GENOTOXIC EFFECT OF PARACETAMOL CONTAINING TABLETS IN CULTURED HUMAN LYMPHOCYTES

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ABSTRACT

Lymphocytes are the key cells in body's defense system against foreign pathogens. Any agent which causes reduction in their number and viability can seriously impair the body's defense system. Paracetamol or acetaminophen is a widely used over the counter drug for many common ailments. Acute or overdoses of commonly used tablets containing paracetamol causes undesired effects in human body often leading to death. Although there are extensive reports about the toxicity of paracetamol in association with liver failure and kidney functions, little is known about its effect in cultured lymphocytes and the effect on human DNA. This study focuses on the genotoxic effects of commonly used tablets containing Paracetamol in cultured human lymphocytes using routine cytotoxic assays viability and counting of cultured lymphocytes.

KEY WORDS: Paracetamol, Genotoxicity, Lymphocyte culture, Viability, DNA laddering, MTT assay.

INTRODUCTION

Paracetamol or acetaminophen is an analgesic and antipyretic drug commonly used for the relief of fever, headaches, other minor aches, and pains and is also used in the management of more severe pain in combination with Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) or opioid analgesics. [1] Paracetamol is a major ingredient in numerous cold and flu remedies. While generally safe for human use at recommended doses, acute or overdoses of Paracetamol can cause potentially fatal liver damage and in rare individuals, a normal dose can do the same. Paracetamol toxicity is the foremost cause of acute liver failure in the Western world [2,3,4,5].

The genotoxic effect of paracetamol was recorded in many *in vitro* test systems such as congenital malformation in embryo[6], inhibition of DNA synthesis and increase of sister chromatid exchange in hamster cells[7]. After the intake of 3grams by i.v. injections paracetamol changed the chromosome structure in human lymphocytes [8]. On the other hand, paracetamol did not cause any type of mutation in Salmonella[9]. There was no chromosomal aberration in lymphocytes of human volunteers who had given repeated i.v. injection (1gram) every 6 hours neither for a week, or in persons attempting suicide by an acute ingestion of very high doses[10].

This study investigates the Genotoxic effects of paracetamol in cultured human lymphocytes using various cytotoxicity and apoptosis assays.

MATERIALS AND METHODS

Preparation of Tissue Culture Media

RPMI 1640 (HIMEDIA - AT 007) medium was prepared in sterile distilled water and the required amount of sodium bi carbonate was added. The pH was adjusted to 7.4 using 1N HCl. The media was sterilized using a sterile membrane filter with a porosity of 0.22 micron or less under negative pressure.

Isolation of Lymphocytes from Whole Blood

From each donor 2-3 ml blood was collected into the blood collection tubes which were pre washed with 100 mM EDTA. The blood collection tubes were closed tightly and mixed by inversion with 2 ml of 1X PBS. The final blood dilution volume was made up to 4ml. The PBS diluted blood was laid over ficoll solution taken in 15 ml centrifuge tubes. While layering the blood over ficoll (Himedia) density solution, the centrifuge tubes were held at 45^0 angle and then the tubes were centrifuged at maximum speed for 25 minutes. After centrifugation the top plasma layer was removed and the mononuclear cell layer in the interphase were scooped up and transferred into fresh centrifuge tubes. The mononuclear cells were washed 2 to 3 times with 1X PBS by centrifuging each time at 1400 RPM for 10 minutes. The pellet was suspended in 1 ml of PBS.

Measurement of Cell Viability

Under sterile conditions 100-200 µl of cell suspension was taken to which an equal volume of Trypan blue was added and mixed gently by pipetting. The haemocytometer the cover slip was moistened with water and was kept over the counting chamber. The chamber was filled with 10-20 µl of the cell suspension then viewed under a light microscope at 40X magnification. The number of cells were counted while viable cells appear colorless and non viable cells were stained blue (Fig: 1).The percentage of viability was calculated.

Culturing of Lymphocytes

The prepared lymphocytes were mixed with 1X PBS and made up to a suitable volume (1ml). The prepared lymphocytes were added to disposable culture vessels containing 4 ml of RPMI 1640 medium enriched with 10% fetal calf serum (Invitrogen Eugene, Oregon, USA). The flasks were shaken gently and kept inside the CO_2 incubator at 37^0C with 5% carbon dioxide.

