aqeel121212121212@gmail.com**

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ABSTRACT

The cytotoxic effects on human lymphocytes of Hemolysin sample (one pure sample produced in the laboratory for this study) were assessed at four different concentrations (25, 50, 100 and 200 µL) using the methylthiazol tetrazolium (MTT) bioassay. The results showed that growth was inhibited by 21.4, 32.3, 48.7, and 79.34 for each of the four concentrations of the pure sample, respectively, Deoxyribonucleic acid (DNA) was extracted from the lymphocytes and analysed by electrophoresis on a 1% agarose gel. Hemolysin appeared to have the ability to degrade or damage the DNA. The present study showed that both the growth inhibition and DNA damage experienced by the human lymphocytes increased linearly with increasing concentrations of the toxin.

INTRODUCTION

Haemolysin is a toxic extracellular protein produced by many gram negatives (e.g., E. coli, Serratia spp). Proteus spp., Vibrio spp., Pasteurella spp., Pseudomonas aeruginosa) and bacteria gram-positive (e.g., streptococcus spp., Staphylococcus aureus, listeria spp., Bacillus cerius, Titanium Clostridium), all possess the potential of a particular nurse. Hemolycin has therefore always been considered virulent, most red blood cell decompositions cause red blood cells to decompose by forming pores with different diameters in the membrane and are assigned as such because they have the ability to decompose red blood cells (RBCs). Many hemolycene can also attack - possibly by a similar mechanism - other mammalian cells. Because of this cellular effect, it is also called cytolysins (Goebel *et al.,* 1988).

Studies in the living body and in the laboratory have provided some evidence that hemolycin leads to excessive secretion of inflammatory mediators by immune cells that promote inflammation caused by other bacterial virulent factors. Hemolycin has been shown to stimulate the secretion of interleukin-1φ and tumor necrosis factor α (TNF-α) from single human cells as early as a few decades ago, partially purified hemolycin has been shown to have metogenic ability However, some speculation has arisen that the toxins used in the studies may be contaminated by other factors that stimulate the glioblastoma of lymphocytes (Adam Bownik *et al.*,2008). Further studies on pure poison have shown that hemolycin actually stimulates the proliferation of human T and non-T lymphocytes.

This property is usually maintained after the poison is disabled at 60°C for 10 minutes but in return, its hemolytic activity is reduced **(**Adam Bownik *et al.,* 2008).

MATERIALS AND METHODS

Investigate the More Efficient Isolation of Hemolysin Production from *S. aureus* Bacteria:

1.Qualitative Measurement:

The presence of hemolysin isolytic activity was tested on a blood agar dish, where the bacterial suspension was prepared from the erythrocyte hydrolyzing isolates that gave the complete decomposition of beta-hemolysis by transferring pure bacterial colonies to the sterile test tube containing the sterile physiological local solution, after which a hole is made in the blood acar dish by means of a sterile steel tube with a diameter of 3.5 mm, and 20 microliters of The bacterial was stuck in the well, and the diameter of the hemolytic zone after incubation was measured at 37‡C for 18 hours. (Takada *et al.*, 2003) (Al-Shammary *et al.*,2012)

2.Quantification Measurement:

The quantification of hemolytic activity was by the method (Bernheimer and Schwartz1963) with minor modifications (Takada *et.,al.,* 2003) blood was taken from donor people after which the blood was washed to obtain erythrocytes by centrifugation using Phosphate buffer saline (PBS) three times (1500 cycles per minute, 5 minutes), 50 µL of bacterial stranded (which was prepared by transferring pure bacterial colonies to a sterile tube containing a sterile solution of sterile physiological sweetener) was added with 20 ml. Müller of dithiothreitol (DTT) to 50 µltr solution prepared as a suspension of red blood cells by 2% with PBS. After incubation at 37 ° C for 1 hour, the suspension was centrally expelled (10,000 rpm, 5 minutes) immediately after adding 300 µL of PBS, and absorption was measured using the spectrophotometer UV-160A spectrophotometer at a wavelength of 540 nm to determine the decay activity (Al-Shammary *et al.*,2012)

3.Protein Concentration (Bradford 1979):

