ORIGINAL ARTICLE

Depending on HPLC and PCR, detection of aflatoxin B1 extracted from Aspergillus flavus strains and it's cytotoxic effect on AFB treated-hematopoietic stem cells obtained from human umbilical cord

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Abstract

Background: Aflatoxin B1 (AFB1) is a carcinogenic metabolite produced by *Aspergillus falvus* which cause several diseases in humans. This study aimed to determinate the role of polymerase chain reaction (PCR) and high-performance liquid chromatography (HPLC) techniques in discrimination between aflatoxigenic and non-aflatoxigenic producer isolates of *A. flavus* and molecular expression for aflatoxin encoding genes (aflM ver-1, aflD, and aflR). Furthermore, to assess the cytotoxic effect of AFB on isolated hematopoietic stem cells (HSCs) obtained from the human umbilical cord. **Materials and Methods:** The extracted aflatoxin was detected by HPLC. Fungal DNA was extracted and submitted to PCR for amplifying AFB encoding genes (nor1 aflD, aflM, and aflR). The collection of umbilical cord blood samples was accomplished using the system close method. CD34 were used for immunophenotypic analysis of HSC which were obtained using buffy coat method. An experimental study was conducted *in vitro* to assess the cytotoxicity effects of AFB with certain concentrations on the viability of AFB treated-HSC. **Results:** Out of 15 *A. flavus*, the genes (AflR, nor1 aflD) were founded in 11 (73%) of *A. flavus* strains, while the (ver aflM) was appeared in 10 (67%) of strains. HPLC technique had been detected efficiently AFB1-producing isolates in 10 (66.7%) out of 15 isolates of *A. flavus* with rates ranged from 0.78 to 45.03 ppm. It was yielded that the inhibition rate of isolated HSC was increased seriously with an increase of aflatoxins concentration leading to decrease in viability of AFB-treated cells. **Conclusions:** Aflatoxin production was directly associated with the appearance and expression of genes (aflM ver-1, aflD, and aflR) by PCR. Furthermore, HPLC is a superior technique in identifying and analyzing aflatoxin with high sensitivity and accuracy. Further, in a cytotoxicity assay, whenever the aflatoxin concentration was high, the rate of HSC growth inhibition increases and viability of AFB-treated cells decreased.

Key words: Aflatoxin, *Aspergillus flavus*, high-performance liquid chromatography, polymerase chain reaction, stem cell

INTRODUCTION

Aflatoxins as effective carcinogenic toxins are secondary metabolites produced by several species of *Aspergillus* like *Aspergillus flavus*.[1] These toxins are important because they can colonize a range of economically important crops such as maize, nuts, tree nuts, and peanuts, it appears in deterioration, bad odor, and discoloration and maybe contaminate them with aflatoxin, featuring these toxins have high acute toxicity.[2] There are many factors affecting aflatoxin production during the storage period, such as temperature, moisture, availability of oxygen, and fat content. There are four types of aflatoxins (B1, B2, G1,

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and G2) while aflatoxin B1 (AFB1) is the toxic type which is more dangerous.[3]

The cluster gene pathway for aflatoxin may allow all of the pathway genes to be expressed rapidly on the appearance of secondary metabolites, therefore, aflatoxins start to accumulate rapidly after 18–20 h of mycelial growth.[4] A distinction between aflatoxigenic and non-aflatoxigenic strains of *Aspergillus* section Flavi has been a matter of controversy.[5] Conventional methods which used for detection of aflatoxins are microbiological identify, high-performance liquid chromatography (HPLC), thin-layer chromatography, or enzyme-linked immunosorbent assay (ELISA). These procedures are time consuming in spite of they are considered reliable techniques in detection of aflatoxins.[6] However, polymerase chain reaction (PCR) is easy to be used, rapid and accurate.[7]

Up to our simple knowledge, there is no report in Iraq what's regard to use of real-time PCR as a cutoff diagnostic tool for differentiation of aflatoxin-producing isolates in addition to molecular screening and expression for aflatoxin encoding genes (aflM ver-1, aflD, and aflR). Thus, the aims of our study were to (1) determinate the capability of *A. flavus* isolated from clinical and environmental sources for production of AFB1, (2) differentiate between aflatoxigenic and non-aflatoxigenic isolates of the above fungus depending on PCR in addition to molecular expression for aflatoxin encoding genes (aflM ver-1, aflD, and aflR), and (3) investigate the role of HPLC in analyzing and identifying AFB1 level produced from *A. favus* obtained from well-extracted samples.[8]