Treatment of Cells with Paracetamol

Paracetamol injections and tablets were purchased from local drug store. The tablets were powdered and dissolved in sterile distilled water to make a 75mg/ml concentration. The 72 hours cultured lymphocytes were treated with 5 different concentrations of paracetamol, (0.05, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml).

Measurement of Cell Viability after Paracetamol Treatment

Under sterile conditions 100-200 µl cell suspension from each concentration were mixed with an equal

volume of trypan blue. 10-20 µl of the cell suspension was filled into the counting chamber. It was viewed under a light microscope at 40X magnification and counting was done as mentioned previously.

DNA Fragmentation Assay

Qualitative Analysis of DNA Fragmentation by Agarose Gel Electrophoresis

0.5 ml of cell suspension were dispensed into 1.5 ml eppendorff tubes and labeled B (bottom). The cells were centrifuged at 2000 rpm at 4°C for 10 min. Supernatant was transferred carefully in to new tubes labeled S (supernatant). To the pellet, 0.5 ml of Tritron Tris EDTA (TTE) (Himedia) solution was added and vortexed vigorously, then centrifuged at 14,000 rpm for 10 min at 4°C. Supernatant was transferred carefully in to new tubes labeled T (top). 0.5 ml of TTE solution was added to the pellet and 0.1 ml of ice-cold 5M NaCl was added to the 0.5 ml volume present in tubes labeled B, S and T and vortex vigorously. 0.7 ml of ice-cold isopropanol was added to each tube and vortex vigorously. All the tubes were kept overnight at -20°C for precipitation of DNA. After precipitation, DNA was recovered by pelleting at 14,000 rpm at 4°Cfor 10 min. The supernatant was discarded by rapidly inverting tubes and carefully removed any drops or fluid remaining adhered to the wall of the tube with a filter paper. The pellet was rinsed by adding 0.5-0.7 ml of ice-cold 70% ethanol to each tube and centrifuged at 14,000 RPM for 10 min at 4°C. The supernatant was discarded. The tubes were air dried. The pellet was dissolved in 50 µl of TE buffer. 10-20 µl of the samples were mixed with equal volume of loading buffer and loaded on to a 1% agarose gel containing ethidium bromide. Appropriate DNA molecular weight markers are also included. The electrophoresis was run in standard Tris Boric acid EDTA (TBE) buffer after setting the voltage to the desired level.

MTT Assay for Cellular Toxicity

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) assay is used to study the cytotoxicity of a compound. Metabolically viable cells convert the MTT into colored insoluble crystal formazan. These crystals can be dissolved in Dimethyl sulphoxide (DMSO) and can be measured spectrophotometrically.

MTT stock solution was prepared by diluting 5mg/ml MTT in RPMI-1640 without phenol red. MTT working solution was prepared by 1:10 dilution of the 5mg/ml MTT. This solution is filtered through a 0.2 µ filter and stored at 2-8°C. The cells were harvested by centrifugation at 5000 RPM for 10 min. The cells were seeded into 96 well flat bottom tissue culture plates containing 200 µl of complete medium and incubated at 37^0C. The percentage of cell viability was found out and a density of 0.5 -1.0 X 10^5 cells/ml were selected. After 24 hours of incubation various concentrations of paracetamol (0.05, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml) was added into the wells. Incubation was continued for 48 hours. Four hours before termination, 20 µl of MTT solution (5 mg/ml) was added to each well. The cultures from each well were then transferred to Eppendorff tubes, and then centrifuged at 1000 rpm for 10 min. The supernatant was removed and 100µl of DMSO was added to each tube. The tubes were incubated at room temperature for 15 minutes. The optical density was measured at 570 nm.

The percentage of dead cells was determined using the formula:-

Percentage of dead cells = 1- O.D of drug treated cells / O.D of control x 100.

RESULTS

Cell Counting and Viability

Cell counting and cell viability were calculated for cells which were treated with increasing concentration of paracetamol and the results are interpreted in Table: 1. Relation between increasing paracetamol concentration and cell viability is expressed in fig: 2. The effect of paracetamol in cell density is shown in figure: 3.

DNA Laddering

The cells treated with maximum concentration of paracetamol gave the maximum number of breaks in DNA characterized by ladders in the agarose gel. Lane 1 was loaded with marker DNA while lanes 2-6 were loaded with cells treated with different concentration of paracetamol ranging from 0.05, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml. Lane 7 was loaded with the control cell suspension which was not treated with paracetamol. There were no breaks in DNA from the cells which were treated with minimum concentration of 0.05 mg/ml. so intact genomic DNA bands were observed similar to those in the control (lane 7). 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml of paracetamol concentration showed laddering pattern in lanes 2-6. The results of the laddering pattern of DNA and the number of fragments formed in each concentrations of paracetamol are given in fig: 4.