Protein concentration was determined according to the (Bradford Method 1979) as follows: Different concentrations (0,2,4,6,8,10 mg/ml) of Bovine serum albumin stock solution (BSA) were prepared. After that, 2.5 ml of the Comassie brilliant blue dye (G-250) was added, mixed and left to stand for 2 minutes at room temperature. absorption was measured at 595 nm; (Blank) 0.45 ml of Phosphate buffer saline (PBS) and 2.5 ml of dye detector. The protein concentration was estimated by mixing 0.05 ml from the test sample, 0.45 ml from Phosphate buffer saline (PBS) and 2.5 ml from Comassie brilliant blue dye (G-250), and left to stand for two minutes at room temperature before measuring absorption at 595 nm.

4.Extract Raw Heamolysin From Bacteria *S.aureus*:

After the isolation of *S. aureus* was activated by preparing its first plant, at the age of 18 hours in the center of Trypton Soya Broth and warmly (37)M, a glass cycle capacity (1000) ml containing (25) 0) From the center of Trypton Soya Broth and (10) ml of activated implant and hug the dork warmly 37 m for 18 hours in a vibrator incubator at a speed of 150 shakes/minutes to provide good ventilation of the implant. Discard the bacterial sticker with a 4-m cooled centrifuge at 5,000 rpm for 30 minutes, and the spray is collected in a sterile container. The effectiveness of the spray was determined by an injection into the blood agar and the concentration of protein.

4. Purification by Ion Exchange Chromatography:

DEAE-Cellulose column was prepared according to Whitaker and Bernard, (1972) by dissolving 20 g of resin in 1L of distilled water, where purified hemolysin was added at the top The column was rinsed with the solution (phosphate buffer) and with a linear saline gradient using sodium chloride with a range (0.15-0.25) müller and at a run-

off speed (0.5) ml/minute, I collected the window parts of the column in 3 ml/tube test tubes, read the absorption of the window parts by the optical spectrophotometer along the wavelength (280nm), Draw the curve of the relationship between absorption and window parts. The effectiveness of the parts that formed the tops in the relationship curve was determined and then collected the tubes that gave effectiveness and were carried against several substitutions (2) of distilled water, focused by polyethylenglycol and estimated protein concentration in the model after its concentration, keeping the concentrated protein at (-20) until use.

5.Preparation of Toxin Concentration:

Hemolysin extract was dissolved in DMSO at 1 mg/ml and used to prepare concentrations of 25, 50, 100 and 200 µL in complete culture media (RPMI-1640 medium supplemented with 10% fetal calf serum, containing a solution of penicillin 100 units/ml and streptomycin 100 lg/ml) (Freshney, 2012).

6. **Collection and Processing of Human Lymphocytes:**

Peripheral venous blood was taken from a healthy 26-year-old male donor and lymphocytes were extracted following (Rafael and Vaclav, 2000). The suspension of cultured human lymphocytes was adjusted until the number of cells was about 1 · 104 - cells/ml. 100 ll of the cell suspension was then dispensed into each of the 96 wells of a microtiter plate to give a final cell count of 1000 cells/well. The plates were then incubated at 37 C in an incubator supplemented with 5% CO2 for 24 h. After incubation, hemolysin was added to each well at different concentrations and incubated for 24 h (Rafael and Vaclav, 2000).

7. Cytotoxicity Assay Using the 3-[4,5-dimethylthiazoyl]-2, 5-diphenyltetrazolium bromide (MTT) Test:

This test was performed by dissolving 3-[4,5-dimethylthiazoyl]- 2, 5-diphenyltetrazolium bromide in phosphate-buffered saline (PBS) at 2 mg/ml, filtrated by a 0.22 lm millipore filter. 50 ll of the MTT dye was added to each of the microtiter plate wells containing human lymphocytes treated with different concentrations of hemolysin for 24 h. The MTT-formazan crystals, which are formed only by live cells, were dissolved in 100 ll Dimethyl sulphoxide (DMSO), enabling the optical density of each well to be measured using an ELISA reader at a transmitting wavelength of 620 nm (Freshney, 1994). The inhibitory rate was measured according to (Wang *et al.,* 2003) as follows:

$$\text{Growth inhibition } \% = \frac{O.D.of\ control - O.D.of\ Sample}{O.D.of\ control} \times 100$$

8. Genotoxicity Assay:

After the incubation period, all the treated human lymphocyte content of the wells was transferred from the well of the microtiter plate by micropipette before staining to a sterile Eppendorf tube for use in the genotoxicity assay. DNA was extracted from the cultured cells using a genomic DNA mini kit (Geneaid Company). The samples were analysed by electrophoresis on a 1% agarose gel, following (Sambrook *et al.*,1989). Visual observations of DNA were recorded with UV camera system (Abraham *et al.,* 2008).

