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Abstract

Cell culture has seen a growing number of uses in recent times for different research purposes. The technique dates back to 1907 after which a number of modifications were made to refine the technique as is used today. It is mostly a precursor to understand the viability and apoptosis pathways in a cell and testing the apoptosis-inducing properties of different substances.

There are a number of different assays that are used for testing the viability of cells. The Alamar blue assay works on the principle of the uptake of the dye by cells undergoing cell death. The haematoxylin and eosin stain are two contrasting dyes used for staining different organelles in the cell. The caspase activity assay and Bradford assay measure the activity of caspases and protein concentrations in solutions of cells undergoing apoptosis. The DNA fragmentation assay is used for the visualization of fragments of DNA of cells undergoing cell death pathways. The lactate dehydrogenase assay is used for the measurement of the enzyme lactate dehydrogenase released by necrotic cells.

For all assays, two cytotoxic agents, etoposide and anisomycin were used to induce cell death and this was analyzed either qualitatively or quantitatively by the various assays mentioned above.

1. Introduction

1.1 Introduction to cell culture

Cell culture has become an indispensable tool for the study of life sciences. It basically involves isolation of cells from their natural environment and their placement in an artificial environment which will support their growth. The growth medium will either be a liquid or semisolid medium which will contain all necessary growth nutrients based on the cell type. Cell culture dates back to 1907 when Ross Harrison made the first attempt to culture animal cells. His technique underwent a lot of modifications in the 1940s and 1950s when it became an integral part of life science research. In the 1960s and 1970s, the technique of cell culture became commercialized and it began contributing to important discoveries in the field of biology (Ryan, 2003).

1.2 Alamar blue assay

The Alamar blue assay is performed for the quantification of viable cells *in vitro*. Alamar blue is a stable, non-toxic, water-soluble dye which is taken up by viable cells facilitating their monitoring and quantification. The oxidized form of the dye is added to the cytosol which gets reduced by mitochondrial enzymes and cytochromes. This reaction gives a fluorescent pink color to the cells as opposed to indigo blue of the culture medium which can be measured by fluorometry or colorimetry (Al-Nasiry et al., 2007).

1.3 Haematoxylin and Eosin (H & E) staining

H & E staining is often used for pathological diagnostic work and is mostly used for staining paraffin sections. It is a robust universal stain and is the primary contrast method in the field of medical diagnostics. It has the ability to distinguish between different features of the cytoplasm, nucleus and the extracellular matrix. Haematoxylin is used as the primary stain and Eosin is used as the contrasting counterstain. The timing used for both the stains determines the level of contrast that will be visible under the microscope and the specificity of the organelles that will be stained (Ankle and Joshi, 2011).

1.4 Caspase activity assay and Bradford assay

Caspase activity assay is an apoptosis assay or cell death assay which measures one of the important markers for cell death – caspases. Caspase activation in a cell is usually a trigger for alterations in cell permeability, DNA damage, and collapse of the cytoskeleton. A fluorescently labelled substrate is usually introduced into the cells where it is cleaved by active caspases (if present). The fluorescent labelled peptides are retained inside the cell while the others are washed off. By means of fluorimetry, the caspase presence and concentration can be determined (Telford et al., 2002). Bradford assay is used for the quantification of proteins by means of absorbance or flourimetry. The Coomassie Brilliant Blue G-250 dye is used which undergoes deprotonation upon binding to a protein molecule and results in an increase in absorbance at 595 nm (Pihlasalo, 2011).

1.5 DNA fragmentation assay

One of the hallmark features of apoptosis is the breakdown of DNA into small 200 bp fragments. Hence, the extraction of the nuclear DNA from a cell and demonstration of its fragmentation is characteristic of apoptosis in that cell. The standard technique used involves the lysis of cells in a hypotonic environment and precipitation of unfragmented DNA by using polyethylene glycol (PEG). If there is any fragmented DNA, it remains in the supernatant and can be demonstrated by agarose gel electrophoresis of a small quantity of the supernatant (Ioannou and Chen, 1996).

