The Investigation of the Effect of the Concentration of *Rhus verniciflua* Herb on the Cell Viability of *Homo sapiens* Liver Cancer Cells

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ABSTRACT

Our lives are based on cells, the functional basic unit of organisms.[1] Our cells constantly divide, and sometimes they divide in an abnormal pattern, which is called cancer. Although some drugs are used to treat cancer, scientists think that using anticancerogenous herbs can also be effective in alleviating the disease. The focus of this study is to test the effect of concentration of an anticancerogenous herb on the viability of cancer cells. It is very difficult to test all of the anticancerogenous herbs and cancer cells, so *Rhus verniciflua* (sumac herb) and human (*Homo sapiens*) hepatocellular carcinoma liver cancer cells are tested. So the aim of this study is to investigate whether the concentration of the *Rhus verniciflua* herb has an effect on the liver cancer cells by measuring cell viability values after giving cells the herb in different concentrations. In the experiment, the herbs were liquefied and given to liver cancer cells via serial dilution and those were then installed into the microplate reader for determining the absorption values. These values were then processed and percent cell viability results were obtained. As the concentration of the herb drug increases, the absorption values, thus the cell viability results decreases.

In this investigation, it was found that increased concentrations of the sumac solution have an effect on the liver cancer cells. The least cell viability is observed in the 20 µM sumac solution and the most cell viability is observed in 1.2 µM sumac solution. Overall, the results of this study advocates the hypothesis that concentration of sumac solution affects the cell viability of hepatocellular carcinoma liver cancer cells and the optimum dosage of the drug is 2.0 µM, which would result in maximum potency.

Word Count: 299

[1] Campbell, N.A. Williamson, B., Heyden, R.J. pg. 6

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INTRODUCTION

I was always interested in cancer cells. My aunt, who is a pathologist, talked about cancer, what it did to ordinary cells and how it had been a detrimental disease for the past years. Although she told that there were not medicaments to cure the disease completely, she talked about some anticancerogenous herbs that could help the patients. I was surprised to hear that some botanical plants could help cure the disease. From that day on, I wondered how herbs could affect cancer cells.

Cell division is a complex process that is normally tightly regulated. In some cases, the cells that undergo mitosis for cell division grow in an unregulated pattern. This type of disease is named as cancer. Cancer occurs when problems in the genes of a cell prevent these controls from functioning properly. These problems may come from damage to the gene or may be inherited, and can be caused by various sources inside or outside of the cell. Cancer may also spread to more distant parts of the body through the lymphatic system or bloodstream.[2] Although it is referred as a deleterious disease, some benign cancer tumors do not spread throughout the body.

The present state of cancer indicates a crucial problem to human health. Especially brain, breast and liver cancers have arisen in several countries due to technological devices that transmit electromagnetic waves.[3] Determining the causes of cancer is complex and it is difficult to assign a specific cause. Many things are known to increase the risk of cancer, including tobacco use, radiation, lack of physical activity, poor diet and environmental pollutants.[4] These can directly damage genes or combine with existing genetic faults to cause the disease. However, approximately five to ten percent of cancers are entirely hereditary.

The type of cancer cell used in this investigation was liver cancer cell. Liver or hepatic cancer is a cancer that originates in the liver and is a tumor that grows on the surface or inside the liver. Liver tumors are discovered on medical imaging equipment or present themselves symptomatically as an

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[2] Anand P, Kunnumakkara A.B., pg 204.
[3] Kinzler, K.W., Vogelstein, B. pg 5.
[4] http://www.cancer.gov/cancertopics/causes (Retrieved on January 20, 2012)

abdominal mass, jaundice, nausea or liver dysfunction.[5] In an article I had read, liver cancer cells were praised for their depiction of accurate results when drugs were tested on them. *Liver cancer cells are common types of cancer cells that are treated with contemporary medicaments in order to perceive the effects of a particular drug and apply it to other cancer cells if applicable.*[6] Even though there were other cancer cells that could have been used, the liver cancer cells showed extraordinary results with other drugs, leading me to use it in my experiment.

Cancer prevention consists of active measures to decrease the risk of cancer.[7] The majority of cancer risk factors are environmental, which are mainly controllable. Thus, cancer is considered a preventable disease.[8] There are various ways to cure this disease to an extent, and it is conceived that anticancerogenous herbs contribute to the remedy of this disease.

There are more types of cytotoxic herbs than there are chemotherapeutic drugs. Scientists researched references to cancer herbs spanning 5000 years and 2500000 herbs. Of these, they determined that at least 3000 herbs possess some anti-cancer properties.[9] However, the mechanisms by which these anti-cancer herbs achieve their effects vary. Some arrest the fermentation process the cancer cells depend for survival, some inhibit cell division and some affect the pH to reduce the development of secondary tumors and metastasis.[10]

The herb used in this investigation was *Rhus verniciflua*. In an article I had read, the significant impact of this herb on cancer was appraised. *Flavonoids from Rhus verniciflua (sumac) exhibit selective antiproliferative and apoptotic effects on SV40-transformed liver tumor cells*.[11] The sumac plant not only has a significant effect on cancer cells but it is also a plant used in fields such as gastronomy and chemistry.