On the other hand, aflatoxins have been commonly classified as a carcinogen by the International Agency for Research on Cancer, (IARC). These toxins have many potentials such as cytotoxicity, carcinogenicity, hepatotoxicity, immunotoxicity, and genotoxicity.[9] Most of the previous studies were concentrated on a study the effects of aflatoxin on mature lymphocytes while there is no study of their toxic effects on precursors hematopoietic stem cells (HSC). Thus, the present study was also aimed to study the cytotoxic effect of aflatoxin on HSC obtained from the human umbilical cord and detect it's toxic dose.[10]

MATERIALS AND METHODS

Identification of fungal isolates

Fifteen *A. flavus* isolates were obtained from two laboratories; 5.0 (33%) clinical isolates were obtained from laboratory of a College of Veterinary Medicine - University of Baghdad while 10.0 (67%) environmental isolates were obtained from laboratories of the Environment and Water Department, Ministry of Science and Technology. These isolates were examined directly under the microscope using 10% KOH and culturing on Sabouraud Dextrose Agar (SDA) and Potato Dextrose Agar (PDA).[11] *A. flavus* isolates were cultured on SDA and with the addition of 0.05 mg/mL chloramphenicol to inhibit the growth of bacteria, then incubated at 28°C and 37°C and examined for 7 days according to Hamed *et al*.[12] Isolates were identified depending on the species level based on macroscopical and microscopical characteristics using SDA[13] and scotch tape preparation.[14] All fungal isolates which identified as *A. flavus* were cultured and maintained in PDA supplemented with chloramphenicol and incubated for 5 days at 28 ± 2°C.[15]

SDA slants were prepared and sterilized according to Jamali *et al*.[16] After that, chloramphenicol of 50 mg/l was added. A loop was used for taking spores to implant in test tubes then incubated at the same temperature. After incubation of 10 mL of sterile distilled water in culture tubes with *A. flavus,*[17] the spores were harvested by sterile loop and suspension of spores was prepared in the tubes. After transferring and dilution of spore suspension, the spores were accounted in 5 µL using a hemocytometer.[18]

According to Levin,[19] each flask was inoculated with million spores and incubated at 28 ± 1°C in the dark for 21 days and shaken once or twice daily for 3 days to aid in even distribution of the inocula. After fermentation, the flasks were placed briefly in the oven at 60°C for 3 h to destroy the fungus.

Extraction and detection of AFB1 by HPLC

AFB1 has been extracted according to AOAC, 2000 procedure with few modifications. AFB1 has been detected by high-performance liquid chromatography (HPLC) with the following conditions: Column: 250 mm × 4.6 mm, particle size 5 µm, ODS (C18), mobile phase acetonitrile:water 40:60 v/v, flow rate 1 mL/min, detector UV-365 nm and compared to standard AFB1 (Sigma) as control.

Extraction of fungal DNA

DNA was extracted from 0.5 g (wet weight) of fungal mycelium/spores according to the instructions of fungal genomic isolation kit, Promega, USA.[19]

Target genes and PCR condition

For the molecular diagnosis of aflatoxin which produced from toxigenic *A. flavus* isolates, PCR was conducted for amplifying three genes, i.e., (ver1, nor1, and aflR). These genes and specific primer sequences have been selected from the already reported data[20] the primer sequences, the name of genes and their product size are listed in Table 1. All PCR reagents including pre-mix had been provided and synthesized by (Bioneer-Korea), primers were scientifically designed thematically, and all PCR attempts were carried out

in PCR thermal cycler (Applied Biosystems, Germany). The PCR reaction was carried out in 25 µl solution containing (5 µl) premix Taq DNA polymerase, 250 µM (each) deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, deoxythymidine triphosphate, and 1.5 mm MgCl2, reaction buffer (PH 9) and loading dye buffer (Trace), 4 µl each of amplification primers, 5 µl target DNA, and 11 µl free nuclease water. Thermal cycle conditions were carried as mentioned in Table 1 except annealing temperature, which was variable (58–62°C) for study three genes. PCR amplified products were checked on 1% gel by gel electrophoresis and visualized under gel documentation system.