MTT Assay

The cells which were exposed to higher concentrations of paracetamol showed higher percentages of cytotoxicity. Percentage of cytotoxicity increased as the concentration of paracetamol was higher. The percentage of cytotoxicity of the cells exposed to different concentrations of paracetamol are found out using the formula,

Percentage of dead cells =

1 - [O.D of Test / O.D of Control] x 100.

MTT assay for determining the cytotoxicity of paracetamol was performed and the results of the assay showed the percentage of cytotoxicity increased with the increasing paracetamol concentrations. All the cells were dead at the maximum concentration of (2.5mg/ml) paracetamol and the percentage of cell viability at that concentration was 0 and the percentage of cytotoxicity was 100 (fig: 5).

DISCUSSION

In this study the effect of paracetamol on cell viability was found out and its was concluded that as the concentration of paracetamol increases, the cell viability decreases (Table 1) and at concentration of 2.5mg/ml of paracetamol the cell viability was 0, which indicates the concentration of 2.5 mg/ml of paracetamol as the lethal dosage for the cultured human lymphocytes.

This result supports the findings that, acetaminophen has a variety of effects upon cells or tissues both *in vitro* and *in vivo* and cell proliferation[11,12,13,14]. Paracetamol at high concentration had inhibitory effect on cell division. The

inhibition could be the results of its direct interaction with DNA leading to broken chromosomes (92.3%) at 5mg/ml concentration in addition to complete cell death and lysis[15].

DNA from the cells treated with paracetamol were isolated and analyzed by electrophoresis in agarose gel for fragmentation, it was found that the cells which were exposed to the highest level of paracetamol concentration had DNA which showed maximum number of fragments, which indicates the extend of DNA damage and initiation of cell death. The control cell sample which was given a placebo dosage did not produce any significant DNA fragmentations (fig: 4).

The genotoxicity effect of paracetamol was recorded in many *in vitro* test systems such as congenital malformation in embryo[6], inhibition of DNA synthesis and increase of sister chromatid exchange in hamster cells[7]. *In vivo* paracetamol changed the chromosome structure in lymphocytes of human volunteers after intake of 3grams by i.v. injections[8]. *In vitro* acetaminophen inhibits DNA synthesis[16], down regulates c-myc and bcl-2 mRNA[11,14], and increases chromosomal breaks[13].

The genotoxicity of paracetamol, including covalent binding to DNA, induction of DNA single-strand breaks (SSBs), and inhibition of replicative and repair of DNA, has been investigated in rodents *in vivo*. The alkaline elution profile from damaged liver nuclei was markedly biphasic, suggesting that breaks were induced in DNA from a subpopulation of liver cells. The non-hepatotoxic paracetamol regioisomer, acetyl-m-aminophenol which binds covalently to proteins, did not cause DNA SSBs[17].

The effect of paracetamol on genetic material was investigated by measuring the mitotic index (MI) and chromosome behavior during cell division in *Allium cepa* root tip cells and human blood cells. Paracetamol significantly increase the rate of cell division at low concentrations (0.05-0.005) at almost time (4, 12, 72 hours). Paracetamol also has inhibitory effect on MI at high concentrations (5-0.5). This effect is time depended. The genotoxic effect of paracetamol which is represented by high percentage of chromosome abnormality in treated cells is time and concentration dependent. The incidence of mutation is significantly higher in cells treated with low concentration (0.05, 0.005) and for long time (72 hours)[15].

MTT assay is a laboratory test and a standard colorimetric assay for measuring the toxicity of a compound on the cellular metabolism. It measures the activity of enzymes that reduce MTT to formazan, giving a purple color. This mostly happens in mitochondria and as such it is in large a measure of mitochondrial activity. It can also be used to determine cytotoxicity of potential medicinal agents and other toxic materials[18].