1.6 Lactate Dehydrogenase (LDH) cytotoxicity assay

This assay is used for the detection of necrosis which is a type of programmed cell death characterized by rupture of organelles, breakdown of plasma membrane and release of cell contents. During this process, one of the cytoplasmic enzymes that is released is lactate dehydrogenase which can be detected as a marker of necrosis. The assay uses lactate which is oxidized to pyruvate by LDH, in the process releasing NADH. This NADH converts a tetrazolium salt into a colored formazan product which is spectroscopically quantified by colorimetry (Chan et al., 2013).

2 Methods

2.1 Alamar blue assay

The procedure for the Alamar blue assay can be found on page 6 of the *in vitro* cytotoxicity manual.

2.2 Haematoxylin and Eosin staining

The procedure for Haematoxylin and Eosin staining can be found on page 10 of the *in vitro* cytotoxicity manual.

2.3 Caspase activity assay and Bradford assay

The procedure for the Caspase activity assay and Bradford assay can be found on page 12 of the *in vitro* cytotoxicity manual.

2.4 DNA fragmentation assay

The procedure for the DNA fragmentation assay can be found on page 15 of the *in vitro* cytotoxicity manual.

2.5 LDH cytotoxicity assay

The procedure for the LDH cytotoxicity assay can be found on page 17 of the *in vitro* cytotoxicity manual.

3 Results

3.1 Alamar blue assay

The Alamar blue assay was performed using two cytotoxic agents etoposide and anisomycin and these were tested in Jurkat cells. After adding resazurin, absorbance was measured at 570 nm. The data for each of the cytotoxic agents is given below.

3.1.1 Etoposide

The data for all groups had equal n numbers, passed homogeneity of variance and Shapiro Wilks test of Normality $p>0.05$. This showed that the data was according to a parametric t-test. So, there were more than two groups - control and different dosages of the drug etoposide. One-way ANOVA was used for statistical analysis of the data.

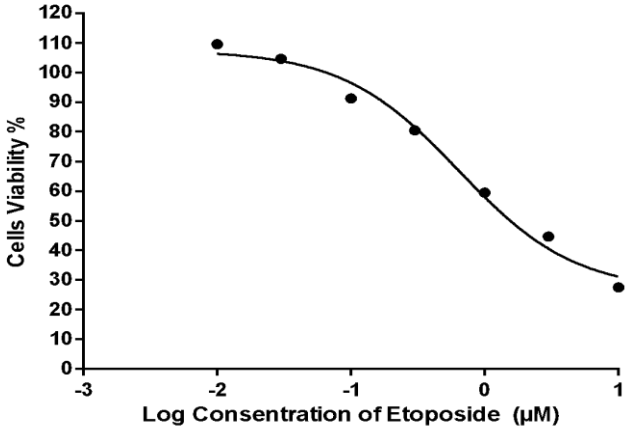


Figure 1: Etoposide dose response – This figure represents a line graph plotting the % viability of cells against the log concentrations of etoposide. As per the graph, the Medina Lethal Dose (LD_{50}) of etoposide at 50% cell viability was found to be 0.6430 µM.

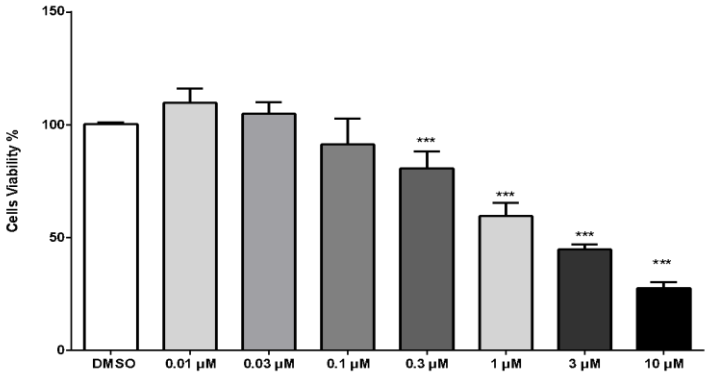


Figure 2: Bar chart showing etoposide dose response – This bar chart shows the data expressed as Mean ± SD of 0.01, 0.03, 0.1, 0.3, 1, 3 and 10 µM of etoposide concentrations, and 0.1 % DMSO acting as control. A one-way ANOVA test showed that there was a significant difference between the groups $F_{(7, 16)} = 73.096$, $p<0.001$***. This was confirmed by SNK test post hoc display which proved that there was a significant deference between 0.3, 1, 3 and10 µM of etoposide and 0.1% DMSO, and this significance was found to have a p value of **p**<0.001*** by applying Dunnett post hoc test.