Umbilical cord blood (UCB) and separation of mononuclear cells (MNCs)

The UCB samples were obtained from 15 full-term infants with the information consent of the mothers. All UCB specimens were freshly collected from the umbilical vein using system close method. The blood was flow by gravity into the bag containing citrate phosphate dextrose adenine-1 as an anticoagulant. During collection, the blood bag had been shaken gently, and the anticoagulant was freely mixed with UCB.[21] The blood was diluted with an equivalent volume of phosphate buffer saline (PBS). 4 ml of diluted blood was transferred to 10 ml round bottom tubes and layered carefully on 3 ml of Ficoll-Hypaque solution and centrifuged at 2200 rpm for 25 min at 4°C to isolate MNCs. Then, four distinct layers were yielded markedly. Using Pasteur Pipette, the MNCs rich zone (buffy coat layer) was removed and transferred into a new 10 ml round bottom tube and washed twice with PBS through centrifugation at 4°C in 2000 rpm for 8 min and 1000 rpm for 10 min.[21] CD34 were used for immunophenotypic analysis of HSC, and they performed according to manufacturer instructions of CD 34 kit (Dako, USA).

The cell count and viability had been determined using Trypan blue stain 0.04 % was accomplished according to procedures laid down by Moubasher *et al*.[22] After determination of HSC count and viability, a number of 1×10^6 cells were cultured in a 25-mm tissue culture flask contains 5 ml of DMEM medium supplemented with 10% FCS and 100 U/ml penicillin, 100 µg/ml streptomycin, and 25 µg/ml amphotericin B and they were plated onto a 25-mm tissue culture dish and incubated overnight at 37°C, with 5% carbon dioxide. After 3 days, the non-adherent cells were harvested by centrifugation (10 min and 300 g). Then, the supernatant was aspirated and was cultured again in a similar condition until a monolayer was obtained. After that MNCs became ready to perform the cytotoxicity assay.

Table 1: Primers used and their sequences

Primers name	Sequence ('5-'3)
Nor (aflD)	nor1 ACCGCTACGCCGGCACTCTCGGCAC nor2 TTGGCCGCCAGCTTCGACACTCCG
Ver (aflM)	ver 1 GCCGCAGGCCGCGGAGAAAGTGGT ver 2 GGGGATATACTCCCGCGACACAGCC
AflR	aflR1 TATCTCCCCCCGGGCATCTCCCGG aflR2 CCGTCAGACAGCCACTGGACACGG

Measurement of the viable lymphocytes by MTT assay

An aliquot of 100 µl of the cell suspension was implanted in each of the 96 well microtiter plates, (104 cell/well). The plate was incubated at least for 2 h in a CO_2 incubator then serial of concentrations from each purified extract (aflatoxin) was prepared from each stock solution (µg/ml) to get (1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625, and 0.007813) µg/ml, and sterilized with 0.22 µm Millipore filter, and then 100 µl from each concentration of the previous extract was added to each well of the lymphocytes implanting plate. The plate was incubated at 37°C in a CO_2 incubator for 24 h and eventually, 50 µl of MTT stain (2 mg/ml) was added to each well. Then, it was incubated for a further 4 h. After centrifugation, the medium was removed gently by fine gauge needle. Then, MTT- formazan crystals that formed only as a result of live cells were dissolved with 100 µl of DMSO and added to all wells. The absorbance at 620 nm was recorded immediately by ELISA reader. A comparison between the results of both extract (aflatoxins) at different concentrations was statistically calculated to pick up the most effective dosages of each concentration that may cause lymphocytes killing.[23]

The Statistical Analysis System (2012) program was used to reveal the effect of different factors in study parameters. Chi-square test was used to significant compare between percentage and least significant difference (LSD) between means.[24]

RESULTS

Out of 15 isolates, 11 (73%) were positive for aflR gene and aflD gene, while 10 (67%) isolates were positive for ver aflM gene. Furthermore, amplification of nor1 gene targeted by PCR and electrophoresis by agarose gel electrophoresis revealed that out of 15 isolates of *A. flavus*, 11 (73%) isolates of them were positive for nor (aflD) gene, while 4 (27%) isolates were negative as reflected in Figure 1.

Amplification of aflM (*ver-1*) gene targeted by PCR showed that out of 15 isolates, 9 (60%) isolates of *A. flavus* were positive for aflM (ver-1) gene as represented in Figure 2. The presence of the aflM ver-1 gene, which in charge of the production of AF had shown in 10 (67%) isolates of the *A. flavus* from a total of 15 isolates, where it has been noticed that the site of the gene appeared in the sequence (600 bp) when the primer aflM ver-1 was used.