Genotoxic effects are hardly assessable in an exposed population but are generally considered to be serious due to their unpredictable effects on subsequent generations and to the link between genotoxicity and cancer. Lack of knowledge about a genotoxic and or carcinogenic potential has to be stated for numerous compounds which are often in pharmaceutical use known for a long time. A thorough testing programme should be done for new compounds and is essential for such compounds that are not completely unsuspicious with respect to being reactive with macromolecules or

that have the potential to generate reactive metabolites in the body. Paracetamol, anthraquinone-containing preparations, and griseofulvin are examples for pharmaceuticals that have been in use for a long time but for which genotoxicity testing revealed a possible deleterious potential only recently. The Federal Health Office/Federal Institute for Drugs and Medical Devices therefore imposed new studies upon companies marketing these compounds in the last years. These studies in part led to a more thorough description of possible adverse effects or even restrictions for use

SUMMARY AND CONCLUSION

In this study, paracetamol tablets and injections were purchased from local drug stores and they were analyzed for their genotoxic and effect on cell growth. Human lymphocytes from healthy volunteers were isolated and cultured in RPMI 1640 medium enriched with 10% fetal calf serum. These cultures were treated with different concentrations of paracetamol.

After incubation the cells were counted for cell viability and it was found that increasing paracetamol concentrations decreased cell viability. 2.5 mg/ml concentration of paracetamol resulted in death of all the cells.

DNA from these treated cells was isolated and analyzed for fragmentation and it was found that the maximum numbers of fragments were in the sample treated with the highest concentration of paracetamol. MTT assay for determining the cytotoxicity of a compound was performed and it was found that the cytotoxicity of cells increased with increasing paracetamol concentrations. From the above results it can be concluded that the compound paracetamol we consume for our minor ailments can affect the cell division and viability of the cells and it is genotoxic when consumed frequently at high concentrations.

Paracetamol exhibits a genotoxic potential *in vitro* and *in vivo* probably via indirect cytotoxicity or enzyme inhibition-mediated effects. Further studies will have to clarify whether a threshold could be established and whether effects do not occur at therapeutic dose levels.

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Table 1: Number of viable cells and viability percentage after Paracetamol treatment

Sl. No:	Paracetamol (mg/ml)	Cell volume (ml)	Total number of cells/20µl	Total number of viable cells/20µl	Percentage of viability
1	0.05	4.0	230	200	86.95
2	0.50	4.0	210	150	71.42
3	1.00	4.0	200	120	60.00
4	1.50	4.0	175	90	51.42
5	2.00	4.0	150	25	16.42
6	2.50	4.0	100	0	00.00
7	0.00	4.0	250	225	90.00

Table 1 sows the total number of cells and the total number of viable cells in the culture after paracertamol treatment, along with the percentage of viability.