	Mean %	**SD %**	**CV %**
0.1% DMSO	100.3355	0.6966	0.6943
0.01 uM of Etoposide	109.6813	6.324	5.7658

0.03 uM of Etoposide	104.7267	5.2059	4.9709
0.1 uM of Etoposide	91.349	11.3857	12.464
0.3 uM of Etoposide	80.578	7.5309	9.3461
1 uM of Etoposide	59.5753	5.7579	9.6649
3 uM of Etoposide	44.758	2.2365	4.9969
10 uM of Etoposide	27.596	2.7057	9.8047

Table 1: Table showing etoposide dose response – This table shows data expressed as Mean ± SD & CV % for control DMSO 0.1%, and 0.01, 0.03, 0.1, 0.3, 1, 3 and 10 µM concentrations of etoposide. There was a significant difference between the groups $F_{(7, 16)} = 73.096$, $p<0.001$***, SNK test post hoc display showed that there was a significant difference between 0.3, 1, 3 and 10 µM of etoposide and 0.1% DMSO, and this significance was found by a p value of $\boldsymbol{p}<0.001$*** by applying Dunnett post hoc test.

3.1.2 Anisomycin

The data for all groups had equal n numbers, passed homogeneity of variance and Shapiro Wilks test of Normality $p>0.05$. This showed that the data was according to a parametric t-test. So, there were more than two groups - control and different dosages of the drug anisomycin. One-way ANOVA was used for statistical analysis of the data.

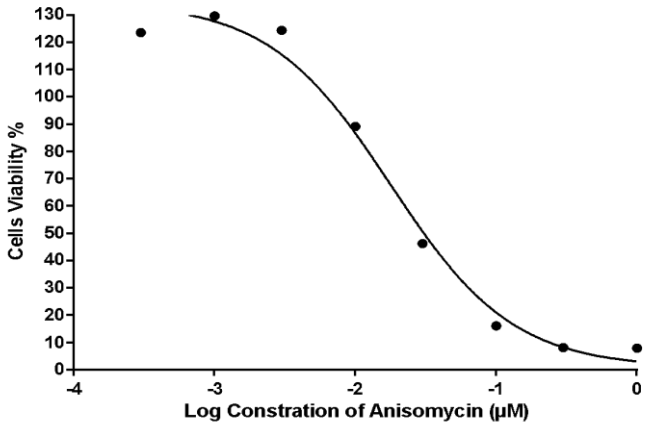


Figure 3: Anisomycin dose response curve – This figure represents a line graph showing the % viability of cells plotted against the different concentrations of anisomycin. The amount of Medina Lethal Dose (LD_{50}) of anisomycin at 50 % cell viability was found to be 0.01797 µM.

