TED ANKARA COLLEGE FOUNDATION PRIVATE HIGH SCHOOL

The Cytotoxic Effects of Wheat Germ Extract on Glioblastoma Cell Line

BIOLOGY EXTENDED ESSAY

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ABSTRACT

The aim of this experiment was to determine whether wheat germ extract has an increasing cytotoxic activity or any cytotoxic activity on the malignant glioblastoma cancer cells. Thus, this paper includes the comparison of cell viability after the exposition to different concentrations of Wheat Germ Extract (WGE) in medium.

My research question was: “How does the cytotoxic activity measured by the percentage of remaining living cells found in Methylthiazol Tetrazolium Assay test (MTT) of glioblastoma multiforme cells differ according to their exposition to different concentrations of wheat germ extract?”

My hypothesis was: “As the concentration of applied WGE increases, the cytotoxic activity in glioblastoma cells will also increase. It is expected that the rapidly proliferating cells will be slowed down and largely killed as the WGE concentration increases.”

To answer the research question, WGE was produced and given to glioblastoma cells in 6 different concentrations starting from 10% to 35%, increasing 5 by 5 in growth medium. Distilled water was given to the control group at the same concentrations in medium as distilled water was used during the production of WGE and it was important to check whether WGE or distilled water had the cytotoxic effect on cells. The cells were left in a CO_2 incubator for 48 hours.

After 48 hours, the medium, distilled water, WGE were extracted from the cells and were replaced with MTT. An MTT test was applied and viability was calculated with the results. It was clear that the cytotoxic activity increased as the WGE concentration increased and more than half of the cells went through apoptosis at 35% WGE exposure. Such a conclusion gives hope towards the future of curing of cancer and raises new questions about the usage of WGE.

Word count: 287

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INTRODUCTION

Even as a toddler, I would identify the most amusing object in our house to be the unshapely skull on our bookshelf. I always had an interest in the human body and its wonders. All the questions I would ask my father, a doctor, and the researches I would make to find out even more, I justify to have led me to this research today. Whenever my father had an upcoming surgery my mother would ask 'What is it?' and if 'glioblastoma' the answer, my mother's shoulders would drop with a glimpse of pity in her eyes; an encouraging pat on my father's back would follow. The reason for that scenery I learned years later when I could pronounce the word glioblastoma: the most malignant brain tumor leaving its victims approximately 12 months of survival time.

Curious, I began to research this fatal disease. My research showed me that glioblastoma multiforme (GBM) in its full name is a grade IV brain tumor deriving from the cancer of astrocyte cells in the brain[1]. (see Appendix 1) According to the grading of the World Health Organization (WHO) the most distinctive characteristics of the grade IV tumor would be its rapid growth so much as to include necrosis regions, blocking the way of other cells forcing them to break down and thus forming a dead area inside of the tumor.

Next, I decided to talk to experts on the issue and ended up speaking to a biologist[2]. He named me some drugs that help eradicating glioblastoma and advised me to observe the antioxidant, oxidant, and cytotoxic activity of these different drugs on the cell line. However, I was not looking for drugs. I was looking for something more natural so I began my own research again. I found that wheat germ extract (WGE) is recently being used to cure cancer. All studies I found were on the impacts of WGE on different cancer types, though I never came across a study which focused only on glioblastoma multiforme in this sense. I decided to use WGE and to choose one of the three activities of antioxidant (inhibiting oxidation), oxidant (promoting oxidation) or cytotoxic (the quality of being toxic to cells) as to make the core of my research question clear and straightforward. I wanted the experiment to be

[1] Ugur HC, Ramakrishna N, Bello L, Menon LG, Kim SK, Black PM, Carroll RS. Continuous intracranial administration of suberoylanilide hydroxamic acid (SAHA) inhibits tumor growth in an orthotopic glioma model.J Neurooncol. 2007 Jul;83(3):267-75. Epub 2007 Feb 20. http://www.ncbi.nlm.nih.gov/pubmed/17310267

[2] Mehmet Taşpınar PhD, Van 100. Yıl University Department of Biology

genuinely mine and desired the least amount of help from professionals. Therefore I resolved to choose cytotoxic activity as it is directly correlated with the amount of cells that die from the exposition to WGE. For the other two; I would have to work with biochemists.

In light of these decisions, I have settled my research question to be: **How does the cytotoxic activity measured by the percentage of remaining living cells found in MTT test (Methylthiazol Tetrazolium Assay) of glioblastoma multiforme cells differ according to their exposition to different concentrations of wheat germ extract?** This paper will display how the experiment was planned and carried out as well as evaluate the results provided by the analyzing of the data obtained.

HYPOTHESIS

Since most studies focus on the cytotoxic activity of Wheat Germ Extract (WGE) on cell lines like ovarian and colon cancer, there is limited information about WGE's effects on glioblastoma cell line. Studies focusing specifically on ovarian cancer[3], colon cancer[4], and leukemia[5] indicate that WGE has anti-proliferative and anti-metastatic effects on cancer cell lines. It is suggested that the high redox potential of 2,6-dimethoxy-p-benzoquinone and 2-methoxy benzoquinone found in wheat germ causes cell cycles to be disturbed, thereby acting as an inhibitor.[6] Apparently, WGE keeps malignant cells from growing by changing their metabolism and induces apoptosis[4] (the triggering of a pathway in a cell which is naturally programmed to kill it). Both apoptosis and the limited growth of a cell are considered as cytotoxic activity for a cell.

Therefore it can be hypothesized that, as the concentration of applied WGE increases, the cytotoxic activity in glioblastoma cells will also increase. It is expected that the rapidly proliferating cells will be slowed down and largely killed as the WGE concentration increases.

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[3] Judson PL, Al Sawah E, Marchion DC, Xiong Y, Bicaku E, Bou Zgheib N, Chon HS, Stickles XB, Hakam A, Wenham RM, Apte SM, Gonzalez-Bosquet J, Chen DT, Lancaster JM. Characterizing the efficacy of wheat germ extract against ovarian cancer and defining the genomic basis of its activity. Int J Gynecol Cancer. 2012 Jul;22(6):960-7. doi: 10.1097/IGC.0b013e318258509d http://www.ncbi.nlm.nih.gov/pubmed/22740002

[4] Farkas E. [wheat germ extract in the supportive therapy of colorectal cancer]. Orv Hetil. 2005 Sep 11;146(37):1925-31. http://www.ncbi.nlm.nih.gov/pubmed/16255377

[5] Comin-Anduix B, Boros LG, Marin S, Boren J, Callol-Massot C, Centelles JJ, Torres JL, Agell N, Bassilian S, Cascante M. wheat germ extract inhibits glycolysis/pentose cycle enzymes and induces apoptosis through poly(ADP-ribose) polymerase activation in Jurkat T-cell leukemia tumor cells. J Biol Chem. 2002 Nov 29;277(48):46408-14. Epub 2002 Sep 25. http://www.ncbi.nlm.nih.gov/pubmed/12351627

[6] Alexander K, Markus H. CAM-Cancer Consortium. wheat germ extract. August 21, 2013 http://cam-cancer.org/CAM-Summaries/Dietary-approaches/Fermented-wheat-germ-extract

METHOD DEVELOPMENT AND PLANNING

Glioblastoma cell line was chosen to work with because it is one of the most malignant cancer cells. Wheat Germ Extract (WGE) was chosen to expose the cells to because it is a natural substance newly being tested on cancer cells. A laboratory was needed as such an experiment could not be conducted at home. Talking to a few doctors, I was lead to the molecular biology department of Ankara University. There, I explained the aim of my experiment and it was shown great interest. I was told that I could receive assistance with using the lab equipment. The glioblastoma cell line was provided from the Neurosurgery Department Neuro-oncology Laboratory of Ankara University. The materials needed to inoculate cells and feed them throughout the experiment such as RPMI-1640, Trypsin Hormone, Fetal Bovine, and L-glutamine were obtained from the medical firm Sigma. (See Appendix 2) I was also lead to a research which explains the method of WGE production.[7] (see Appendix 7)