RESULTS AND DISCUSSION

Investigate the More Efficient Isolation of Hemolysin Production from *S. aureus* Bacteria:

1. Qualitative Measurement:

The presence of heamolysin-producing isolation activity of *S. aureus* of 32 isolations was tested at 29% on a blood-dendritic dish, where bacterial trappers are prepared from heamolysin-producing insulation that is transported by bacterial colonies. Pure to the sterile test tube container on the local sterile vesal solution, after which

drilling is made in the dish of blood dens by a sterile steel tube with a diameter of 3.5 mm, and 20 microliters of bacterial trappers were placed in the well, the results showed the appearance of Clear areas of hemolysis after the end of the incubation period around developing colonies in different countries (Table 1). In the 32 isolations of *S. aureus*, the most efficient and productive isolation was identified as the S.a 27, which gave the highest diameter of 17mm to the hemolytic region as shown in Table 1.

Table 1: Countries of the hemolycin hemolycin-induced hemolycin region resulting from *S. aureus* insulation grow on blood rents after 24 hours of incubation at 37 degrees M.

Isolation number and code	Diameters of the decomposition zones (mm)
S.a 1	15
S.a 2	11
S.a 3	6
S.a 4	10
S.a 5	11
S.a 6	14
S.a 7	10
S.a 8	12
S.a 9	13
S.a 10	11
S.a 11	9
S.a 12	11
S.a 13	13
S.a 14	16
S.a 15	9
S.a 16	11
S.a 17	12.5
S.a 18	6
S.a 19	4
S.a 20	11
S.a 21	9
S.a 22	7
S.a 23	12
S.a 24	14
S.a 25	13
S.a 26	11
S.a 27	17
S.a 28	13
S.a 29	13
S.a 30	11
S.a 31	9
S.a 32	10

2.Quantification Measurement:

After testing the 32 *S. aureus* insulation in paragraph (Qualitative Measurement), the isolations that gave higher diameters to the 10 analyzed areas were taken and examined in a measured manner. The quantum of hemolytic activity in the Bernheimer and Schwartz method with minor modifications, and absorption was measured using spectrophotometer optical spectrophotometer UV-160A at a wavelength of 540 nm to determine hemolytic activity as shown in (Table 2).

Table 2: Hemolycene activity resulting from *S. aureus* insulation by quantification using a spectrophotometer.

Isolation number and code	(U/ml)Activity
S.a 1	320
S.a 6	165
S.a 9	160
S.a 13	180
S.a 14	320
S.a 24	160
S.a 25	40
S.a 27	320
S.a 28	160
S.a 29	160

The results showed out of 10 *S. aureus* isolations, S.a 27 isolation was selected based on qualitative measurement and ironing because it gave the highest decomposition rate (17mm) and the highest activity using a spectrophotometer (320 ml/U) compared to the rest of the isolations**.**

3.Purification by Ion Exchange Chromatography:

Haemolysin was produced from the isolation *S. aureus* by developing it on the center of Trypton Soya Broth pH equal to 7 and hugging this medium at a temperature of 37 m for 18 hours in a rocking incubator at a speed of 150 tremors/minutes and was extracted by separating the scarves (haemolysin) for sediment (bacteria) using cooled central ostracism. Hemolycene was estimated to be effective using the method as in paragraph (2.1.1) The amount of protein in it was estimated using the Bradford (1976) method of 6.65 mg/ml, (Table 3) shows hemolycene purification steps, which included deposition using Ammonium Sulphate, which is one of the initial steps for protein purification because of its high efficiency in protein deposition due to sulfur capacity. Ammonium on the equation of charges on the surface of the protein due to salt and disruption (Disruption) to the water layer that surrounds protein molecules which leads to a decrease in protein melting and thus deposition, proteins are soluble in aquatic circles because they contain side chains for water-loving amino acids provided by essential amino acids, acid amino acids and water-loving neutral amino acids. Salt such as ammonium sulfate interferes with these interactions between amino acid side chains and water, by reducing available water and reducing protein meltability, this allows protein reactions rather than protein-water interactions and will remove protein from the solution. Depending on the water's love of protein, different proteins will separate at different ammonium sulfate saturation levels. A higher water-loving and higher concentration of ammonium sulphate will be needed to break down the interactions between protein and water to enable access to raw tyvan **(**Wingfield, 2001; Burgess R, 2009). Many methods have been used for the purpose of obtaining raw lefants from bacterial implant leakers in an attempt to reach an efficient method of isolating raw tails in large quantities for their remote use in purification, and from commonly used deposition methods for obtaining bacterial toxins deposited with ammonium sulphate at a final concentration of 70%, which was used by researcher (De Saxe *et al.*, 1982) and researcher.(Al-Shammary *et al.*,2012), The resin used in this step is diethylaminoethylcellulose (DEAE-cellulose), which is a weak base. The nature

of the resin enables anionic proteins to bind while allowing cationic proteins to pass through. The binding of the protein to the resin depends upon the pI value of the protein and the pH of the buffer solution in which the DEAE-Cellulose is equilibrated. DEAE-cellulose resin has many advantages, including good separation, high-resolution power, easy handling, high capacity, the possibility of reactivation using many times and the simplicity of the separation principle which was based on the charge difference **(**Al-hussan.,2016), Purification by DEAE-cellulose chromatography has led to a further increase in enzyme activity.

Table 3: Stages of Hemolycene Purification of *S. aureus* Bacteria.

Purification stages	Crude hemolysin	Precipitation with ammonium sulfate	Dialysis	Ione exchange
Size (ml)	250	100	50	25
Protein concentration (mg\ml)	6.65	3.4	2.16	1.9
Effectiveness	+	+	+	+

After collecting the window parts and reading the absorption of these parts at a wavelength of 280 nm where two peaks (Fig. 1) were observed, the hemolycene event appeared at the second summit at parts (48) & (47) which was given after assembly and Dales Its concentration and concentration of protein is equal to 1.9 mg/ml and has shown obvious effects on the blood agar when using the method (Qualitative Measurement), and the naCl concentration required for recovery was 0.25 müller.

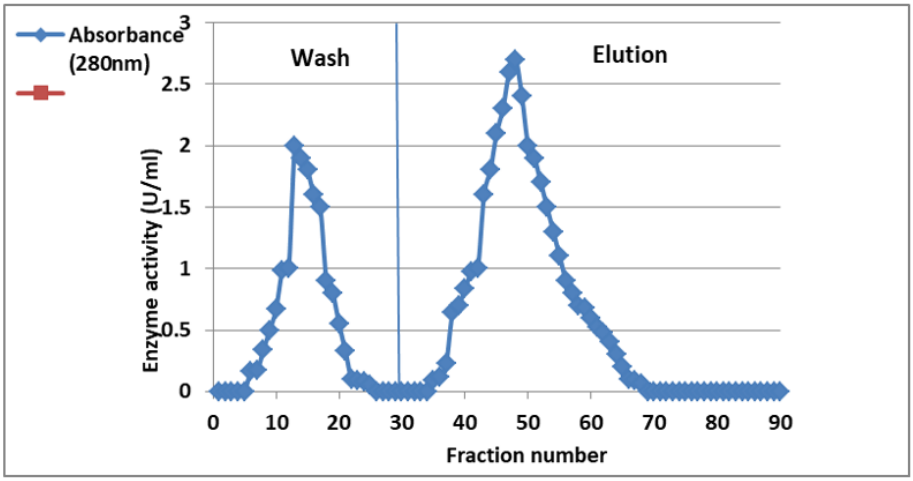

Fig.1: Ion exchange chromatography on DEAE cellulose column (3x 20cm).