In addition to the research conducted by scientists, I desired to work on this herb that could help cells overcome cancer. **Does increasing the concentration of the liquiefied anticancerogenous**

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[5] Rosen, H.R. pg. 2429.
[6] http://cancer.gov/cancertopics/types/liver (Retrieved on March 26, 2012)
[7] Cancer. The Journal of World Health Organization.
[8] O'Dell, Michael W., pg 983.
[9] www.cancerslaves.com/botanical_approaches/individual_herbs.html (Retrieved on May 14, 2012)
[10] Cancer prevention: 7 steps to reduce your risk, Mayo Clinic Journal.
[11] Harris, T. Toxicol Journal 2005 Jan 15;155(1):115-25.

***Rhus verniciflua* given to cultures of human hepatocellular carcinoma cancer cells affect the viability of them under identical growth conditions by measuring the absorbance values of the cancer cells at 490nm after the addition of the sumac herb and calculating cell viability with an iMark Microplate Absorbance Reader?** This research question, along with the detailed biological aspects of this herb will be discussed throughout this paper.

HYPOTHESIS

The cell is the basic structural unit of organisms.[12] Cells strive for the regulation of daily activities and obtain energy to accomplish their tasks by the intake of nutrition. Some of these cells play a crucial role in growth and metabolism, whereas some function in cell division or protein synthesis.[13] Cells are created by cell division, which is the process by which a parent cell divides into two or more daughter cells. A healthy individual's cells divide regularly, but there are cases when cells can divide in an unregulated pattern.

Cancer is a term used for diseases in which abnormal cells divide without control and invade other tissues. There are several types of cancer treatment, including the consumption of anticancerogenous herbs. In this experiment, the type of cancer cell and herb and the proliferation conditions were stabilized so that the effect of the concentration of the herb can be observed. Since research has shown that *Rhus verniciflua* has anticancerogenous properties, the aim of this experiment is to observe whether the concentration of the anticancerogenous herb would affect the viability of the malignant *Homo sapiens* liver cancer cells.[14]

As the herb is given to cancerous cells that are stained with a specific solution, the cell viability starts to change. This process might be accelerated by increasing the concentration of the herb drug, which could even cure the disease to an extent. In light of this information, it can be hypothesized that **there is a relationship between the concentration of the extracted *Rhus verniciflua* and the *Homo sapiens* hepatocellular carcinoma cancer cells that the herb is given to.** The herb dilution with the highest concentration of *Rhus verniciflua* is expected to have the least survival percentage of hepatocellular carcinoma liver cancer cells.

[12] Campbell, N.A., Williamson, B., Heyden, R.J., pg 294.
[13] Maton, A., Hopkins, J.J., LaHart, S., Warner, D.Q., Wright, M., Jill, D., pg. 41
[14] http://gradworks.umi.com/33/21/3321691.html (Retrieved on February 4, 2012)

METHOD DEVELOPMENT AND PLANNING

To test the research question "Does increasing the concentration of the liquefied anticancerogenous sumac herb given to human hepatocellular carcinoma cancer cells affect the viability of the cancer cells that are in identical growth phases by measuring the absorbance values of the cancer cells at 490nm with a iMark Microplate Absorbance Reader under laboratory conditions?", different concentrations of sumac solution should be added to identical liver cancer cells.

I investigated this research question at Bilkent University and acquired laboratory access from Rengül Atalay, a scientist working at Bilkent. The equipment and the solutions used in the experiment were supplied by the Department of Molecular Biology and Genetics.

In order to observe the effect of different concentrations of sumac, one should obtain extracts from a single sumac plant during the liquefying process (Appendix 1) of the plant. By doing this, one can acquire his testing groups. I obtained the extract HUH7-a5-6 from the sumac plant by using maceration, a technique that involves using an organic solvent to extract compounds.[15] I used an organic solvent called DMSO (Appendix 1) to extract HUH7-a5-6 from the *Rhus verniciflua*. As for the different concentrations, I was doing research when I discovered a process called serial dilution (Appendix 2), which is the stepwise dilution of a substance in solution.[16] In this experiment, the concentrations of the extract were halved each time by using RPMI media (Appendix 3) in order to observe the outcome of different concentrations. As I used microtiter plates and planted liver cancer cells to each well for the experiment to work properly, this method was suitable for the examination of the concentration effect.