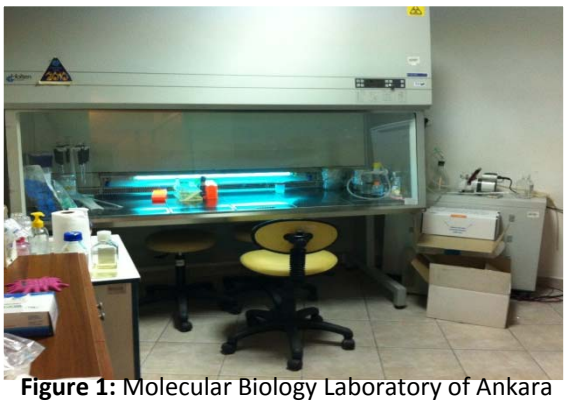


Figure 1: Molecular Biology Laboratory of Ankara University

Next came designing the experiment. The cells were too valuable to waste. To find an interval of WGE concentration which would probably contain the cytotoxic activity threshold of glioblastoma cells was needed to determine the amount of WGE to give the cells. With this aim, the threshold levels of other malignant cancer cells were researched as there were not many articles on glioblastoma cell lines. It was found that there is a general IC50 (half maximal inhibitory concentration is a measure of the effectiveness of a compound in inhibiting biological or biochemical function[8]) interval from 10% to 35% in various cancer cell

[7] Oya Sena AYDOS, Aslıhan AVCI, Tülin ÖZKAN, Aynur KARADAĞ, Ebru GÜRLEYİK, Buket ALTINOK, Asuman SUNGUROĞLU. Antiproliferative, apoptotic and antioxidant activities of wheatgrass (Triticum aestivum L.) extract on CML (K562) cell line. Turk J Med Sci 2011; 41 (4): 657-663 © TÜBİTAK doi:10.3906/sag-0912-425 http://journals.tubitak.gov.tr/medical/issues/sag-11-41-4/sag-41-4-13-0912-425.pdf

[8] "IC50." Wikipedia, The Free Encyclopedia http://en.wikipedia.org/wiki/IC50

lines[9], IC50 being the value that causes 50% of cells to stop growing or go through apoptosis which is interpreted as cytotoxic activity. So, the WGE concentrations given to cell lines were determined as 10%, 15%, 20%, 25%, 30%, 35%.

Now, knowing how long the cells would be exposed to WGE was needed. It was found that 48 hours is usually the best fit for cells to react to WGE as 24 hours can sometimes be insufficient for significant change in cell activity.[10] As glioblastoma cells are considered malignant cells, 48 hours was chosen for the duration of WGE exposure to cells before conducting a viability test.

At the end of the experiment, there was the need to carry out a viability test to measure what percentage of the cells had died to see cytotoxic activity. As earlier mentioned, cell apoptosis is cytotoxic activity. Research for possible viability assays showed that tetrazolium salts (reflect a purple color when the enzymes in a living cell reduce them to their insoluble formazan dye) are used in experiments in which cells go through apoptosis. During apoptosis, deformations in the mitochondria occur and the mitochondria die.[11] These tetrazolium salts can be reduced by an enzyme present in only metabolically active cells, thus, cells with healthy mitochondria.[12] Among these tests with tetrazolium salts, were the commonly used MTS, MTT, and XTT tests. As the laboratory only had MTT assay available, MTT assay[13] was

Figure 2: MTT assay dye

[9] Mueller T, Jordan K, Voigt W. Promising cytotoxic activity profile of wheat germ extract (Avemar®) in human cancer cell lines J Exp Clin Cancer Res. 2011 Apr 16;30:42. doi: 10.1186/1756-9966-30-42 http://www.ncbi.nlm.nih.gov/pubmed/21496306

[10] Marcsek Z, Kocsis Z, Jakab M, Szende B, Tompa A. The efficacy of tamoxifen in estrogen receptor-positive breast cancer cells is enhanced by a medical nutriment. Cancer Biother Radiopharm. 2004 Dec;19(6):746-53. http://www.ncbi.nlm.nih.gov/pubmed/15665622

[11] Green DR, Reed JC. Mitochondria and apoptosis. Science. 1998 Aug 28;281(5381):1309-12 http://www.ncbi.nlm.nih.gov/pubmed/9721092

[12] Cory AH, Owen TC, Barltrop JA, Cory JG (July 1991). "Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture". *Cancer communications* **3** (7): 207–212. ISSN 0955-3541 http://www.ncbi.nlm.nih.gov/pubmed/1867954

[13] Ghoddusi J, Tavakkol Afshari J, Donyavi Z, Brook A, Disfani R, Esmaeelzadeh M Cytotoxic effect of a new endodontic cement and mineral trioxide aggregate on L929 line culture. Iran Endod J. 2008 Spring;3(2):17-23. Epub 2008 Apr 2 http://www.ncbi.nlm.nih.gov/pubmed/24171015

determined as the test to be conducted to measure cell viability in this experiment. MTT was provided the firm Roche in Germany.

To reach viability statistics after the viability assay, a control group to which the cytotoxic activity of cells exposed to WGE can be compared was needed. Since distilled water was used as a solvent in the production of WGE, it was appropriate to give the same percentages of distilled water and medium of all wells to a new set of wells which would form the control group. So 10%, 15%, 20%, 25%, 30%, and 35% distilled water would be given to cells in 90%, 85%, 80%, 75%, 70%, and 65% medium respectively.

At this state, determining the controlled variables was the issue. To make sure the same amount of cells was inoculated into each well I learned to use the technique used in the laboratory of the university called the Thoma cell counting chamber which allows one to count the number of cells in $1mm^3$ on the chamber under the microscope and then reach the total number of cells per ml by direct correlation.

A 96 well plate as was going to be used as need 60 wells for 5 trials in 6 different concentrations and their control groups were needed. Research showed that 30,000 to 40,000 cells should be inoculated in each well to reach a 90% confluence after incubation.[14] So, 40,000 were inoculated in each well.

Figure 3: CO_2 incubator

Another important controlled variable was temperature. The CO_2 incubator in the laboratory was used and was set to 37^oC as it is the body temperature.

Naturally, pH levels could not be a controlled variable as

Degen M, Alexander B, Choudhury M, Eshghi M, Konno S. Alternative Therapeutic Approach to Renal Cell Carcinoma: Induction of Apoptosis with Combination of Vitamin K3 and D-fraction. J Endourol. 2013 Nov 7. [Epub ahead of print] http://www.ncbi.nlm.nih.gov/pubmed/24195738

[14] Düzgüneş, Nejat. Methods in Enzymology Volume 509 Nanomedicine: Infectious diseases, Immunotherapy, Diagnostics, Antifibrotics, Toxicology and Gene Medicine. Page 231. First edition. San Diego: Elsevier Inc. 2012 http://books.google.com.tr/books?id=88XS1FDIo1QC&pg=PA231&lpg=PA231&dq=40000+cells+96+well+plate+MTT&source=bl&ots=34czkw5V6L&sig=fHixwgdxFapFljRPkWv-ejR7HFM&hl=tr&sa=X&ei=tRiqUs6yOsWctAbuioHQDw&ved=0CF4Q6AEwBw#v=onepage&q=40000%20cells%2096%20well%20plate%20MTT&f=false

the difference in WGE concentrations would also affect the pH levels. The amounts of any liquid put into the wells would be performed by pipettes which are professional tools to prevent errors and get precise amounts of liquids. The wells are also all the same sizes so any other cells in another well will not have more space to grow as sometimes, inadequate place to grow may also cause of cell deaths. The kind of serum given to the cells for them to feed on will be the same but the amounts will change due to the concentrations of WGE. This serum would be the one always used by the laboratory for cell cultures. It contains 89% RPMI 1640 Medium, 10% Fetal Bovine serum which is rich of proteins and further supports the growth and survival of cells, and 1% L-glutamine which is an amino acid which also assists cell growth.