Amplification of *aflR* gene targeted by PCR and electrophoresized by agarose gel electrophoresis showed that *A. flavus* was yielded in 11 (73%) as reflected in Figure 3. The results appeared that the ability of 10 isolates out of 15 isolates to produce AF through the gene aflR which is responsible for regulating the AF biosynthetic process, especially the site 1000 of this gene

The study isolates were examined using HPLC techniques with the ultraviolet radiation detector at a wavelength of 365 nm. The results showed the ability of most isolates of *A. flavus* to produce AFB1, as it gained the value of the retention time for each of the inoculated sample extract and the sample of the standard solution B1 (7.30) min [Figure 4].

The analysis of HPLC results indicated that *A. flavus* in peanut produced the highest concentration of AFB1 at a rate of 45.03 ppm while the rate of *A. flavus* in crude oil was 0.78 ppm (least rates of AFB1) [Figure 4].

There is a variation in the level of production of AFB1 extracted from *A. falvus* which was collected from clinical and environmental sources as observed in Table 2. Furthermore, tere was high significant correlation and agreement between study result of HPLC technique and gene expression for study target genes amplified by PCR with the exception of isolate no. AFL1.

Regard in the study of the cytotoxic effect of AFB on hematopoetic stem cells; the results revealed that the inhibition rate of HSC increases seriously with the increase aflatoxins concentration. At other meaning, the viability of AFB treated cells was decreased while the toxin concentration increased as represented in Figure 5.

It has been observed that the concentration of 1 µg/mL was completely inhibited HSC when it's compared with other concentrations with a percentage of inhibition (100%). The study concentrations of 0.125, 0.25, and 0.5 µg/ml had been given different rates of inhibition reached to 3.32%, 24.4%, and 28.72%, respectively, while no inhibition effect appeared with other used concentrations. Statistically, high significant observed between the concentration used and growth inhibition rate at which $P < 0.05$. LSDs were detected for all variables.

DISCUSSION

It is well recognized that aflatoxint causes immunosuppression, mutagenesis, and teratogenic effect at which they were classified as the Group I carcinogens by IARC. The Food and Agriculture Organization indicated that there is no less than 25% of the world's food contaminated with mycotoxins.[25] In this study, molecular techniques have been used to differentiate between the complexity of the *A. flavus*, and to determine the phylogenetic analysis. The gene aflD plays a great role in the early conversion of the norsolorinic

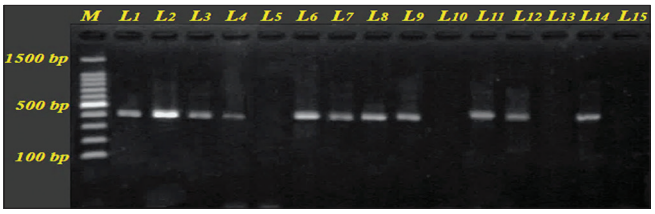

Figure 1: Polymerase chain reaction product with *nor* primer (specific for *aflD*) on 1% agarose gel electrophoresis with ethidium bromide, M: 100 bp DNA ladder. Lanes: AFL1, AFL2, AFL3, AFL4, AFL5, AFL6, AFL7, AFL8, AFL9, AFL10, AFL11, AFL12, AFL13, AFL14, and AFL15

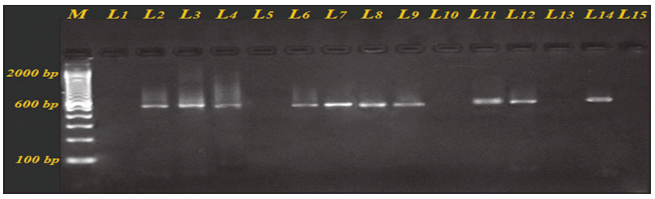

Figure 2: Polymerase chain reaction product with *ver*1 primer (*aflM* gene) on 1.5% agarose gel electrophoresis with ethidium bromide, M: 100 bp DNA ladder. Lanes: AFL1, AFL2, AFL3, AFL4, AFL5, AFL6, AFL7, AFL8, AFL9, AFL10, AFL11, AFL12, AFL13, AFL14, and AFL15