The cells that I planned to use were *Homo sapiens* cells and had 'hepatocellular carcinoma', a specific type of human liver cancer cell numbered as CRL-2233 in the ATCC catalog (Appendix 4).[17] These cancerogenous cells were obtained from a 42-year old male that was diagnosed with this disease

[15] http://www.unido.org/fileadmin/user_media/Publications/Pub_free/Extraction_technologies_for_medicinal_and_aromatic_plants.pdf (Retrieved on December 5, 2011)
[16] http://en.wikipedia.org/wiki/Serial_dilution (Retrieved on January 3, 2012)
[17] http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx?ATCCNum=CRL-2233&Template=cellBiology (Retrieved on January 9, 2012)

in Hacettepe Oncology Hospital. The liver tissue removed from the patient was used as the liver cell stock and was ready after the viability and contamination control steps. These cells were also adherent cells that stuck to the bottom of the wells, which was beneficial for screening. For comparing the effectiveness of the differently concentrated sumac extracts, RPMI solutions were prepared. RPMI medium is a form of medium used in cell culture experiments. The most important property of RPMI is that it protects the cells from freezing or burning, inhibits undesired reactions and is a suitable media for diluting cells.[18] So if one cultures the liver cancer cells from the stock to the microtiter plate with RPMI media and different concentrations of sumac herb, together with the control well that only contains RPMI, he can compare the effectiveness of the differently concentrated sumac medication and calculate the cell viability from the absorption values, for one shall assume that the cells with only RPMI would be all living and have not reacted or contaminated. A good medication should also be present to observe the optimum effect of the medication. While I was researching for the control drug, I came across CPT, also called camptothecin (Appendix 5), which showed remarkable anticancer activity.[19] So I presumed that two rows of wells with CPT would enable me to compare my absorbance values from the sumac solutions, providing knowledge about the effectiveness of the herbal drug. During the experiment, I vortexed the solutions 20 times. Vortexing is also an important factor, since homogeneity plays a crucial role in the investigation.

After researching the approximate volume of RPMI and CPT I will be using, I found out that using 10.00mL RPMI and 3.00mL CPT for the whole investigation would be suitable.[20] Apart from the drugs used, the concentration of the stock solution, thus the number of cells should be found in *105*cells/mL[21], the ideal cell concentration at which the effects of the drugs could be detected. After researching a suitable method, I found the Trypan Blue Exclusion Assay (Appendix 6). Trypan blue is a stain used to color dead cells blue. The stained cells would be put on counting slides and the counting could be performed in a Countess Cell Counting Chamber (Appendix 6) that would give the concentration of total, live and dead cells on the slides. One can then assure that the number of cells in

[18] http://www.chem.wisc.edu/areas/reich/DMSO (Retrieved on March 7, 2012)
[19] Samuelsson, G., pg. 372.
[20] http://www.i-cancer.org/bioinformatics2011 (Retrieved on March 7, 2012)
[21] http://www.cellsignal.com/products/5723.html (Retrieved on March 8, 2012)

each well would be the same per mL, for the number of cells can change the absorption values acquired from the plate reader.

In addition, for the iMark Microplate Reader to find out the optical densities, one must stain the cells in a colorful solution that should bind to the cells and change their optical densities, enabling a correlation between the drug concentrations and the cell absorbance values. I found out that the best colorful solution would be SRP.[22] SRP (Appendix 7) is an abundant protein-RNA complex that targets specific proteins in eukaryotes.[23] SRP is also suitable for its adherent nature and its affinity of proteins. The more cells alive, the more stain it will produce, thus affecting the optical densities (Appendix 8) of the cells. Although there were some more colorful solutions that I could have used, the laboratory only offered this solution and told me that it would be more reliable. Only the staining process was conducted in a dark room outside the culture hood. If one kept the cells under sunlight during staining, light would damage the volatile SRP and inhibit its activity, altering the optical densities read by the iMark Microplate Reader (Appendix 8).

In order to find the effect of the concentration of the sumac extracts on the absorbance values of the cancer cells at 490nm, a cell stock should be obtained and its concentration should be determined by the Trypan Blue Exclusion Assay. After loading and counting the cells on a Countess cell counter, one should calculate the volumes of RPMI and the original stock solution required to 105cells/mL of solution to start setting up the 96-well plate (Appendix 8). Once the plate is filled with 6 rows and 7 columns consisting of wells filled with only RPMI media and cells with RPMI, it should CO_2 for 24 hours in a tissue culture incubator for cell proliferation. After the time elapses, the drug dilutions for both the sumac and CPT should be prepared, starting with 40.0µM and ending at 1.25µM, using the serial dilution technique and added to the appropriate rows and columns of the plate. After 48 hours in the incubator, SRP can be added into the wells and incubated for another hour. In the last step, the plate should be put in the iMark Microplate Absorbance Reader and the cell absorbance values at 490nm should be calculated and processed.