It is important to note that a different pipette end must be used for each different substance handled by the pipette. The experiment must be conducted in a sterilized laboratory and the person conducting must wear medical gloves and a face mask. The tools used, the area that the experiment will be conducted in, and the gloves should be sterilized with a 75% ethanol solution before the experiment starts and every time an object like the incubator or a chair that is outside the area of experiment is touched.

MATERIALS AND METHOD

- Organic Wheat seeds (by Işık organic agricultural products)
- Plastic mortar
- 0.2 µm pore sized filter
- CO_2 incubator
- Glioblastoma cell line
- 1 ml Trypsin hormone
- Centrifuge
- Pipette station
- Medical gloves
- Face mask
- 75% ethanol solution
- 96-well plate
- Thoma cell counting chamber
- Light Microscope
- 1 L Distilled water
- 6 ml MTT[1]
- Spectrophotometer
- 100 ml medium (contains 10 ml Fetal Bovine, 1 ml L-glutamine, and 89 ml RPMI-1640)

To begin the experiment, firstly, WGE is produced. (See Appendix 7)

For the steps after this point, it is important to note that a different pipette end must be used for each different substance handled by the pipette. The experiment must be conducted in a sterilized laboratory and the person conducting must wear medical gloves. The tools used, the area that the experiment will be conducted in, and the gloves should be sterilized with a 75% ethanol solution before the experiment starts and every time an object like the incubator or a chair that is outside the area of experiment is touched.

Figure 4: Flask containing glioblastoma cells

The glioblastoma cells are by nature, cells that grow by sticking to surfaces. So when there is not enough surface area, some cells begin to die. That is why, when the flask full of glioblastoma cells is first taken out, 1 ml of the hormone Trypsin is added to the flask and kept in the incubator with 37°C for 3 minutes. The hormone Trypsin helps separate the cells from the surface so that they can be inoculated in wells. After 3 minutes, the flask is put into a centrifuge which spins 2000 times per minute. This makes the healthy cells precipitate to the bottom so that the dead cells can easily be extracted from the top of the liquid with a pipette. When the liquid formed of the Trypsin hormone and dead cells is extracted, 2 ml of medium is added to the healthy cells. The cells and the medium are mixed with pipetting technique.

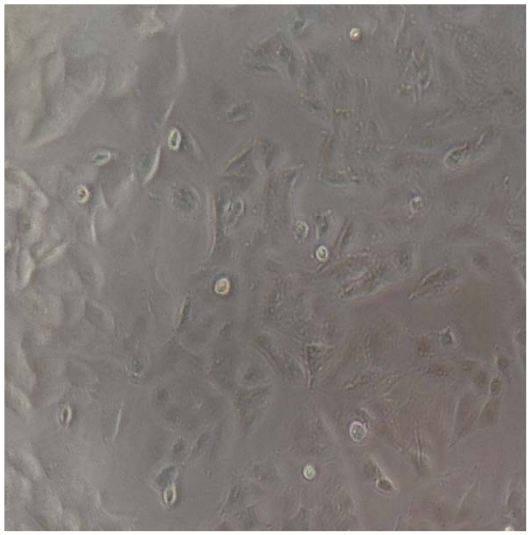


Figure 5: Glioblastoma cells under light microscope. The clearly outlined ones are the ones that have died and been dispatched to the top.

100 µl of the cell solution is put on the Thoma Cell Counting Chamber (see Appendix 3) using the pipette. This chamber is composed of microscopic square areas on which cells can be counted under the microscope. Numbers of cells in two squares are counted and their average is taken. This number is multiplied by 10^4 which give the expected number of cells in 100 µl. The two squares counted gave the numbers 12 and 13. Their average, 12.5, multiplied by 10^4 made 50,000 cells in every 100 µl. In the 96-well plate, a 6 by 10 rectangular area in the middle of the set is selected to plant the cells in. This is to further prevent any temperature changes or air circulation that might happen in the outer wells that are closer to the outside environment. In order to plant 40,000 cells per well, 80 µl is put into each well that is selected. Then 200 µl of medium is added to each well and the well plate is left in the incubator for 24 hours for the cells to stick to the bottom and grow.

After 24 hours, it is time to expose the cells to WGE. For %10, %15, %20, %25, %30, %35 concentrations of WGE, 20, 30, 40, 50, 60, and 70 µl of WGE respectively is needed for every 5 wells as there will be 5 trials of each concentration and the same amount of distilled water for their control group. This makes the total need of WGE 1.35 ml and the total need for distilled water also 1.35 ml. In correspondence to WGE, 180, 170, 160, 150, 140, 130 µl of medium respectively is needed for every 10 wells for both the variable group and the controlled group making the total amount 9.3 ml medium. 6 rows are allocated for 6 different concentrations. The first 5 in a row is for the variable group with WGE and the second 5 is for the controlled group corresponding to that concentration. Taking these into consideration, the used up medium is quickly removed from wells and the new appropriate amount of medium is put into the wells with the order that they have been emptied. This is important because the cells may die due to dryness caused by the outtake of the medium. The cells must be left dry for as short a time period as possible. Then the appropriate amount of WGE is put into the wells that are in the variable group and the same amount of distilled water is put instead, in the controlled group. Afterwards, the plate is left in the incubator for 48 hours.

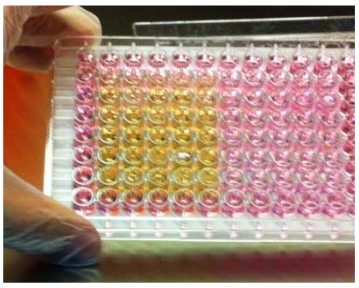


Figure 6: 96 well plate after inoculation and the addition of WGE, medium, distilled water. The yelloe wells are the ones with WGE.

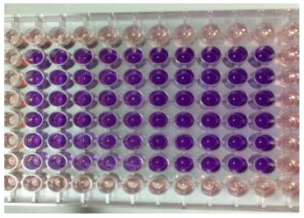


Figure 7: 96 well plate after MTT test, the darkest purple colors showing the highest viability.

After 48 hours, the plate is taken out of the incubator and the wells are again cleaned of the used up medium, WGE and distilled water as quickly as possible. 10 µl of MTT is added into each well along with 100 µl of fresh medium. The plate is left in the incubator for 1 hour. After an hour has passes the plate is taken out and put into a Spectrophotometer which will calculate the viability (See Appendix 4) of living cells according to the wavelengths received by the living mitochondria. The wavelength of the color that viable cells reflect is 550 nm. However, the background plate absorbance is 690 nm. This is why the MTT test requires the results obtained at 690 nm to be subtracted from those obtained at 550 nm to reach the accurate results. When the results have been obtained the experiment is over. The remaining plate with the cells and the pipette ends must be thrown to a medical waste bin.