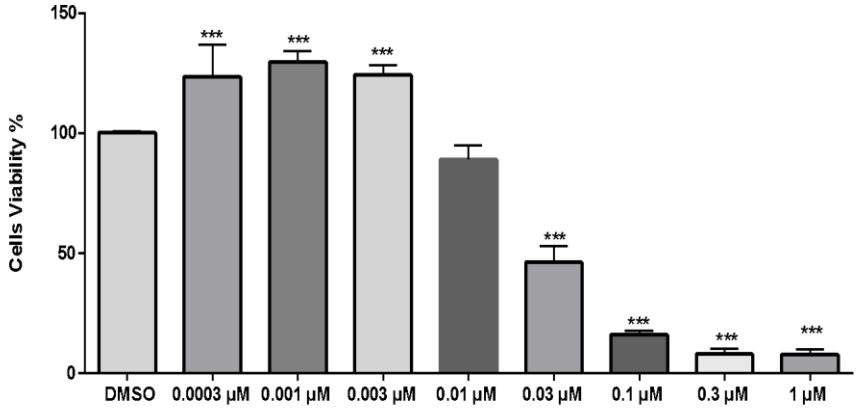


Figure 4: Bar chart showing anisomycin dose response – This bar chart shows data expressed as Mean ± S of (0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 and 1 µM of anisomycin and 0.1% DMSO acting as control. The test One-way ANOVA proves that there was a significant difference between the groups $F_{(8, 18)} = 243.543$, $p<0.001$*** and Tuerky post hoc test showed that there was difference between 0.0003, 0.001, 0.003, 0.03, 0.1, 0.3 and 1 µM of anisomycin and 0.1% DMSO, and this significance was found at a p value of $p<0.001$***. This was also confirmed by Dunnett post hoc analysis.

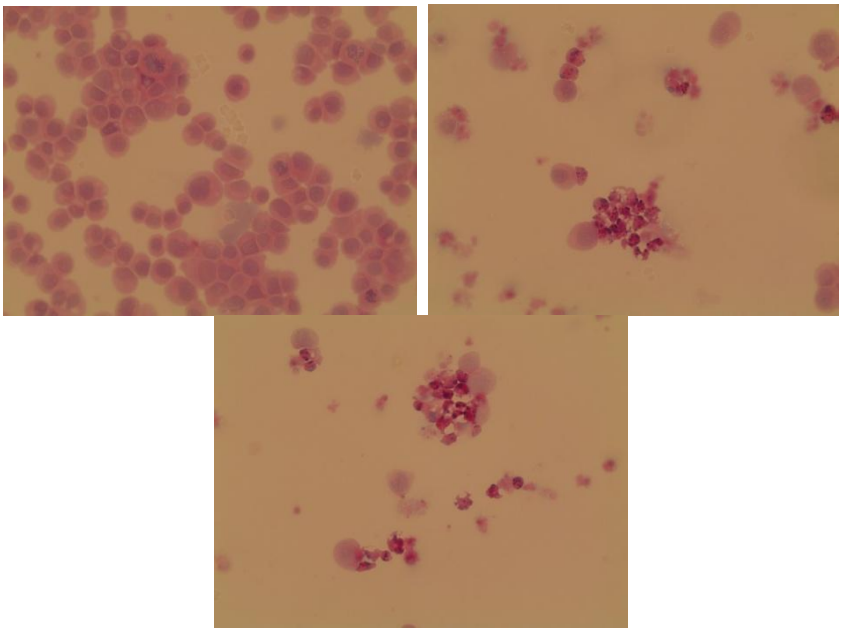
	Mean %	**SD %**	**CV %**
0.1% DMSO	100.3355	0.6966	0.6943
0.0003 uM of Anisomycin	123.6397	13.269	10.732
0.001 uM of Anisomycin	129.7523	4.4728	3.4472
0.003 uM of Anisomycin	124.4817	4.0937	3.2886
0.01 uM of Anisomycin	89.2433	5.7454	6.4379
0.03 uM of Anisomycin	46.3573	6.6103	14.2595
0.1 uM of Anisomycin	16.162	1.7518	10.839
0.3 uM of Anisomycin	8.209	2.2013	26.8157
1 uM of Anisomycin	7.9833	2.0384	25.5333

Table 2: Table depicting anisomycin dose response – This table represents data as Mean ± SD & CV % of 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 and 1 µM of anisomycin and 0.1% DMSO acting as control. The test One-way ANOVA proves that there was a significant difference between the groups $F_{(8, 18)} = 243.543$, $p<0.001$*** and Tuerky post hoc test proves that there was difference between 0.0003, 0.001, 0.003, 0.03, 0.1, 0.3 and 1 µM of anisomycin and 0.1% DMSO and this significance was found at a p value of $p<0.001$***. This was also confirmed by Dunnett post hoc analysis.

3.2 Haematoxylin and Eosin staining

The haematoxylin and eosin stains were used for staining of Jurkat cells, and as seen in figure 5, nuclei were stained blue by haematoxylin and all other organelles were stained pink by

eosin. Cells treated with either DMSO, etoposide and anisomycin were used in this assay. The stain is picked up more robustly by cells treated with etoposide and anisomycin as compared to DMSO due to the cytotoxic nature of these agents.