[22] http://mcb.berkeley.edu/courses/mcb110/210X/4-Halic-ribo-srp.pdf (Retrieved on March 10, 2012)
[23] http://www.ncbi.nlm.nih.gov/pubmed/21330537 (Retrieved on February 19, 2012)

This method will be a constant procedure and will be repeated 3 times in order to achieve more accurate results.

METHOD

Materials Used in the Experiment

- 3.00mL 40.0µM liquid HUH7-a5-6 extract from *Rhus verniciflua*
- 3.00mL liquid *Homo sapiens* 'Hepatocellular carcinoma' liver cancer cell stock of unknown concentration
- Vortexer

  CO2
- 96-well plate
- 2 microfuge tubes with 0.4% Trypan Blue
- P20,P200 and P1000 micropipeters and tips
- 30 1.50mL microfuge tubes
- 30 15.00mL conical centrifuge tubes
- 30 sterile pipets
- 2 microfuge tube racks
- Countess cell counter and 2 counting slides
- 10.00mL RPMI media
- 3.00mL 40.0µM liquid CPT
- 1.50mL liquid SRP
- iMark Microplate Absorbance Reader
- Kimwipes
- Marker
- 3 Biohazard bags

Procedure

1) Perform the Trypan Blue Exclusion Assay (Appendix 6).

   105 cells/mL. (Appendix 6).

3) Set up the microtiter plate (Appendix 8) with cells and RPMI.

4) Obtain the sumac stock of 40.0μM and the CPT stock of 40.0μM and prepare the dilutions according to Table 1.

Two-fold Dilution Series (Appendix 2)				
Tube	**Final drug concentration (±0.5 μM)**	**Volume of RPMI added (±0.05 μL)**	**Initial drug concentration (±0.5 μM)**	**Volume of drug added (±0.05 μL)**
1	20.0	200.00	40.0	200.00
2	10.0	200.00	20.0	200.00
3	5.0	200.00	10.0	200.00
4	2.5	200.00	5.0	200.00
5	1.2	200.00	2.5	200.00

Table 1: The final drug concentration, volume of RPMI added, concentration of drug originally added and the volume of the drug added, along with their appropriate units. Note that the stock solution is 40.0μM, there is a control tube (#6) that will only contain cells and media, not the drug and there is a column with only media, so there will be a total of 8 full columns on the 96-well plate.

5) Add the sumac solutions and the CPT drug to the microtiter plate with cells (Appendix 2).

6) Perform the Cell Viability Assay (Appendix 8).

RESULTS

Concentration	15 wells (3	Volume of RPMI media	Absorption	Volume of sumac drug	Type of plat	Pressure of the	Temperature	Type of Incubator	Conditions of the Incubator	Type of	Absorption	Concentration	Volume of	Volume of
20.0	1	50.00	0.163	50.00	*Rhus verniciflua*	1067.0	22.00	Tissue Culture Incubator	37°C 2		0.151	20.0	50.00	20.00
	2	50.00	0.175	50.00							0.162	20.0	50.00	20.00
	3	50.00	0.119	50.00							0.167	20.0	50.00	20.00
10.0	1	50.00	0.184	50.00	*Rhus verniciflua*	1067.0	22.00	Tissue Culture Incubator	37°C 2		0.172	10.0	50.00	20.00
	2	50.00	0.168	50.00							0.165	10.0	50.00	20.00
	3	50.00	0.176	50.00							0.174	10.0	50.00	20.00
5.0	1	50.00	0.207	50.00	*Rhus verniciflua*	1067.0	22.00	Tissue Culture In	37°C 2		0.186	5.0	50.00	20.00
	2	50.00	0.209	50.00							0.194	5.0	50.00	20.00

	3	50.00	0.206	50.00				cubator			0.192	5.0	50.00	20.00
2.5	1	50.00	0.215	50.00	*Rhus verniciflua*	1067.0	22.00	Tissue Culture Incubator	37°C 2		0.210	2.5	50.00	20.00
	2	50.00	0.210	50.00							0.198	2.5	50.00	20.00
	3	50.00	0.224	50.00							0.203	2.5	50.00	20.00
1.2	1	50.00	0.362	50.00	*Rhus verniciflua*	1067.0	22.00	Tissue Culture Incubator	37°C 2		0.341	1.2	50.00	20.00
	2	50.00	0.353	50.00							0.336	1.2	50.00	20.00
	3	50.00	0.356	50.00							0.348	1.2	50.00	20.00

Table 2: The final concentration of the sumac plant drug, concentration and volume of drug originally added, the absorbance values acquired from the different concentrations of sumac, volume of RPMI media added, type of drug, type of incubator and its conditions, along with the concentration and volume of the control drug CPT, the absorbance values recorded for CPT, the volume of SRP and room conditions depicted explicitly with the appropriate uncertainties and units.