Wavelength at which the Spectrometer is making measurement nm	Concentration of WGE or distilled water in medium (%)	Spectrometer Measurement of Absorbance photons/sec (± 0,001)									
		WGE Group					Control Group				
		Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
550	10	1,311	1,277	1,246	1,205	1,226	1,531	1,497	1,494	1,485	1,464
	15	1,111	1,153	1,135	1,071	1,039	1,486	1,445	1,449	1,464	1,450
	20	1,097	1,098	1,112	1,125	1,027	1,423	1,412	1,423	1,414	1,462
	25	1,130	1,130	1,111	1,096	1,116	1,359	1,342	1,326	1,368	1,373
	30	0,898	0,873	0,800	0,890	0,888	1,299	1,260	1,291	1,255	1,358
	35	0,476	0,605	0,618	0,622	0,655	1,194	1,189	1,178	1,223	1,231
690	10	0,054	0,055	0,054	0,054	0,060	0,058	0,059	0,059	0,059	0,058
	15	0,053	0,061	0,056	0,054	0,053	0,059	0,058	0,059	0,060	0,060
	20	0,054	0,054	0,056	0,055	0,053	0,059	0,060	0,060	0,058	0,061
	25	0,056	0,056	0,054	0,054	0,054	0,058	0,059	0,059	0,058	0,059
	30	0,052	0,053	0,052	0,051	0,051	0,057	0,058	0,058	0,057	0,059
	35	0,050	0,050	0,051	0,050	0,050	0,055	0,057	0,057	0,056	0,057

Table 1: Raw data table of measured absorbance of each well at 550 nm and 690 nm wavelengths measured by Spectrometer

WGE Concentration	Calculated Absorbance after 550nm-690nm photons/sec (± 0,001)									
	WGE Group					Control Group				
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
10%	1,257	1,222	1,192	1,151	1,166	1,473	1,438	1,435	1,426	1,406
15%	1,058	1,092	1,079	1,017	0,986	1,427	1,387	1,390	1,404	1,390
20%	1,043	1,044	1,056	1,070	0,974	1,364	1,352	1,363	1,356	1,401
25%	1,074	1,074	1,057	1,042	1,062	1,301	1,283	1,267	1,310	1,314
30%	0,846	0,820	0,748	0,839	0,837	1,242	1,202	1,233	1,198	1,299
35%	0,426	0,555	0,567	0,572	0,605	1,139	1,132	1,121	1,167	1,174

Table 2: Calculated absolute absorbance of wells by subtracting the absorbance of each well at 690 nm from that of 550 nm (see Appendix 4)

	WGE Concentration in Medium (%)						Distilled Water Concentration in Medium (%)					
	10	**15**	**20**	**25**	**30**	**35**	**10**	**15**	**20**	**25**	**30**	**35**
Mean	1,253	1,102	1,092	1,117	0,870	0,595	1,436	1,400	1,367	1,295	1,235	1,147
Standard Deviation	0,042	0,047	0,038	0,014	0,040	0,069	0,024	0,017	0,020	0,020	0,041	0,023
Standard Error	0,043	0,044	0,037	0,013	0,040	0,069	0,024	0,017	0,020	0,020	0,041	0,023
t	2,776	2,776	2,776	2,776	2,776	2,776	2,776	2,776	2,776	2,776	2,776	2,776
95% CL	0,116	0,129	0,105	0,040	0,111	0,192	0,068	0,046	0,054	0,055	0,113	0,064

Table 3: Descriptive statistics of calculated absorbance shown on Table 2

Concentrations of WGE or distilled water in medium (%)	Means of trials		Viability (%)
	WGE Group	**Control Group**	
10	1,198	1,436	83,449
15	1,046	1,400	74,736
20	1,037	1,367	75,848
25	1,062	1,295	82,008
30	0,818	1,235	66,246
35	0,545	1,147	47,532

Table 4: Viability of glioblastoma cells after being exposed to WGE or distilled water for 48 hours

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Mean*	*Variance*
10%	5	5,988	1,1976	0,001831
15%	5	5,232	1,0464	0,001947
20%	5	5,187	1,0374	0,001376
25%	5	5,309	1,0618	0,000178
30%	5	4,09	0,818	0,001623
35%	5	2,725	0,545	0,004769

ANOVA

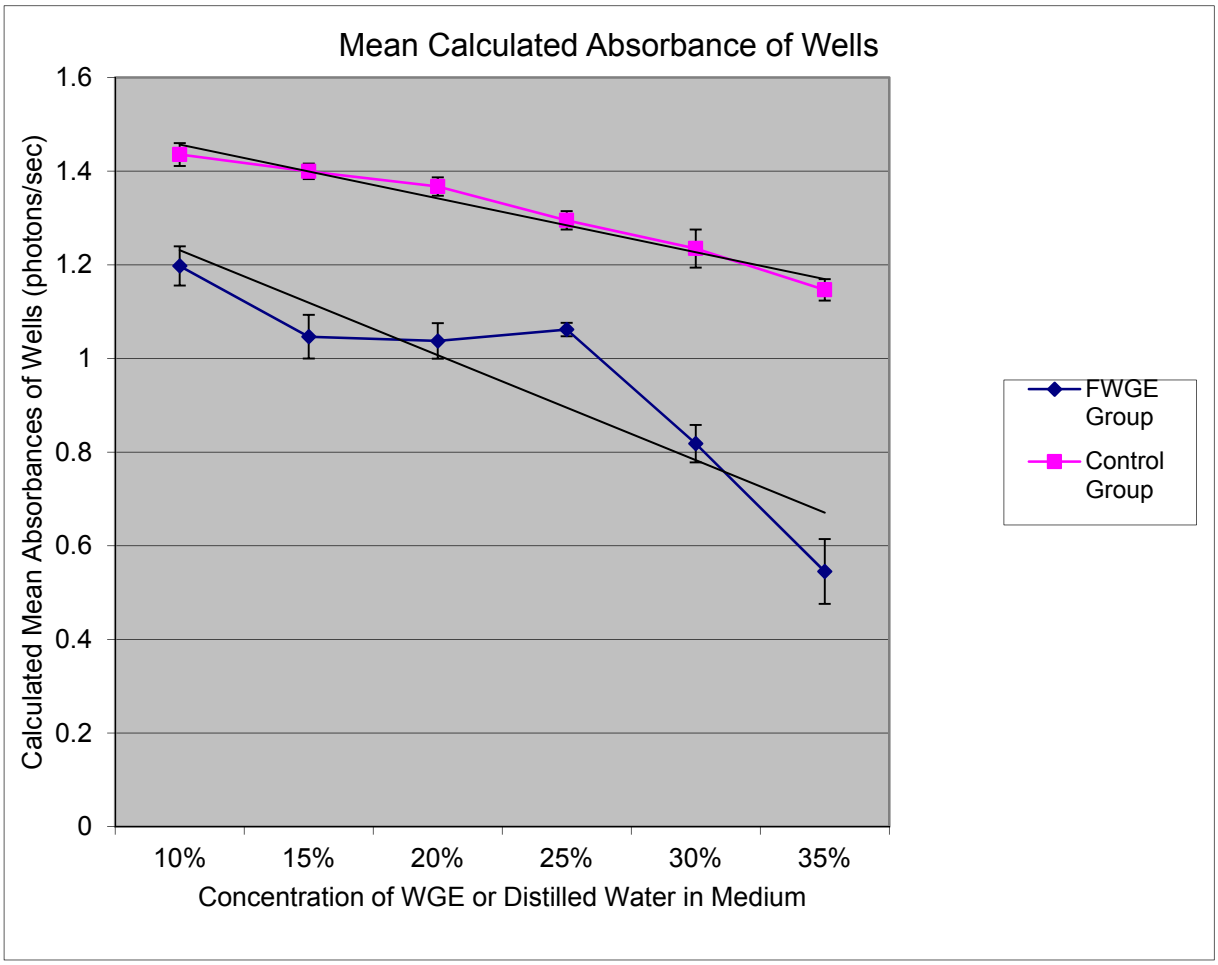
Source of Variance	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1,360897	5	0,272179	139,2982	6,46E-17	2,620654
Among Groups	0,046894	24	0,001954			
Sum	1,407791	29				

Table 5: Anova Single-Factor table of calculated absorbance of cells exposed to different concentrations of WGE in medium.