A. (B) (C)

Figure 5: Haematoxylin and Eosin staining of Jurkat cells – (A) Cells treated with 0.1 % DMSO (B) Cells treated with 10 µM etoposide (C) Cells treated with 1 µM anisomycin. All images are zoomed to 40 x resolution.

3.3 Caspase activity assay and Bradford assay

The data for all groups had equal n numbers, passed homogeneity of variance and Shapiro Wilks test of Normality $p>0.05$. This showed that the data was in accordance with a parametric t-test. So, there were more than two groups - control and different dosage of each drug etoposide and anisomycin. A One-way ANOVA test was used for statistical analysis of the data.

A Krustal Wallis was used to determine the significant difference between the groups control, etoposide and anisomycin, so that all sets of data could be comparable. K = 6.615, $p<0.05$*.

A Mann Whitney U test was used as a post hoc to test for significance between the groups. There was no significant difference between the groups control and etoposide U = 2,000, p = 0.083, but there was a significant difference between the groups control and anisomycin U = 000, $p<0.05$*.

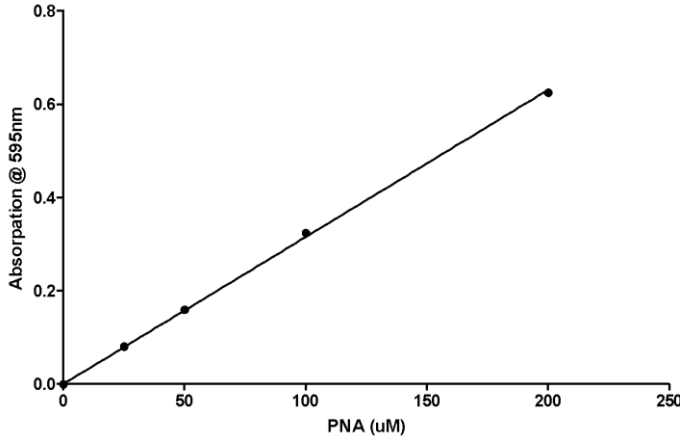


Figure 6: This figure represents the caspase activity assay with the pNA calibration plotted against the optical density with 1/slope = 316.927. The value of pNA (uM) which is released in each of the treated groups 10 µM etoposide, 1µM anisomycin, and 0.1% DMSO (control) as compared to untreated groups was found to have a significant difference. This was confirmed by measuring the average pNA (uM) for control group and treated – untreated groups. The average pNA (uM) in 0.1 % DMSO = 115.837 (uM) whereas the average pNA (uM) in 10 µM etoposide = 161.07 (uM). The average pNA (uM) in 1µM anisomycin = 176.053 (uM). The average pNA (uM) in 0.1 % DMSO was lowest as compared with the average pNA (uM) in each treated – untreated groups. As the value of pNA (uM) released increased, the absorption at 450 nm was also found to increase.

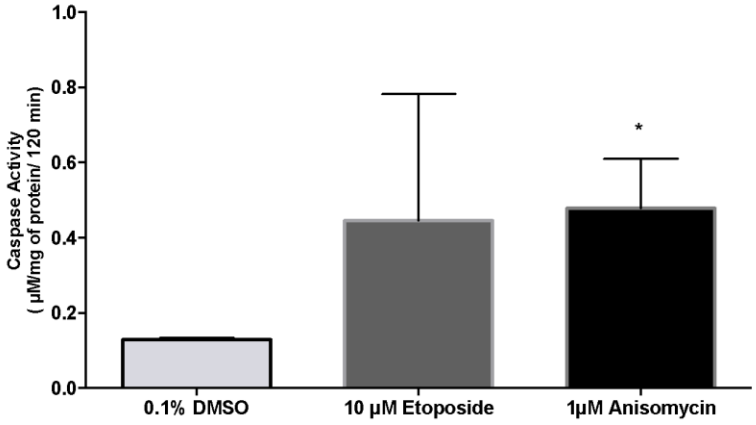


Figure 7: This figure shows the value of caspase-3 activity in µM/mg of protein/120 min. Data is expressed as Mean +/- SD of caspase-3 activity in each untreated (0.1% DMSO) and treated (10 µM etoposide and 1 µM anisomycin) groups.