During the experiment, one can realize some changes with his senses. These qualitative data can be:

- The production of bubbles after the drugs are added to the wells, indicating a reaction or an error related to pipetting
- The plate being hot and moist and the cells releasing an odor when acquired from the incubator.
- The production of a yellow color after the SRP incubation.

Absorption Values for the Liver Cancer Cells Treated with Sumac and CPT at 490 nm (± 0.005 AU)					
	Concentration of Sumac Drug Given to the Cells (± 0.5 µM)				
Trials (Well Rows)	**20.0**	**10.0**	**5.0**	**2.5**	**1.2**
1	0.163	0.184	0.207	0.215	0.362
2	0.175	0.168	0.209	0.210	0.353
3	0.119	0.176	0.206	0.224	0.356
	Concentration of CPT Drug Given to the Cells (± 0.5 µM)				
Trials (Well Rows)	**20.0**	**10.0**	**5.0**	**2.5**	**1.2**
1	0.151	0.172	0.186	0.210	0.341
2	0.162	0.165	0.194	0.198	0.336
3	0.167	0.174	0.192	0.203	0.348

Table 3: The absorption values obtained from the iMark microplate reader for cells in each of the different concentrations of drugs under identical conditions such as temperature, pressure and volumes of SRP at 490 nm wavelength. The difference between the numbers indicates the distinct rates of survival, thus cure for this type of liver cancer.

Percentage Cell Viability Values for the Liver Cancer Cells Treated with Sumac and CPT at 490 nm (%)					
	Concentration of Sumac Drug Given to the Cells (± 0.5 µM)				
Trials (Well Rows)	**20.0**	**10.0**	**5.0**	**2.5**	**1.2**
1	1.104	1.627	2.694	3.162	13.384
2	1.396	1.248	2.709	3.234	12.762
3	0.612	2.349	2.687	4.367	13.195

	Concentration of CPT Drug Given to the Cells (± 0.5 μM)				
Trials (Well Rows)	**20.0**	**10.0**	**5.0**	**2.5**	**1.2**
1	0.103	1.347	1.419	3.134	12.637
2	0.134	1.294	1.613	2.872	11.594
3	0.149	1.365	1.561	2.941	12.682

Tables 4 and 5: The percentage cell viability values obtained from the iMark microplate reader for cells in each of the different concentrations of drugs under identical conditions such as temperature, pressure and volumes of SRP using the absorption values in Table 3. The values are calculated by taking the cells with no drugs as a reference, since it should have 100% viability. The different values show that the drug has a major effect on this type of cells, depending on the concentration of the drug.

Using the Microsoft Excel 2007 program, one can determine the statistical relationship between the sumac concentration and cell viability. The following formulas are used to obtain the values in Table 6:

Mean:

$$\bar{X} = \frac{\sum_{i=1}^{n} X_i}{n}$$

where;

n is the largest number of trials (for this experiment *n*=3)
is the absorbance value at 490 nm for the first trial

Standard Deviation:

$$\sigma = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(x_i - \bar{x})^2}$$

where;

n is the largest number of trials (for this experiment *n*=3)
is the absorbance value at 490 nm for the first trial

is the mean value of the corresponding group/data

Standard Error:

=

where;

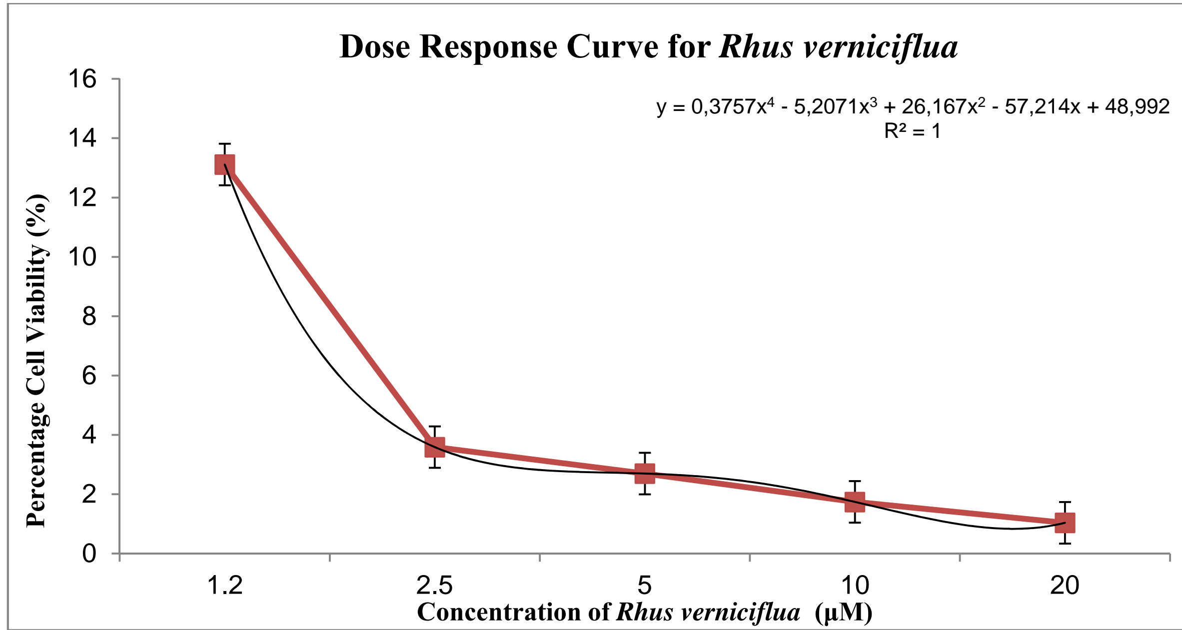
n is the largest number of trials (for this experiment *n*=3)