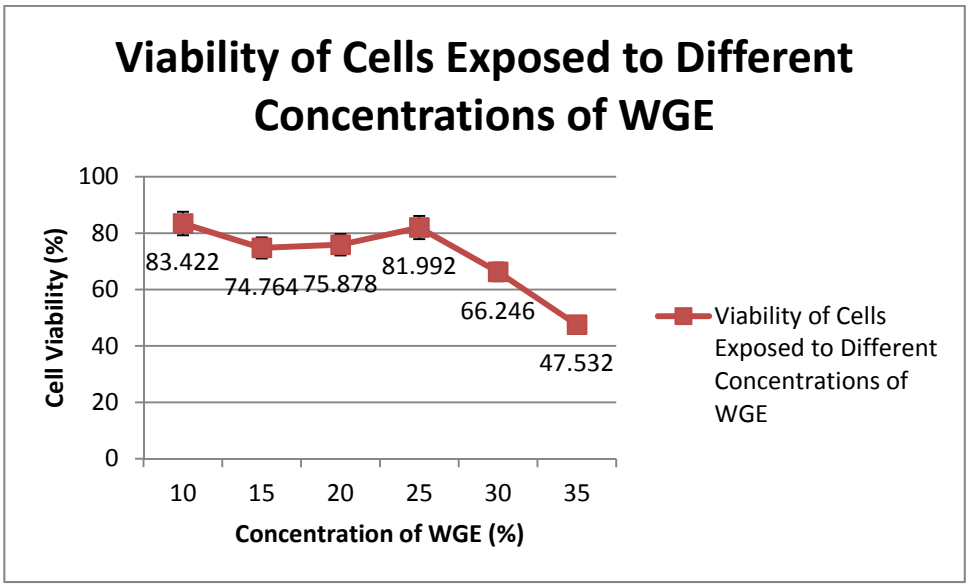
H_0: There is not a statistically significant mean difference between the absorbance of cells exposed to WGE in different concentrations in medium.

H_1: There is a statistically significant mean difference between the absorbance of cells exposed to different concentrations of WGE in medium.

Since P<0, 05 (6, 46E-17), H_0 is rejected and H_1 accepted. This shows that there is a statistically significant mean difference between the absorbance of cells exposed to different concentrations of WGE in medium.



Graph 1: Line Graph of Mean Calculated Absorbance of Wells in both WGE and Control Groups



Graph 2: Line Graph of Viability of Cells exposed to WGE at Different Concentrations

EVALUATION

How does the cytotoxic activity measured by the percentage of remaining living cells found in MTT test (Methylthiazol Tetrazolium Assay) of glioblastoma multiforme cells differ according to their exposition to different concentrations of wheat germ extract?

The results support the hypothesis that as the concentration of applied WGE increases, the cytotoxic activity in glioblastoma cells also increases. The results that support this hypothesis are firstly the mean calculated absorbance of cells (See Table 3). It is important to note that the more absorbance the cells have their viability is. When comparing the calculated absorbance of cells exposed to least amount of WGE to more (1,253 1,102 1,092 1,117 0,870 0,595), it is seen that the ones exposed to less WGE have much more absorbance (1,253) that the ones exposed to the most amount (0,595). Also, compared to the mean absorbance of cells exposed to distilled water, (1,436 1,400 1,367 1,295 1,235 1,147) the cells exposed to WGE have much less absorbance. This shows that the cells haven't only died due to less medium, but also due to the exposition of WGE. For example the mean absorbance of cells exposed to 35% WGE is 0,595 whereas those exposed to 35% distilled water have the absorbance mean 1,147. The viability results also support the hypothesis. (See Table 4) The viability of cells exposed to WGE from 10% to 35% are 83,449 74,736 75,848 82,008 66,246 and 47,532 respectively. This shows that even though there are some minor inconsistencies between the correlations of cell viability as the WGE concentration increases, there is obvious cytotoxic activity at 35% as the cell viability is below 50% (47,532%) which is considered as cytotoxic activity.

The graphs better illustrate the decrease in absorbance means and viability. As observed on Graph 1, mean absorbance of cells exposed to WGE decrease much more than tan those exposed to distilled water. The absorbance decreases very rapidly as the concentration of WGE increases from 25% to 35%.

To further show that as the concentration of WGE increases, that the cytotoxic activity also increases, the Anova Single Factor test is considered. (See table 5) Since P<0, 05 (6, 46E-17), H_0 is rejected and H_1 accepted. The fact that there is a statistically significant mean

difference between the absorbance of cells exposed to different concentrations of WGE in medium is accepted.

To evaluate the reliability of data, the standard deviation, standard error, and 95% confidence level numbers on Table 3 should be taken into consideration. The standard deviation numbers are generally low, the lowest being 0, 014 in 25% WGE concentration and highest being 0, 069 in 35% WGE concentration. This can also be observed on Graph 1 on the standard deviation error bars. The lowest standard error number, 0, 013, is also at 25% WGE concentration and highest at 35% WGE concentration with 0, 069. The highest number being at this concentration may be due to the fact that 35% concentration is a breaking point for crossing the IC50 line. Thus, results may be varying the most at his point back and forth on the line. Predictably, the 95% Confidence level shows most confidence in 25% WGE concentration results with the confidence level at 0,040, and the lowest at 35% WGE concentration with the number 0,192. Overall, the reliability of data is high with low numbers of standard deviation, standard error, and 95% confidence level.

When decrease in viability and absorbance means are examined, it is seen that cell viability exposed to 25% WGE increases significantly after 20% and 15% concentration, having a viability of 82,008 very close to that of 10% WGE concentration which is 82,008. (See Table 4) The discrepancy between means could also be studied on all other tables and graphs. It is seen that viability also slightly increases from 15% WGE concentration to 20% concentration from 74,736% viability to 75,848% viability. Though this does not affect the conclusion of the experiment as 35% WGE concentration has shown concrete cytotoxic activity, it is a significant error to be considered. This error may be caused by too many cells inoculated in some wells. The cells may have been exposed to less WGE per cell due to miscalculated population. To prevent this, cells in more square areas on the Thoma cell counting chamber could be counted to take the average of so that a more accurate number is reached. The gradually decreasing mean absorbance seen on Graph 1 for the control group also supports the idea of randomly inoculated overpopulated wells. Also, to decrease the chance of erring, the trial number could be increased from 5 to 10 or 15.

Another important factor that may have caused errors is the decrease in PH levels as the cells use the WGE and the medium up. The decrease in PH levels may have been a factor in

cell apoptosis. To prevent this, the medium, distilled water, and WGE could be replaced in every well every 6 or 12 hours before the PH levels differ too much.

Research showed that the XTT assay (another form of viability test) has been proposed to replace the MTT assay as it has higher sensitivity and a higher dynamic range. One of the limitations in the experiment was that XTT assay wasn't available in the laboratory used. More accurate results could have been reached using this alternative assay.

Despite erring possibilities, the results can be considered valid as the conclusion reached also abides by the results reached in other published researches[15] on the similar topics. All of these laboratory researches have observed that WGE increasingly has a cytotoxic effect on cancer cells. This experiment has come to the conclusion that as the concentration of applied WGE increases, the cytotoxic activity in glioblastoma cells also increases.