	Mean	SD	CV %
	(µM/mg of protein/ 120 min)	(µM/mg of protein/ 120 min)	
0.1% DMSO	63.738	3.735	5.86
10 uM of Etoposide	242.428	249.882	103.075
1 uM of Anisomycin	256.722	117.992	45.961

Table 3: This table depicts caspase-3 activity in µM/mg of protein/120 min. Data is expressed as Mean +/- SD & % CV of caspase-3 activity in each untreated (0.1% DMSO) and treated (10 µM etoposide and 1 µM anisomycin) groups.

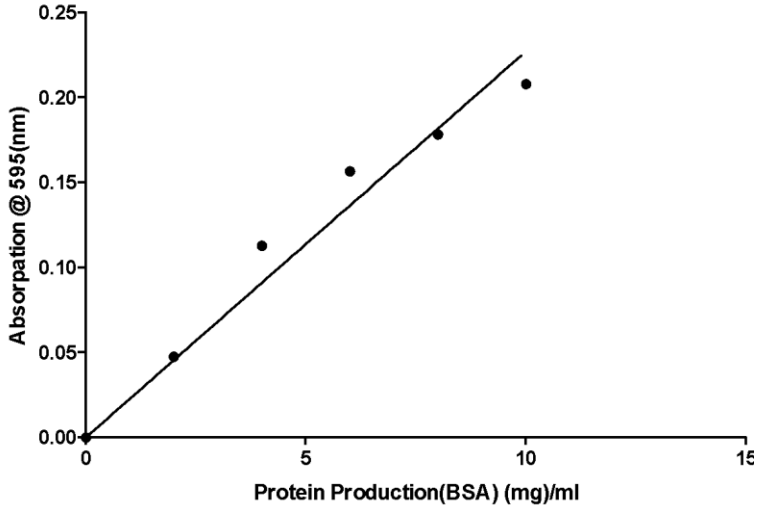


Figure 8: This figure represents the Bradford standard curve which shows the production of BSA in mg/ml plotted against the optical density with 1/slope = 44.05286. The value of BSA in 0.1 % DMSO (control) as untreated group, (10 µM etoposide and 1 µM anisomycin) as treated groups according to the standard curve is found to be significantly different as explained by the average BSA production in 0.1 % DMSO which was 6.130 mg/ml that was the highest as compared with the average BSA production which was 4.185 mg/ml in 10 µM etoposide and 3.186 mg/ml in 1 µM anisomycin. As the value of BSA production (mg/ml) increased, the absorption at 595 nm also increased.

3.4 DNA fragmentation assay

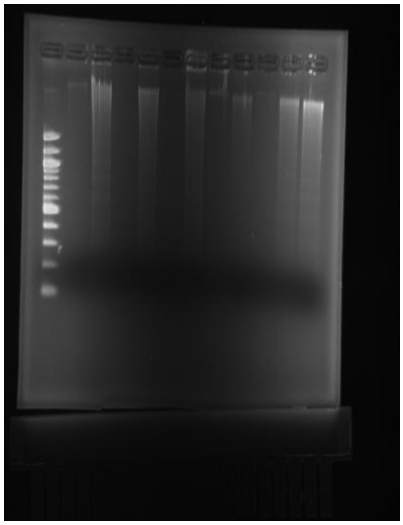


Figure 9: This figure shows the DNA fragmentation profile of cells treated with DMSO, etoposide and anisomycin. Lane 1 – 100 bp ladder, lane 2 and 3 – control, lane 4 and 5 – cells treated with 10 µM etoposide, lane 6 and 7 – cells treated with 1 µM anisomycin.

3.5 LDH cytotoxicity assay

The data for all groups showed a significance in homogeneity of variance. The Shapiro Wilks test of Normality showed $p>0.05$ for all groups. There were equal n numbers for all groups. The data was shown to be non-parametric and a Krustal Wallis was used so that all sets of data would be comparable, K= 46,089, $p<0.001$***.