is the absorbance value at 490 nm for the first trial

is the standard deviation of the corresponding group/data

STATISTICAL ANALYSIS					
Mean	1.037	1.741	2.697	3.588	13.114
Median	1.104	1.627	2.694	3.234	13.195
Range	0.784	1.101	0.022	1.205	0.622
Variance	0.157	0.313	0.000	0.457	0.102
SD	0.049	0.059	0.011	0.076	0.029
SE	0.077	0.050	0.005	0.002	0.043
t	2.571	2,571	2.571	2.571	2.571
95%CI(SE X T(0,05,df))	0.456	0.643	0.013	0.777	0.367
95%CI(EXCEL)	0.347	0.490	0.010	0.592	0.280

Table 6: The mean median, range, variance, SD, SE, t, 95% CI(SE X $T_{(0,05,df)}$) and 95% CI(EXCEL) calculations of the data of Tables 4 and 5.



Graph 1: A dose response curve that depicts the relationship between the *Rhus verniciflua* (sumac) concentration and the percentage viability of the human liver cancer cells under identical conditions. The significant decrease in between 1.2 and 2.5 indicates the drug's effective point (LC_{50}), the dose required to kill half the members of a tested population after test duration. Other concentrations after the LC_{50} form a plateau as expected. Percentage error bars are shown by using the 95% CI in the experiment.

Anova: Single Factor						
SUMMARY						
Groups	***Count***	***Total***	***Average***	***Variance***		
Column 1	3	3.112	1.037333	0.156997		
Column 2	3	5.224	1.741333	0.312854		
Column 3	3	8.09	2.696667	0.000126		
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	293.577	4	73.39426	356.8105	9.73E-11	3.47805
Within Groups	2.056953	10	0.205695			
Total	295.634	14				

Table 7: The Anova: Single Factor results obtained from the data in Table 4. The P-value being numerically less than the alpha value of 0.05 indicates the truth of the hypothesis.

CONCLUSION AND EVALUATION

In this experiment, the effect of the concentration of the anticancerogenous herb *Rhus verniciflua* on the cell viability of *Homo sapiens* hepatocellular carcinoma cancer cells was investigated. Different concentrations of the sumac herb were prepared and added to identical human hepatocellular carcinoma cancer cells to observe the ability of the herb to act as an anticancerogenous drug by eradicating malignant cancerous cells.

These herbal drugs were prepared from the *Rhus verniciflua* herb. DMSO solutions were used to dissolve the extract (HUH7-a5-6) and the cell concentration was found by the Trypan Blue Exclusion Assay. After finding the initial cell concentration and diluting it to the desired concentration 105cells, different concentrations of sumac were prepared with RPMI media using serial dilution. The temperature and pressure of the room, which were 25.0°C and 1067hPa respectively, were stabilized and 50.00μL sumac and 50.00μL RPMI were prepared before the dilutions. The volume of the control drug camptothecin was also maintained at 50.00μL in order to uniquely measure the effect of the concentration of the herbal solution.

The sumac and camptothecin dilutions were then added to identical cultures of liver cancer cells and the cells were stored in an incubator after the addition for 24 hours. Afterwards, the cells were stained with 20.00μL of signal recognition protein and the absorbance values of these cells at 490nm were found by an absorbance reader. The absorbance values were further processed into percentage cell viability values, which were compared to find the relationship between the concentration of *Rhus verniciflua* and the cell viability of the cancer cells.