[15] Mueller T, Jordan K, Voigt W. Promising cytotoxic activity profile of wheat germ extract (Avemar®) in human cancer cell lines J Exp Clin Cancer Res. 2011 Apr 16;30:42. doi: 10.1186/1756-9966-30-42 http://www.ncbi.nlm.nih.gov/pubmed/21496306

Judson PL, Al Sawah E, Marchion DC, Xiong Y, Bicaku E, Bou Zgheib N, Chon HS, Stickles XB, Hakam A, Wenham RM, Apte SM, Gonzalez-Bosquet J, Chen DT, Lancaster JM. Characterizing the efficacy of fermented wheat germ extract against ovarian cancer and defining the genomic basis of its activity. Int J Gynecol Cancer. 2012 Jul;22(6):960-7. doi: 10.1097/IGC.0b013e318258509d http://www.ncbi.nlm.nih.gov/pubmed/22740002

Comin-Anduix B, Boros LG, Marin S, Boren J, Callol-Massot C, Centelles JJ, Torres JL, Agell N, Bassilian S, Cascante M. Fermented wheat germ extract inhibits glycolysis/pentose cycle enzymes and induces apoptosis through poly(ADP-ribose) polymerase activation in Jurkat T-cell leukemia tumor cells. J Biol Chem. 2002 Nov 29;277(48):46408-14. Epub 2002 Sep 25. http://www.ncbi.nlm.nih.gov/pubmed/12351627

CONCLUSION

The evaluation of the results obtained from the experiment show that WGE viability of glioblastoma cells over 50%, at 35% concentration of WGE in medium in 48 hours. As WGE concentration in media increases, the cytotoxic activity of cells also increases, proving the hypothesis right.

The cytotoxic effect of WGE on these cancer cells begets a promising possibility of curing cancer in the future. WGE is a product obtained from the smashing and filtering of wheat germ with no additives. Thus, it is expected to have no side effects to the body as wheat is already a product people consume. However, it would be interesting to investigate whether WGE has any side effects on the human body.

Secondly, WGE has been cytotoxic to the glioblastoma cell line when directly exposed to it at a high level of concentration (30% - 35%). However, if WGE is to be consumed orally, it would not reach the targeted tissues at such high percentages. After digestion, it would be dispersed in the body by the transport system. That is why, techniques to concentrate WGE at a certain tissue, with or without surgery, must be investigated.

Cytotoxic activity to cancer cells is the only known effect of WGE in the body. However, the usage of WGE is newly developed. Thus, not many effects in the body are known. It is given in some countries to patients to help relieve pain. There are promising benefits of using consuming WGE but they should be further investigated.

APPENDIX 1:

What is Glioblastoma?

There are two types of cells in the brain: neurons and glial cells. Glial cells are responsible for supporting the neurons and can be divided into three: astrocytes, oligodendrocytes, and ependyms. Each of these types of glial cells has different responsibilities. The ones that I am concerned with, astrocytes, are responsible for regulating the transmission of electrical impulses within the brain, providing the nervous tissue with nutrients, supporting the formation of blood-brain barrier, maintaining extracellular ion balance, and repairing the brain and spinal cord. The reason for me to be interested in this particular type of glial cells is that the glioblastoma multiforme is a brain tumor deriving from the cancer of this particular cell type. However, not all tumors deriving from astrocytes are glioblastoma. Tumors are graded to classify cancer cells according to how abnormal they look. Glioblastoma multiforme is the particular name given only to the most malignant grade; grade IV, of astrocyte-sourced tumors.[16]

[16] Glioblastoma multiforme. Wikipedia the Free Encyclopedia. http://en.wikipedia.org/wiki/Glioblastoma_multiforme

APPENDIX 2:

Formulations and Ingredients:

RPMI-1640 Medium[17]

Component	R0883 [1x] g/L	R1145 [10x] g/L	R1383 g/L	R1780 [1x] g/L	R2405 [1x] g/L	R6504 g/L	R8758 [1x] g/L
Inorganic Salts							
Calcium Nitrate • $4H_2O$	0.1	1	0.1	0.1	0.1	0.1	0.1
Magnesium Sulfate (anhydrous)	0.04884	0.4884	0.04884	0.04884	0.04884	0.04884	0.04884
Potassium Chloride	0.4	4	0.4	0.4	0.4	0.4	0.4
Sodium Bicarbonate	2	—	—	2	2	—	2
Sodium Chloride	6	60	6	6	6	6	6
Sodium Phosphate Dibasic (anhydrous)	0.8	8	0.8	0.8	0.8	0.8	0.8
Amino Acids							
L-Alanyl-L-Glutamine	—	—	—	—	0.4344	—	—
L-Arginine	0.2	2	0.2	—	0.2	0.2	0.2
L-Asparagine (anhydrous)	0.05	0.5	0.05	0.05	0.05	0.05	0.05
L-Aspartic Acid	0.02	0.2	0.02	0.02	0.02	0.02	0.02
L-Cystine • 2HCl	0.0652	0.652	0.0652	0.0652	0.0652	0.0652	0.0652
L-Glutamic Acid	0.02	0.2	0.02	0.02	0.02	0.02	0.02
L-Glutamine	—	—	0.3	0.3	—	0.3	0.3
Glycine	0.01	0.1	0.01	0.01	0.01	0.01	0.01
L-Histidine	0.015	0.15	0.015	0.015	0.015	0.015	0.015
Hydroxy-L-Proline	0.02	0.2	0.02	0.02	0.02	0.02	0.02
L-Isoleucine	0.05	0.5	0.05	0.05	0.05	0.05	0.05
L-Leucine	0.05	0.5	0.05	—	0.05	0.05	0.05
L-Lysine • HCl	0.04	0.4	0.04	—	0.04	0.04	0.04
L-Methionine	0.015	0.15	0.015	0.015	0.015	0.015	0.015
L-Phenylalanine	0.015	0.15	0.015	0.015	0.015	0.015	0.015
L-Proline	0.02	0.2	0.02	0.02	0.02	0.02	0.02
L-Serine	0.03	0.3	0.03	0.03	0.03	0.03	0.03
L-Threonine	0.02	0.2	0.02	0.02	0.02	0.02	0.02
L-Tryptophan	0.005	0.05	0.005	0.005	0.005	0.005	0.005
L-Tyrosine • 2Na • $2H_2O$	0.02883	0.2883	0.02883	0.02883	0.02883	0.02883	0.02883
L-Valine	0.02	0.2	0.02	0.02	0.02	0.02	0.02
Vitamins							
D-Biotin	0.0002	0.002	0.0002	0.002	0.002	0.0002	0.0002

[17] RPMI-1640 Media Formulation. Sigma-Aldrich. http://www.sigmaaldrich.com/life-science/cell-culture/learning-center/media-formulations/rpmi-1640.html

Choline Chloride	0.003	0.03	0.003	0.003	0.003	0.003	0.003
Folic Acid	0.001	—	0.001	0.001	0.001	0.001	0.001
myo-Inositol	0.035	0.35	0.035	0.035	0.035	0.035	0.035
Niacinamide	0.001	0.01	0.001	0.001	0.001	0.001	0.001
p-Aminobenzoic Acid	0.001	0.01	0.001	0.001	0.001	0.001	0.001
D-Pantothenic Acid (hemicalcium)	0.00025	0.0025	0.00025	0.00025	0.00025	0.00025	0.00025
Pyridoxine • HCl	0.001	0.01	0.001	0.001	0.001	0.001	0.001
Riboflavin	0.0002	0.002	0.0002	0.0002	0.0002	0.0002	0.0002
Thiamine • HCl	0.001	0.01	0.001	0.001	0.001	0.001	0.001
Vitamin B_{12}	0.000005	0.00005	0.000005	0.000005	0.000005	0.000005	0.000005
Other							
D-Glucose	2	20	—	2	2	2	2
Glutathione (reduced)	0.001	0.01	0.001	0.001	0.001	0.001	0.001
Phenol Red • Na	0.0053	0.053	0.0053	—	0.0053	0.0053	0.0053