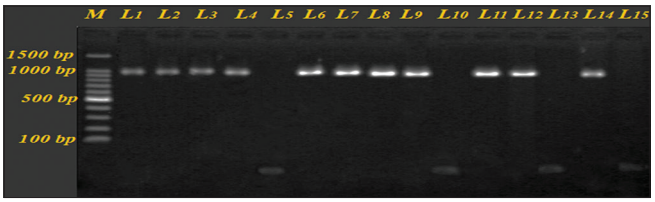

Figure 3: Polymerase chain reaction product with aflR primer on 1% agarose gel electrophoresis with ethidium bromide, M: 100 bp DNA ladder. Lanes: AFL1, AFL2, AFL3, AFL4, AFL5, AFL6, AFL7, AFL8, AFL9, AFL10, AFL11, AFL12, AFL13, AFL14, and AFL15

acid to averantin (in the middle of AF biosynthetic pathway), while other genes are involved in converting sterigmatocystin to AFB1 in the last step of the aflatoxin pathway.[26]

The absence of the genes encoding for aflatoxin production in *A. flavus* genome indicates the inability of this isolate to produce aflatoxin. In appearance one of the encoding aflatoxin production set of genes as appeared in isolate no. (AFL1) at which gene expression was occurred with flR and aflD but lacked with the aflM gene [Table 2] may stopped the production of toxins. This may be due to that the ver-1 (aflM) gene encodes an enzyme that encodes a ketoreductase that is required for the conversion of VERA to demethylsterigmatocystin in the aflatoxin biosynthetic pathway.

The presence of the aflM ver-1 gene, which in charge of the production of aflatoxins had shown in 10 isolates of the *A. flavus* from a total of 15 isolates as shown in Figure 2, where it has been noted that the site of the gene appeared in

the sequence (600 bp) when the primer aflM ver-1 was used. This result is corresponding with the findings laid down. The findings of these studies were in agreement with the results of previous studies on the chain of DNA sequence that belongs to the gene aflM (ver-1) appropriate design significant primer to diagnose fungi producing aflatoxins.[27-32]

The aflR primer pairs were selected to detect the aflatoxigenic isolates of *A. flavus* in the PCR technique as a marker because this gene has an importance in the biosynthesis of aflatoxin. Even though these specific primers are amplified, the expected bands from the isolates, great variability was usually found in their aflatoxinogenic capacity tested by the HPLC method which shows that it was not a sufficient marker for differentiation between aflatoxinogenic and some non-aflatoxinogenic isolates. The lack of aflatoxin production could also be due to simple mutations (substitution of some bases) which lead to the formation of nonfunctional products. Lack of aflatoxin production apparently can be related also to the incomplete pattern obtained in PCR as represented in Figure 3.

Table 2: Distribution the results of HPLC and gene expression by PCR according to study fungal isolates and their clinical and environmental origin

Isolate No.	Isolate origin	Co. AFB1 in HPLC (ppm)	Aflatoxin genes		
			aflR	aflD	aflM
AFL1	Fish	0.00±0.00	+	+	-
AFL2	Lung of animal	11.66±0.75	+	+	+
AFL3	Spices	12.55±0.87	+	+	+
AFL4	Rice	8.35±0.63	+	+	+
AFL5	Animal waste	0.00±0.00	-	-	-
AFL6	Corn grain	33.86±2.04	+	+	+
AFL7	Crude oil	0.78±0.01	+	+	+
AFL8	Barley grain	6.24±0.58	+	+	+
AFL9	Wheat	29.89±1.93	+	+	+
AFL10	Bovine milk	0.00±0.00	-	-	-
AFL11	Fruits	17.39±1.26	+	+	+
AFL12	Nuts	12.66±0.73	+	+	+
AFL13	Poultry lung	0.00±0.00	-	-	-
AFL14	Peanut seeds	45.03±2.59	+	+	+
AFL15	Vegetables	0.00±0.00	-	-	-
	LSD	6.593*			

HPLC: High-performance liquid chromatography,
PCR: Polymerase chain reaction. (+) means PCR amplification signal is present, (-) means PCR amplification signal is absent

HPLC has been recognized as a standard and superior method in the identification and analyzing aflatoxin with higher sensitivity and accuracy in comparison with other methods. The study results obtained from HPLC revealed that non-aflatoxigenic isolates were less detected than aflatoxigenic isolate. Furthermore, aflatoxin production is directly associated with the appearance of genes (aflM ver-1, aflD, and aflR). The analysis HPLC results indicated that *A. flavus* in peanut produced the highest concentration of AFB1 at a rate of 45.03 ppm while the rate of *A. flavus* in crude oil was 0.78 ppm (least rates of AFB1) [Figure 4].