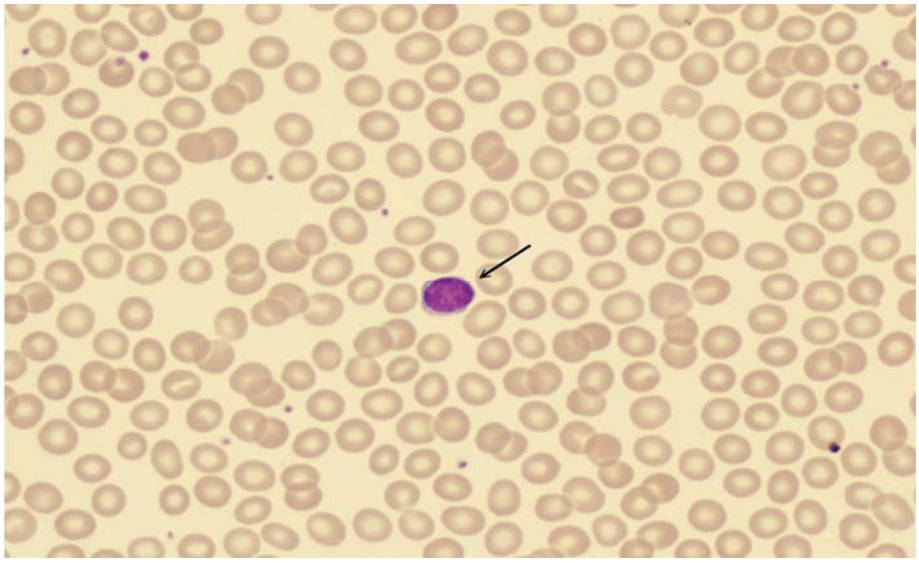


Fig: 1. A Non Viable Lymphocyte stained with Trypan blue

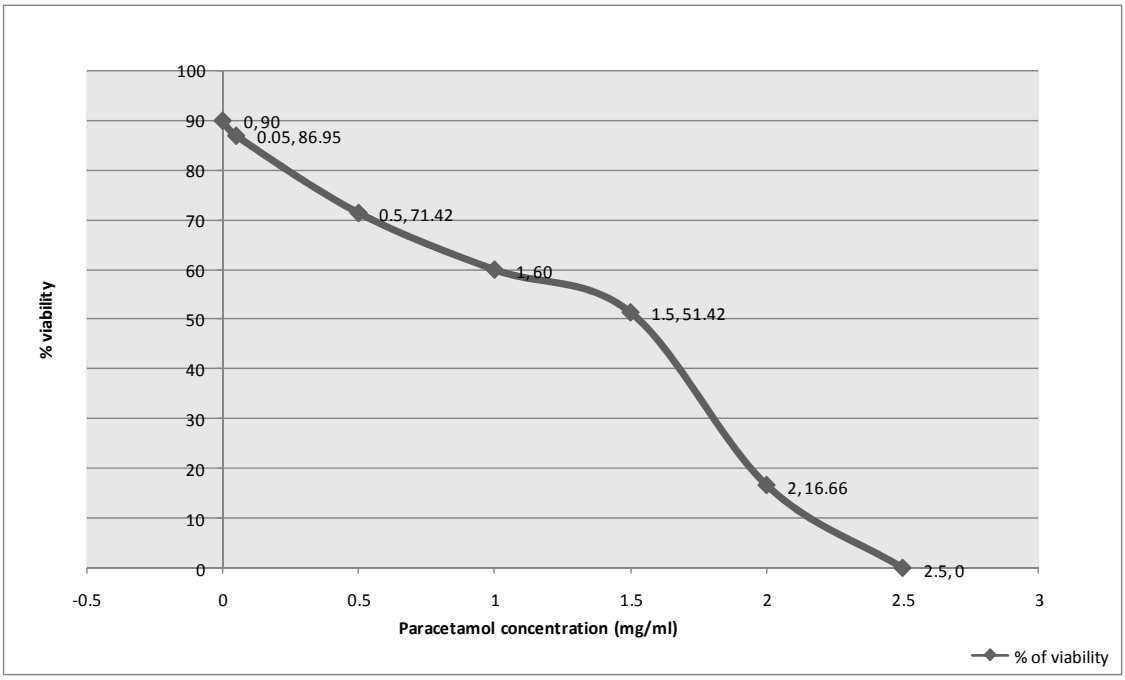


Fig: 2. Cell Viability and its Relation to Increasing Paracetamol Concentrations

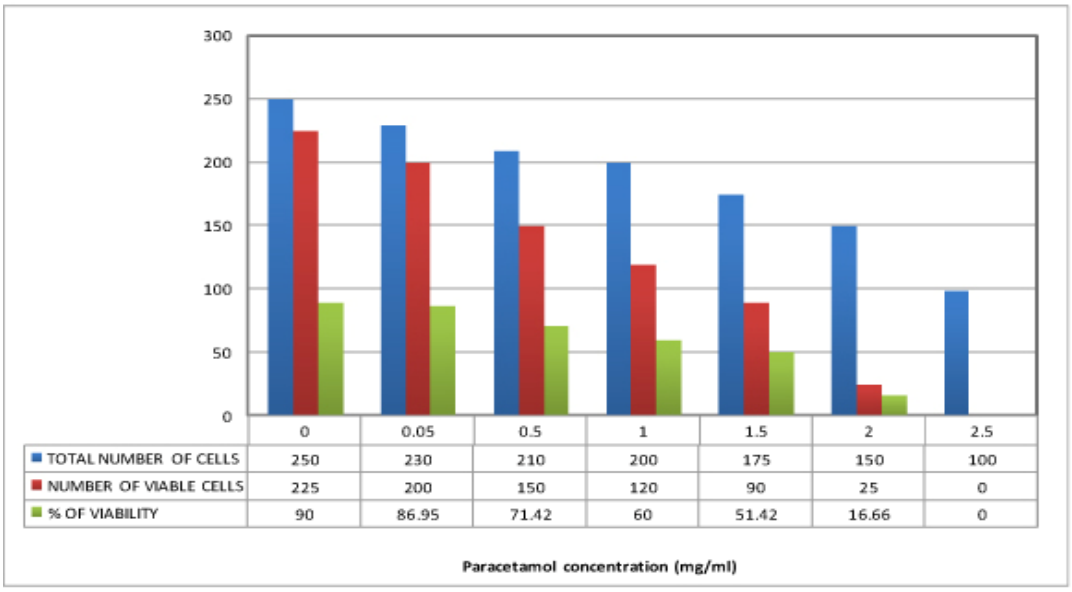
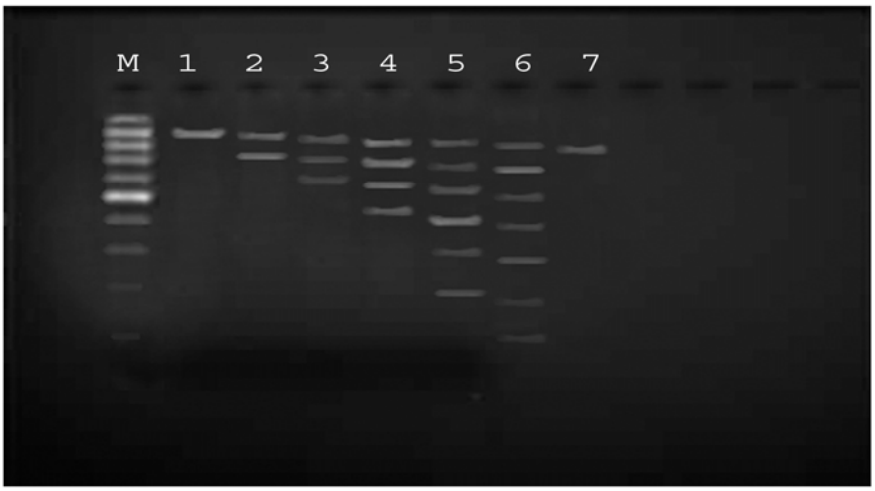


Fig: 3. Effect of Paracetamol concentration on cell density and percentage of viability



M-Marker DNA