A Mann Whitney U test was used as a Turky post hoc to test for significant differences between groups (control and anisomycin), (1:2 control and etoposide), (1:2 control and anisomycin), (1:5 control and anisomycin), U = 0.000, $p<0.01$**. Also, there was a significant difference between group control and etoposide, but there was no significant difference between group control and 1:5 etoposide, U = 11.500, $p = 0.297$.

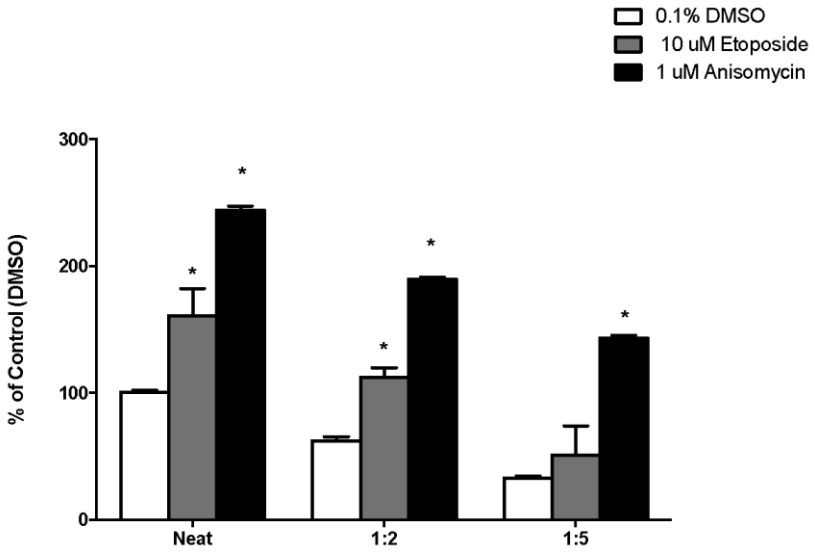


Figure 10: This figure shows the lactate dehydrogenase assay of 100 % DMSO, and etoposide and anisomycin in a ratio of 1:2 and 1:5.

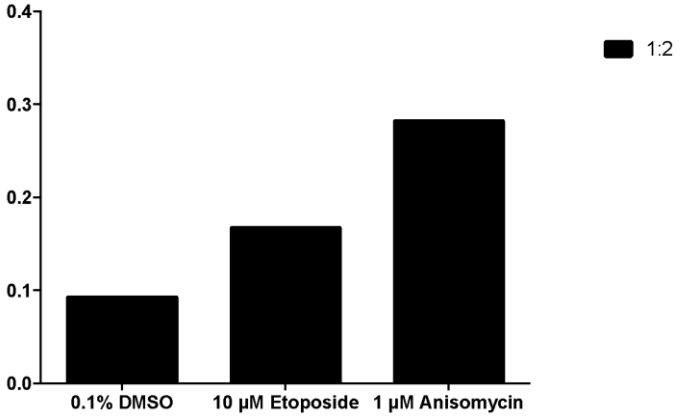


Figure 11: This figure represents the lactate dehydrogenase assay of 1:2 (DMSO, etoposide and anisomycin). The bar chart shows absorbance at 490 nm plotted against 1:2 DMSO and between each group (treated and untreated).

Ctrl 100	100.673	(97.4776-104.2040)
Etoposide 100	163.901	(109.4170-209.4731)
Anisomycin 100	245.292	(239.9103-250.6726)
Ctrl 1:2	62.3318	(54.9327-70.2354)
Etoposide 1:2	109.081	(96.6368-128.4193)
Anisomycin 1;2	189.462	(186.6031-193.4978)
Ctrl 1:5	33.4081	(28.3632-36.6031)
Etoposide 1:5	109.081	(96.6368-128.4193)
Anisomycin1:5	143.049	(140.5269-147.5897)

Table 4: This table shows data of the lactate dehydrogenase assay of 100 % (DMSO, etoposide and anisomycin), 1:2 (etoposide and anisomycin), and 1:5 (etoposide and anisomycin). Data shows median and interquartile range for all groups (N = 9). A Krustal-Wallis test showed that there was significant difference between the groups K = 46,089, $p<0.001^{***}$.