Cytotoxicity And Genotoxicity Effects of Hemolysin on Human Lymphocytes:

1.Cytotoxicity Effects of Hemolysin on Human Lymphocytes:

The toxic effect of cells on *S. aureus* isolated hemolycin toxins on lymph cell growth was observed when incubated at different concentrations of 25, 50, 100 and 200 µL for 24 hours, and the growth inhibition rate for lymphocytes was 13%, 29.3%, 42.6% and 34.79% after the inhibition equation was applied, the results showed that the rate of inhibition of growth was focused, and there was an increase in the inhibitory effect when compared to the negative control (Fig. 2).

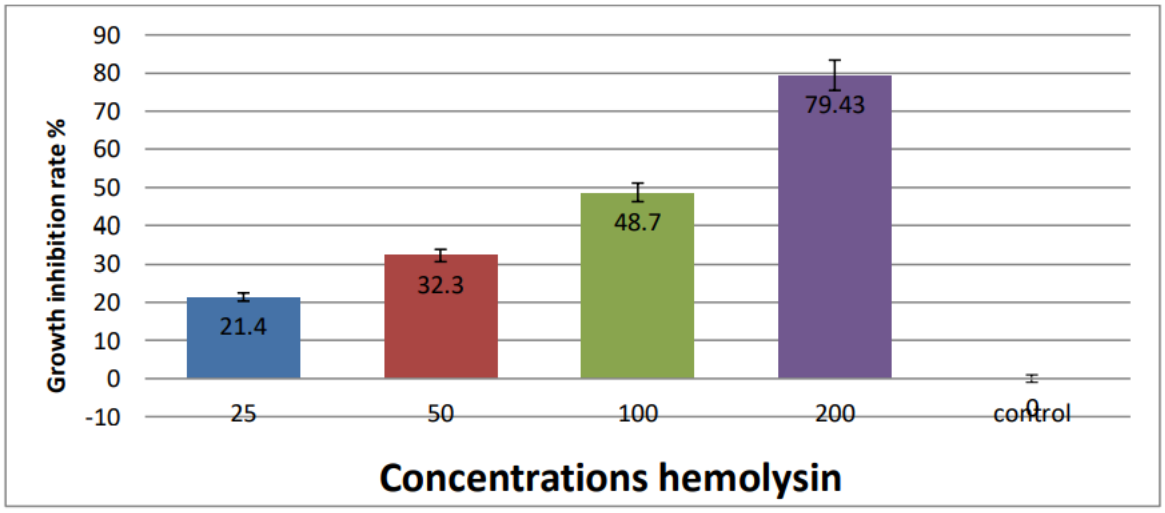

Fig. 2: Cytotoxicity effects of hemolysin on human lymphocytes

Through the results, the treatment of lymphocytes with concentration (200, 100, 50, 25) microliters has led to the activation of lymphocytes compared to control, where the number of lymphocytes decreased with increased concentration and obsolescence, this explanation is not consistent with research conducted by (Bohach *et al.,* 1990) &(Torres *et al.,* 2001) When lymphocytes are treated with low tsst-1 toxins, it leads to a reproductive response by these cells, where lymphocytes decrease with increased concentration of toxins, It is compatible with (Jonas *et al.,* 1993) and also corresponds to (Tsuiji *et al.,* 2019).

Researchers (Jonas *et al.,* 1993) also explained that bacterial toxins can have a direct toxic effect on cells (Cytotoxicity) due to the presence of qualitative receptors on the surface of lymphocytes associated with the poison leading to gradual damage to cell membranes due to this association, which leads to their death, which may explain the low rate of lymphocytes. The sharp decline that occurred when lymphocytes were treated with high toxic toxins is an important observation to be noted.

A dimethylthyazole-2-yl-2.5-divinyl tetrazoleum (MTT) test is a color test to assess cell viability. Nadph-based cellular oxidoridoctaz enzymes may, under specific circumstances, reflect the number of viable cells present. These enzymes are able to reduce ttrazolyum MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltrazolium bromide to insoluble formazan, which has a purple color (Berridge *et al.,* 2005). MTT color cellular toxicity testing is widely chosen as a measure of cell validity for an optimal endpoint. MTT dye is reduced by living cells, but not dead cells to an insoluble purple formazan product in a water solution. The amount of MTT-formazan produced light spectrally can be determined once it melts into a suitable solvent after cells have been subjected to a different concentration at different time intervals for that worrisome substance (Abraham *et al.,* 2008**)**.