HPLC study results were agreed completely with those obtained by molecular technique, PCR. There was a relationship between study result of PCR and HPLC technique stated that the lack of aflatoxin production can be as a result of substitution of some nitrogen bases. Also, it is showed that various physiologic conditions can be effective in aflatoxin biosynthesis. In this study, it was observed that we can refer to PCR as a screening test for initial isolation regarding high sensitivity and speed (100%). The positive samples should be more investigated by efficient technique such as HPLC.

The study result of the cytotoxic effect of AFB on hematopoetic stem cells revealed that the presence of higher concentrations of AF can lead to a significant reduction in the number of lymphocytes. At other meaning, the viability of

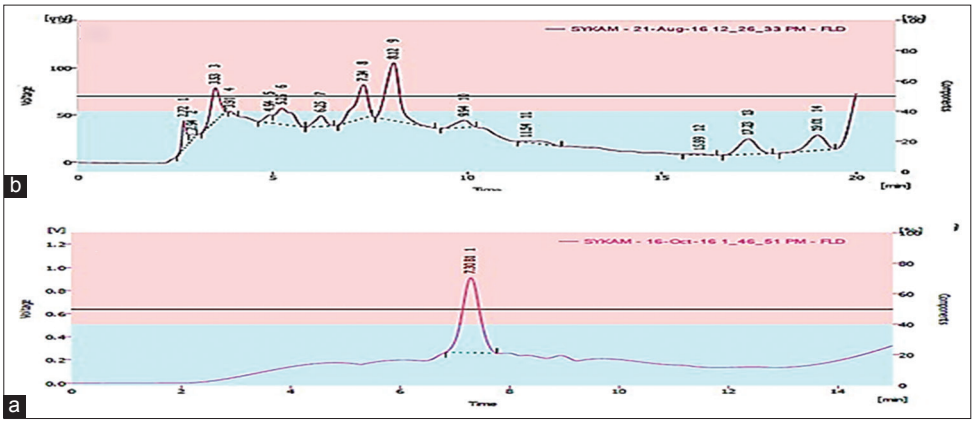

Figure 4: Detection of aflatoxin B1 extracted from *Aspergillus flavus* by high-performance liquid chromatography, standard chromatogram of aflatoxin (total run time: 14 min and retention time for AFB1: 7.30 min) (a) chromatogram of various aflatoxin in *Aspergillus flavus* (b)

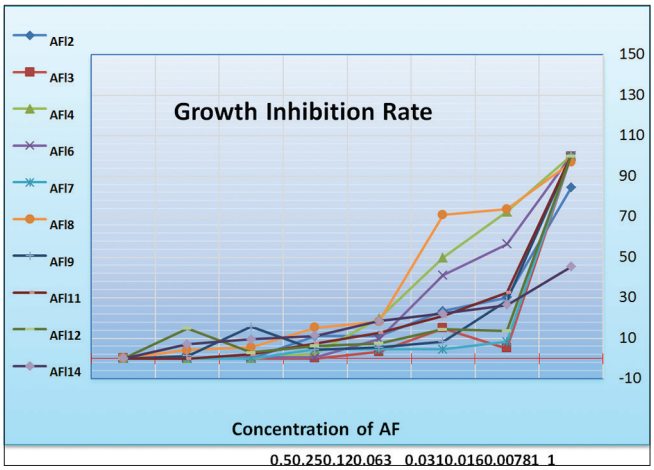

Figure 5: Inhibition rate of mononuclear cells exposed to AF which measured through cytotoxicity assay

AFB treated cells was decreased while the toxin concentration increased. In addition to that, the decline of the lymphocytes mitochondrial activity may possibly lead to weakness of mitochondria and eventually decrease in AFB treated cells viability. This result was in agreement. On the other hand, this study result was not agree with who documented that an increase in the viability of AFB1 treated cells was observed in cells exposed to the highest concentration (20 µg/ml).

The study suggested that PCR technique has proved high efficiency in discrimination between aflatoxigenic and non-aflatoxigenic strains of *A. falvus* on the basis of amplification of the target DNA fragments that are involved in aflatoxin production**.** Further, aflatoxin production is directly associated with the appearance and expression of genes (aflM ver-1, aflD, and aflR). Further, HPLC is a standard and superior technique in identifying and analyzing aflatoxin with high sensitivity and accuracy. Furthermore, aflatoxins had high toxic effects on human lymphocyte precursors, (HSC) and whenever the aflatoxin concentration was high, the rate of stem cell growth inhibition increases and viability of AFB-treated cells decreased.

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