4 Discussion

4.1 Alamar blue assay

Alamar blue assay is used to test the viability of cells after exposing them to a cytotoxic environment (Lewinski et al., 2008). In this experiment, two cytotoxic agents, etoposide and anisomycin were used to induce cytotoxicity and DMSO was used as control. As expected, with increasing concentrations of etoposide and anisomycin, the viability of cells decline whereas they are 100 % viable in the presence of DMSO indicating that etoposide and anisomycin are cytotoxic to the cells. The LD_{50} of etoposide was found to be 0.6430 µM and the LD_{50} of anisomycin was found to be 0.01797 µM indicating that anisomycin is more cytotoxic as compared to etoposide as its activation dose is much lesser as compared to that of etoposide.

4.2 Haematoxylin and Eosin staining

H & E staining is frequently used for staining of cell organelles. Haematoxylin stains the nuclei blue and eosin stains all other organelles pink, thus allowing in-depth visualization of the nucleus and its related structures (Ankle and Joshi, 2011). In this experiment, cells treated with either DMSO, etoposide and anisomycin were stained with H & E and viewed under the microscope. As seen in the results, cells treated with DMSO were stained mildly whereas cells treated with etoposide and anisomycin were stained darker. This is because etoposide and anisomycin induce apoptosis in the cells which allows them to take up the stains more vividly due to plasma membrane leakage giving them a darker color (Ankle and Joshi, 2011).

4.3 Caspase activity assay and Bradford assay

Both these assays are used to test for the induction of apoptosis in cells. Caspases are enzymes released when cell death pathways are activated in a cell and can be measured to show the percentage of cell death in a culture (Telford et al., 2002). The Bradford assay measures the amount of protein in a given solution. When cell death occurs, all membranes and organelles are broken down releasing a lot of proteins into the solution. Hence, more the amount of protein measured by the Bradford assay, higher the percentage of cell death in the culture (Pihlasalo, 2011).

In both assays, etoposide and anisomycin were used to induce cell death in Jurkat cells and caspase activity and protein concentrations were measured and DMSO was used as control. As is evident from the graphs and tables, aliquots of cells treated with etoposide and anisomycin showed a higher level of caspase activity and protein concentration indicating that cell death had occurred upon treatment with these agents. On the other hand, cells treated with DMSO showed a lower level of caspase activity and lower protein concentration indicating the absence of apoptosis in these cells.

4.4 DNA fragmentation assay

This assay is used to assess cell death by measuring the presence of fragmented DNA in the solution. Fragmented DNA is an important feature of cells undergoing apoptosis and this can be easily visualized by agarose gel electrophoresis (Ioannou and Chen, 1996). In this assay, apoptosis is induced by the cytotoxic agents, etoposide and anisomycin, and cells treated with these two agents show the presence of fragmented DNA as evident from the results. When the DNA samples are compared with a standard 100 bp ladder on an agarose gel, the fragmentation of the DNA in the sample is clearly visible in lanes where cells have been treated with the two cytotoxic agents.

4.5 LDH cytotoxicity assay

Lactate dehydrogenase enzyme is an important marker for a different type of programmed cell death pathway – necrosis. In necrosis, leakage of cell contents is a very important feature and an enzyme that is released in this process is lactate dehydrogenase. The level of activity of this enzyme in a solution is indicative of the amount of necrosis in the cells (Chan et al., 2013). Cells treated with etoposide and anisomycin were used for this assay and the activity of LDH was measured. As is evident from the results, the presence of etoposide and anisomycin in a culture medium resulted in a higher amount of LDH activity as compared to control indicating that the two cytotoxic agents resulted in necrosis and release of LDH in the culture medium.

5 Conclusions

All assays were performed using two cytotoxic agents, etoposide and anisomycin, and their cell death-inducing properties were proved by each of the five assays performed. The Alamar blue assay showed conversion of the blue dye into a pink colored product by apoptotic cells. The Haematoxylin and Eosin stain were able to differentiate organelles in paraffin-fixed cells. The caspase activity assay and Bradford assay demonstrated caspase activity and presence

of protein in cells undergoing apoptosis. The DNA fragmentation assay demonstrated DNA fragments of apoptotic cells on agarose gel. The LDH assay proved the presence of the enzyme lactate dehydrogenase in necrotic cells.

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