There is evidence of different effects that hemolycene exerts on pharyngeal cells. It turns out that only human cells are exportable even for low concentrations (Adam Bownik *et al.,* 2008). Studies in the living body and in the laboratory have provided some evidence that hemolycin leads to excessive secretion of inflammatory mediators by immune cells that promote inflammation caused by other bacterial virulent factors. Hemolycin has

been shown to stimulate the secretion of interleukin-1φ and tumor necrosis factor α (TNF-α) from single human cells [4 as early as a few decades ago, partially purified hemolycin has been shown to have metogenic ability However, some speculation has arisen that the toxins used in the studies may be contaminated by other factors that stimulate the glioblastoma of lymphocytes (Adam Bownik *et al.,* 2008).

Further studies of pure poison have shown that hemolycin actually stimulates the proliferation of human T and non-T lymphocytes. This property is usually maintained after the poison is disabled at 60°C for 10 minutes, but in contrast, its hemolytic activity is reduced (Adam Bownik *et al.,* 2008) mediates the activation of lymphocytes through the cell membranes. Hemolycin is known to cause subtle damage to the human fibrous cell membrane in the laboratory (Thelestam, M. 1976.).

2. Genotoxicity Effects of Hemolysin on Human Lymphocytes:

DNA was extracted from lymphocytes treated at different concentrations of hemolycin toxins 25, 50, 100 and 200 µL. Then analyzed by electric nomads on agarose gel 1%. Hemolysin toxins appear to have the potential to decompose or damage DNA. The results showed that DNA damage gradually increased with an increased concentration of hemolycene toxins (Fig. 3).

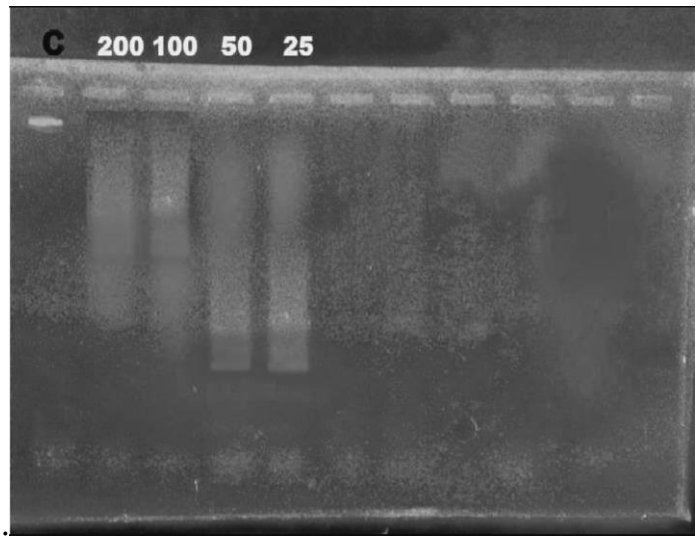

Fig. 3: Agarose gel electrophoresis of the DNA extracted from human lymphocytes after being treated with different concentrations of hemolysin showing degradation of lymphocyte DNA when compared with control., C: control, Numbers.: concentrations of purified hemolysin

In a previous study**)** Jonas *et al.,* 1993) noted that DNA degradation is associated with hemolycin concentration and the time where it affects ATP, if DNA degradation is a process that occurs in cells that are interspersed and dead, it will occur in the same cell group. The non-penetrable cells will retain their ATP and will be simulatable using PHA. In contrast, if DNA degradation is a process that occurs in non-permeable cells, these should eventually lose their ability to integrate [3H] thimidin when stimulating. Hemolysin causes a sudden drop in ATP levels after 3 hours and thus leads to DNA destruction, this study proved that human lymphocytes are quickly killed by hemolysin and this process is accompanied by DNA degradation.

In other studies, dose-related DNA damage has been observed when toxins increase, leading to DNA in mice (Nieminen *et al.,* 2002 b). The interaction of toxic DNA has also been investigated in some laboratory studies (Eichner *et al.,* 1988), which proves that reactive oxygen types produced by toxins are capable of triggering DNA damage. This damage to DNA was observed in toxins-treated cells within 24 hours. Higher concentration of toxins (0.8-1.6 ppm) (Nieminen *et al.,* 2002 b).

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