L-Glutamine:

L-glutamine is an unstable essential amino acid required in cell culture media formulations. Most commercially available media are formulated with free L-glutamine which is either included in the basal formula or added to liquid formulations at time of use. L-glutamine is unstable at physiological pH in liquid media. It breaks down to ammonium and pyroglutamate at rates that make it a problem in many biomanufacturing applications. Today several proprietary media used in biomanufacturing are supplemented with L-glutamine in dipeptide forms, such as alanyl-l-glutamine and glycyl-l-glutamine.[18]

Fetal Bovine Serum:

Fetal bovine serum (FBS) is the most widely used growth supplement for cell culture media because of its high content of embryonic growth promoting factors. When used at

[18] Glutamine in Cell Culture. Sigma-Aldrich. http://www.sigmaaldrich.com/life-science/cell-culture/learning-center/media-expert/glutamine.html

appropriate concentrations it supplies many defined and undefined components that have been shown to satisfy specific metabolic requirements for the culture of cells.[19]

MTT Assay: The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity. Since for most cell populations the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure the in vitro cytotoxic effects of drugs on cell lines or primary patient cells.[20]

APPENDIX 3:

Thoma Cell Counting Chamber[21]

The frame of the counting chamber contains a large central square (with a 1 mm2 which can be seen in its entirety with the 10X objective.

This large central square is divided into 16 medium squares (with the 40X objective the medium squares can see completely), each with 25 small squares inside (9 of them are divided in half) .

. When we put the sample under the coverslip, the cell suspension reaches a height of 0.1 mm. Taking these data into account, and considering one of the large squares, the volume will be:

1 x 1 x 0,1 = 0,1 mm3 = 10-4 ml

With the 10X objective of the microscope the counting area must be located. To count the cells the microscope must be switched to 40X objective. All the cells in the 16 medium squares must be counted according to the following criteria:

[19] Fetal Bovine Serum (FBS). Sigma-Aldrich. http://www.sigmaaldrich.com/life-science/cell-culture/cell-culture-products.html?TablePage=9628642

[20] van Meerloo J, Kaspers GJ, Cloos J. Cell sensitivity assays: the MTT assay. Methods Mol Biol. 2011;731:237-45. doi: 10.1007/978-1-61779-080-5_20 http://www.ncbi.nlm.nih.gov/pubmed/21516412

[21] Thoma cell counting chamber. http://insilico.ehu.es/counting_chamber/thoma.php

All the cells within each medium square and those that are over the top and right sides of the square (even when they are partially out) are counted. Following this approach, in the figure the cells in green will be counted, but not the cells in red. If we have counted N cells in one of the large squares (that is, in 16 medium squares), the concentration of our sample will be:

N x 104 cel/ml

When prior to counting we concentrated or diluted the initial sample, we must take into account the concentration-dilution factor (f):

N x 104 x f cel/ml

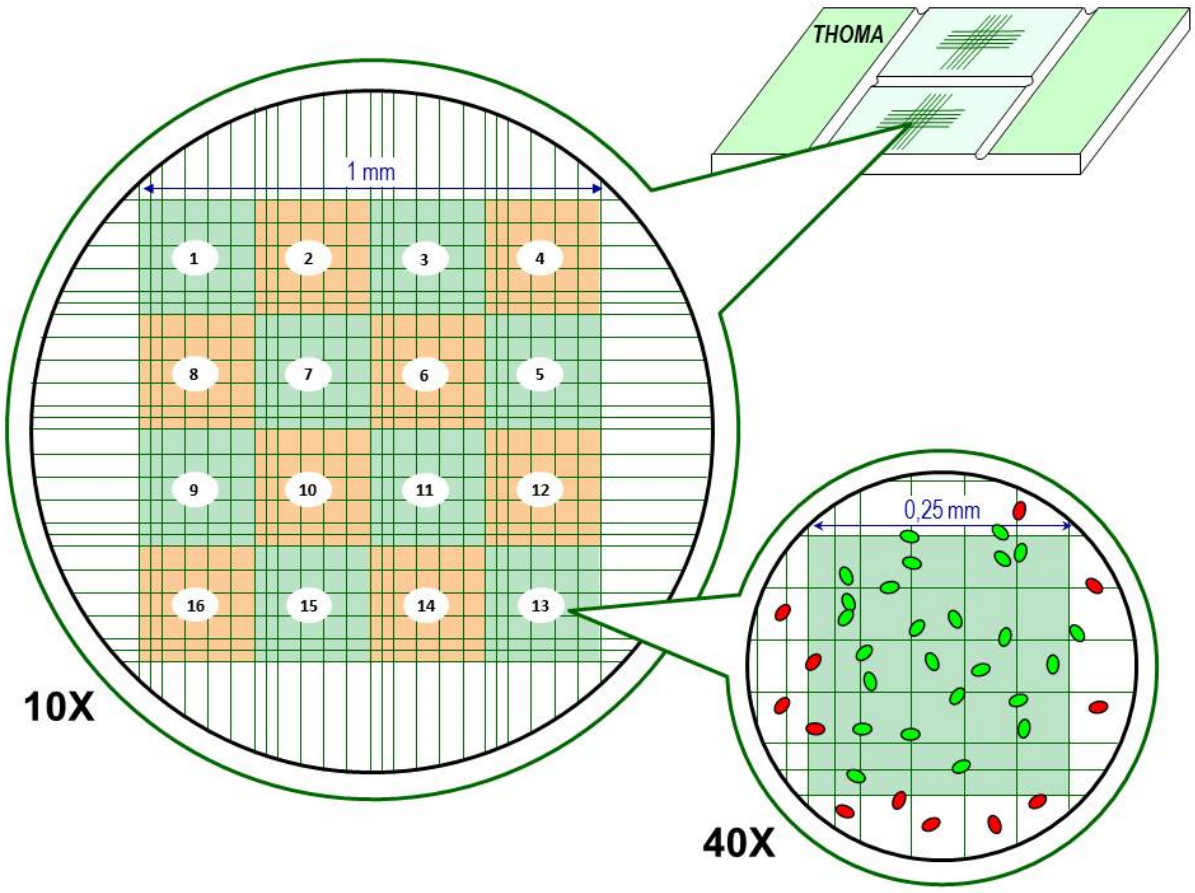


Figure 8: Thoma Cell Counting Chamber

APPENDIX 4:

Calculating Cell Viability and Calculated Absorbance

In order to reach a percentage of cell viability, the results obtained from the spectrophotometer are used. The absorbance of wells exposed to WGE is multiplied by 100 and then divided to the absorbance of control groups because the control group is taken as the normal life span of cells. For example:

The mean absorbance of wells with 35% WGE in medium: 0,545

The mean absorbance of wells with 35% distilled water in medium: 1,1466

Cell viability at 35%:

0,545 x 100 / 1,1466 = 47,53183325 = 47,6% (3 sf)

The calculated absorbance values are reached by subtracting the absorbance value of each well at 690 nm from those at 550 nm. So if a well with 10% WGE and 90% medium has an absorbance of 1,311 photons/sec at 550 nm and an absorbance of 0,054 photons/sec at 690 nm, the equation would be 1,311 – 0,054 = 1,257 photons/sec. The reason for such calculation is to eliminate the background absorbance of the plate which is measured at 690 nm.