1-Cells treated with 0.05 mg/ml Paracetamol
2-Cells treated with 0.50 mg/ml Paracetamol
3-Cells treated with 1.00 mg/ml Paracetamol
4-Cells treated with 1.50 mg/ml Paracetamol
5-Cells treated with 2.00 mg/ml Paracetamol
6-Cells treated with 2.50 mg/ml Paracetamol
7-Control(0.0 mg/ml Paracetamol)

Fig: 4. DNA Laddering Assay

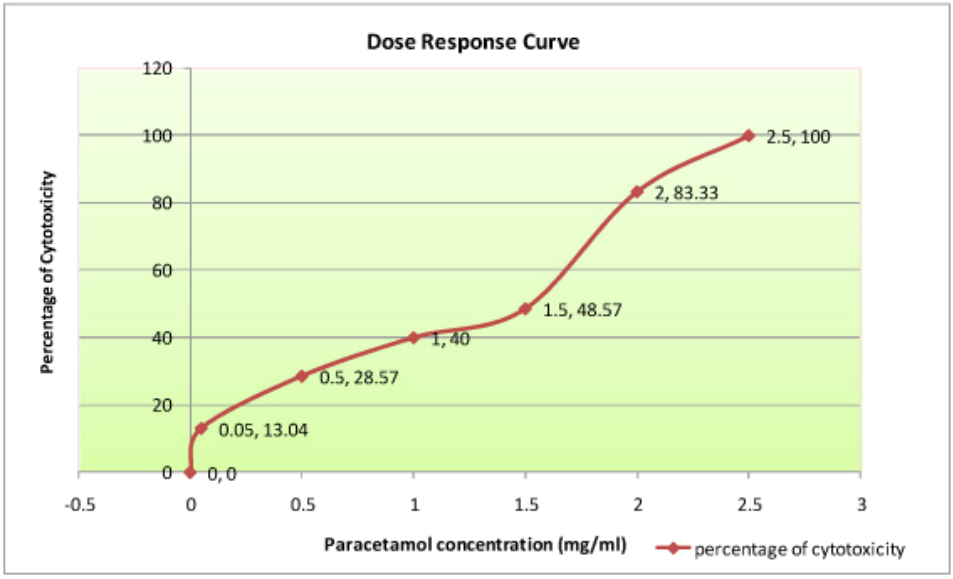


Fig: 5. MTT Assay for Cellular Cytotoxicity