The results of the experiment depict that cell viability decreases as the concentration of sumac increases, which can be inferred from the data in Tables 4 and 5. The mean values of cell viability of the concentrations 20, 10, 5, 2.5 and 1.2 are 1.037, 1.741, 2.697, 3.588 and 13.114 respectively. As observed, there is a peak in between the mean values 2.5 and 1.2, which can also be seen from the

absorbance values of 0.215 and 0.362 in the first trial. So it can be deduced that in this experiment the median lethal dose, LC_{50}, of sumac is approximately 2.0μM, which is also the optimum dose of this particular drug that would result in maximum efficiency (See Graph 1).[24]

Furthermore, it can be seen that the data in Tables 3, 4 and 6 support the hypothesis that there is a relationship between the concentration of the *Rhus verniciflua* solution added and the percent cell viability of *Homo sapiens* hepatocellular carcinoma cancer cells. In each concentration, one can observe the effectiveness of the sumac solution by comparing it to the control drug CPT, an efficient anticancerogenous agent (See Tables 4 and 5). In addition to finding a direct relation from Graph 1, one can assert that the effectiveness starts to degrade after the LC_{50} of 2.0μM and the higher concentration points reach a plateau, which indicates that increasing the concentrations after the LC_{50} did not have a significant effect on the cell viability of the cancer cells.

Additionally, the hypothesis is advocated by the source, which explicitly stated "*Flavonoids from Rhus verniciflua (sumac) exhibit selective antiproliferative and apoptotic effects on SV40-transformed liver tumor cells*."[25] The literature value in this source is numerically close to the value found in this investigation.

According to the P- value of 9.73E-11 obtained from the anova statistical analysis, the hypothesis is also proven to be true since the value is smaller than the alpha value of 0.05 (See Table 7). In short, one can state that as the concentration of the sumac solution changes, the number of the stained liver cancer cells and the absorbance values changes, which also connotes to a change in cell viability.

The Error and Uncertainty

In the investigation, there were some errors and uncertainties due to the equipment used or the environment the experiment was performed in. Although digital devices such as electronic scales, the

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[24] Hodgson, E. pg 52.
[25] Harris, T. Toxicol Journal 2005 Jan 15;155(1):115-25.

absorbance reader and the vortexer minimized the uncertainty of the results, there were some factors that posed digression. After doing the statistical analysis, one can see that the standard errors are very small in value, even smaller than 10%, connoting the accuracy of the results in the study. As one can infer, the 20.0μM concentration has the highest standard error of 0.077 and the 2.5μM concentration has the lowest standard error of 0.002 (See Table 6).

When looked at Tables 4 and 6 there is a regular pattern the data follow, indicating little variability between the absorbance values of different concentrations. There is a 4th power correlation between the cell viability results even though the data group seems scattered (See Graph 1).

The statistical analysis also gives information about precision. The standard deviations are 0.049, 0.059, 0.011, 0.076 and 0.029 respectively for the concentration values. Since smaller values of deviation indicate higher precision of the data group, it can be deduced that the investigation is precise.

Although the standard error and standard deviation values are small, indicating accuracy and precision, and one can state that the investigation is reliable, there are both systematic and random errors in the experiment that can be inferred from the error bars in Graph 1, though the percentage error is not a great value.

The errors and the uncertainties in the investigation were minimized by stabilizing the controlled variables such as:

- The volume of RPMI media put in each dilution and well is constant in each trial so that contamination can be tested and the dilutions can be compared in identical media.
- The volume of sumac drug initially added to each dilution is maintained and the concentration is changed with the addition of RPMI media.
- The type of plant is kept constant, for the anticancerogenous effect of that herb only is investigated.

- The conditions of the incubator are maintained due to their effects in cell proliferation and viability.
- The type, concentration and volume of the control drug are kept constant. CPT is used for comparing the anticancerogenous effect of the sumac herb and the concentrations and volumes of the drug are identical to those of the sumac solution.
- The volume of SRP is maintained so that it would not change the absorbance readings and give inaccurate results.
- The type of cancer cell is kept constant so that identical liver cells are affected by the drug.
- The time required for cell growth and development was maintained, first for an hour, then 24 hours, lastly 48 hours in the incubator.

Even though these factors were controlled, it is perceived that an experiment has error-posing components as long as it consists of measurements and one cannot acquire infallible results.

- Contamination by microorganisms or other non-sterile equipment could have affected the lifespan and nature of the tumor cells, diverting the values of the absorbance reader and resulting in the incorrect calculation of cell viability.
- The cells were prepared in a tissue culture hood. The culture hood has a light source, so the cells could have absorbed the incoming light from the source during the preparation of the plate or the solutions, changing the absorbance values.

In order to improve the investigation, one has to decrease the errors for increasing the accuracy of the experiment. The constancy of the controlled variables was crucial even though some were not stabilized completely.

- The light source could be maintained by performing the whole experiment in a dark room, preventing the absorption of different wavelengths of light.

Further Investigations

After observing the results and attaining an answer to the question "Does increasing the concentration of the extracted anticancerogenous *Rhus verniciflua* given to *Homo sapiens* liver cancer cell and the cell viability of the liver cancer cells that are in identical phases found by measuring the absorbance values of the cancer cells at 490nm after the addition of the sumac herb by the help of an iMark Microplate Absorbance Reader?", a new question arises: Does the concentration of the extracted anticancerogenous *Rhus verniciflua* affect all *Homo sapiens* cancer cells? Research can be conducted to investigate the effect of this particular herb on other cancer cells like breast or heart cancer cells.