APPENDIX 5:

Spectrophotometer:

A spectrophotometer is employed to measure the amount of light that a sample absorbs. The instrument operates by passing a beam of light through a sample and measuring the intensity of light reaching a detector. The beam of light consists of a stream of photons. When a photon encounters an analyte molecule (the analyte is the molecule being studied), there is a chance the analyte will absorb the photon. This absorption reduces the number of photons in the beam of light, thereby reducing the intensity of the light beam.[22]

[22]Blauch, David N. Spectrophotometry.html version 2.01 © 2000-2001,2009
http://www.chm.davidson.edu/vce/spectrophotometry/Spectrophotometry.html

APPENDIX 6:

Units and Abbreviations:

Cel:	Cells
CO_2:	Carbon dioxide
IC50:	half maximal inhibitory concentration
L:	Liters
ml:	milliliters
mm^3:	cubic millimeters
MTT:	Methylthiazol Tetrazolium Assay
MTS:	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
nm:	nanometers
sec:	seconds
WGE:	Wheat Germ Extract
XTT:	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
µl:	microliters
µm:	micrometers

APPENDIX 7:

How to Produce WGE

In order to produce wheat germ extract, wheat seeds are soaked in distilled water for 8 hours. However, as much as 10 ml is left of the 1 L distilled water for later usage. The seeds are then planted in soil and harvested after 10 days. The wheat grass was then cut and mashed using a plastic mortar. A few drops of distilled water is added as a solvent for wheat germ to help get the extract out. The liquid is taken through the 0.2 µm pore sized filter, which does not let bacteria pass through, over and over again until a liquid extract is reached.

BIBLIOGRAPHY (A-Z)

Alexander K, Markus H. CAM-Cancer Consortium. wheat germ extract. August 21, 2013 http://cam-cancer.org/CAM-Summaries/Dietary-approaches/Fermented-wheat-germ-extract

Blauch, David N. Spectrophotometry.html version 2.01 © 2000-2001,2009 http://www.chm.davidson.edu/vce/spectrophotometry/Spectrophotometry.html

Comin-Anduix B, Boros LG, Marin S, Boren J, Callol-Massot C, Centelles JJ, Torres JL, Agell N, Bassilian S, Cascante M. wheat germ extract inhibits glycolysis/pentose cycle enzymes and induces apoptosis through poly(ADP-ribose) polymerase activation in Jurkat T-cell leukemia tumor cells. J Biol Chem. 2002 Nov 29;277(48):46408-14. Epub 2002 Sep 25. http://www.ncbi.nlm.nih.gov/pubmed/12351627

Cory AH, Owen TC, Barltrop JA, Cory JG (July 1991). "Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture". *Cancer communications* 3 (7): 207–212. ISSN 0955-3541 http://www.ncbi.nlm.nih.gov/pubmed/1867954

Degen M, Alexander B, Choudhury M, Eshghi M, Konno S. Alternative Therapeutic Approach to Renal Cell Carcinoma: Induction of Apoptosis with Combination of Vitamin K3 and D-fraction. J Endourol. 2013 Nov 7. [Epub ahead of print] http://www.ncbi.nlm.nih.gov/pubmed/24195738

Düzgüneş, Nejat. Methods in Enzymology Volume 509 Nanomedicine: Infectious diseases, Immunotherapy, Diagnostics, Antifibrotics, Toxicology and Gene Medicine. Page 231. First edition. San Diego: Elsevier Inc. 2012 http://books.google.com.tr/books?id=88XS1FDIo1QC&pg=PA231&lpg=PA231&dq=40000+cells+96+well+plate+MTT&source=bl&ots=34czkw5V6L&sig=fHixwgdxFapFljRPkWv-ejR7HFM&hl=tr&sa=X&ei=tRiqUs6yOsWctAbuioHQDw&ved=0CF4Q6AEwBw#v=onepage&q=40000%20cells%2096%20well%20plate%20MTT&f=false

Farkas E. [wheat germ extract in the supportive therapy of colorectal cancer]. Orv Hetil. 2005 Sep 11;146(37):1925-31. http://www.ncbi.nlm.nih.gov/pubmed/16255377

Fetal Bovine Serum (FBS). Sigma-Aldrich. http://www.sigmaaldrich.com/life-science/cell-culture/cell-culture-products.html?TablePage=9628642

Ghoddusi J, Tavakkol Afshari J, Donyavi Z, Brook A, Disfani R, Esmaeelzadeh M Cytotoxic effect of a new endodontic cement and mineral trioxide aggregate on L929 line culture. Iran Endod J. 2008 Spring;3(2):17-23. Epub 2008 Apr 2 http://www.ncbi.nlm.nih.gov/pubmed/24171015

Glioblastoma multiforme. Wikipedia the Free Encyclopedia. http://en.wikipedia.org/wiki/Glioblastoma_multiforme

Glutamine in Cell Culture. Sigma-Aldrich. http://www.sigmaaldrich.com/life-science/cell-culture/learning-center/media-expert/glutamine.html

Green DR, Reed JC. Mitochondria and apoptosis. Science. 1998 Aug 28;281(5381):1309-12 http://www.ncbi.nlm.nih.gov/pubmed/9721092

“IC50.” Wikipedia, The Free Encyclopedia http://en.wikipedia.org/wiki/IC50

Judson PL, Al Sawah E, Marchion DC, Xiong Y, Bicaku E, Bou Zgheib N, Chon HS, Stickles XB, Hakam A, Wenham RM, Apte SM, Gonzalez-Bosquet J, Chen DT, Lancaster JM. Characterizing the efficacy of wheat germ extract against ovarian cancer and defining the genomic basis of its activity. Int J Gynecol Cancer. 2012 Jul;22(6):960-7. doi: 10.1097/IGC.0b013e318258509d http://www.ncbi.nlm.nih.gov/pubmed/22740002

Marcsek Z, Kocsis Z, Jakab M, Szende B, Tompa A. The efficacy of tamoxifen in estrogen receptor-positive breast cancer cells is enhanced by a medical nutriment. Cancer Biother Radiopharm. 2004 Dec;19(6):746-53. http://www.ncbi.nlm.nih.gov/pubmed/15665622

Mueller T, Jordan K, Voigt W. Promising cytotoxic activity profile of wheat germ extract (Avemar®) in human cancer cell lines J Exp Clin Cancer Res. 2011 Apr 16;30:42. doi: 10.1186/1756-9966-30-42 http://www.ncbi.nlm.nih.gov/pubmed/21496306

Oya Sena AYDOS, Aslıhan AVCI, Tülin ÖZKAN, Aynur KARADAĞ, Ebru GÜRLEYİK, Buket ALTINOK, Asuman SUNGUROĞLU. Antiproliferative, apoptotic and antioxidant activities of wheatgrass (Triticum aestivum L.) extract on CML (K562) cell line. Turk J Med Sci 2011; 41 (4): 657-663 © TÜBİTAK doi:10.3906/sag-0912-425 http://journals.tubitak.gov.tr/medical/issues/sag-11-41-4/sag-41-4-13-0912-425.pdf

Thoma cell counting chamber. http://insilico.ehu.es/counting_chamber/thoma.php

Ugur HC, Ramakrishna N, Bello L, Menon LG, Kim SK, Black PM, Carroll RS. Continuous intracranial administration of suberoylanilide hydroxamic acid (SAHA) inhibits tumor growth in an orthotopic glioma model.J Neurooncol. 2007 Jul;83(3):267-75. Epub 2007 Feb 20. http://www.ncbi.nlm.nih.gov/pubmed/17310267

van Meerloo J, Kaspers GJ, Cloos J. Cell sensitivity assays: the MTT assay. Methods Mol Biol. 2011;731:237-45. doi: 10.1007/978-1-61779-080-5_20 http://www.ncbi.nlm.nih.gov/pubmed/21516412