Additionally, it would be intriguing to examine the optimum dosages of sumac for each type of cancer cell. By this investigation, one can acquire more knowledge about the nature of this herb and use it for cancer treatment. There are several types of cancer cells that can be experimented in order to proceed and find an answer to the question stated.

Cancer is a malignant disease that has been present in our lives since the advancement of technology. As scientists try to find a solution to this disease, there are oncologists who believe that the cure for cancer can come from nature itself and the herbs found could prove quite useful. In this investigation, it is found that the concentration of the sumac herb affects the cell viability of the hazardous cancer cells. As people start discovering more of these herbs, they will be inclined to consume more herbs in their daily lives and use the herbs' benefits in cancer treatment to prevent major diseases perceived as fatal today.

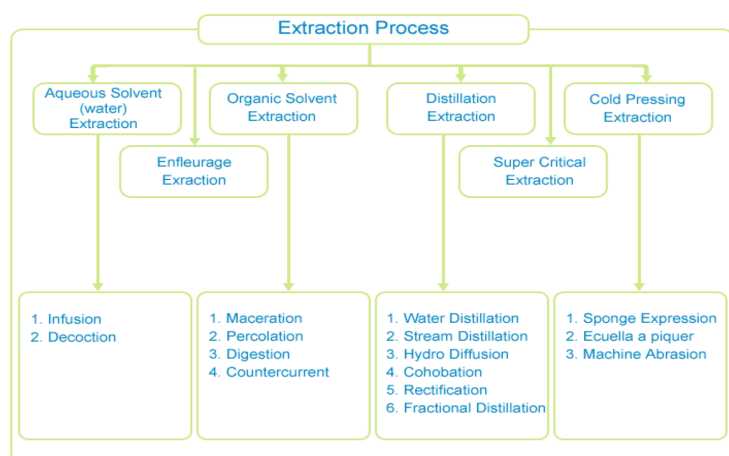
APPENDICES

Appendix 1: Liquefying (Extraction) Process of the Herbs and the Use of DMSO

Extraction is the process in which the plant tissues are treated with specific solvents whereby the medicinally active constituents are dissolved out, cell tissues and most of inactive or inert components remain undissolved. The choice of the plant material for extraction depends on its nature and the components are required to be isolated. The solvents used for extraction purposes is known as "Menstruum" and residue left after extracting the desired constituents is known as "Marc".[26]

From the stand point of pharmacy, the purpose of a solvent is to remove from a solid, either in part or in its entirety such substances that may be rendered to a liquid. When the material has extracted, the "Menstruum" is known as "Vehicle" or "Carrier" of the extracted materials. Solvents differ widely from each other, not only in differing boiling points, but how they act or react with substances in which they come in contact.[27]

CH3)2SO.[28] It was first synthesized in 1866 by the Russian scientist Alexander Zaytsev, who reported his findings in 1867. This colorless liquid is an important polar solvent that dissolves both polar and nonpolar compounds and is commonly used as an extracting reagent for a wide range of organic solutes and solvents in techniques such as batchwise single stages, ion pair extractions and in extraction procedure involving maceration in plants.[29] So for the investigation, DMSO was used to extract the particular sumac extract used in this experiment.



Figures 1 and 2: The extraction process (maceration was used in the experiment) and the DMSO used in the investigation.

[26] http://www.motherearthliving.com/health-and-wellness/herbal-extracts.aspx (Retrieved on April 3, 2012)
[27] http://www.satveda.com/extraction-system.html (Retrieved on March 24, 2012)
[28] Novak, K. M, p. 619.
[29] http://www.cancertutor.com/Cancer/DMSO.html (Retrieved on March 24, 2012)

Appendix 2: Serial Dilution

Serial dilution is the stepwise dilution of a substance in solution.[30] Usually the dilution factor at each step is constant, resulting in a geometric progression of the concentration in a logarithmic fashion.[31] This technique was found by Robert Koch in 1882 at the Imperial Health Office in Berlin.[32] Serial dilutions are used to accurately create highly diluted solutions as well as solutions for experiments resulting in concentration curves with a logarithmic scale.[33]

Procedure for adding the serial dilutions to a microtiter plate with cells:

1. After the preparation of the sumac (*Rhus verniciflua*) dilutions, move the 96-well plate from the tissue culture incubator.
2. Line up the drug dilutions for the sumac herb first in a single row in a microfuge tube rack. Then line the CPT dilutions at the back of the rack to not get confused.
3. Slide the top off the plate to uncover column #8 on the right hand side of the 96-well plate. It is important that the remainder of the left side is kept covered.
4. Starting with the 40 µM Sumac herb stock tube, add 50 µL each to rows A, B and C of column #8. Do not forget to mix the contents of the well up and down gently with the pipet.
5. Change the pipet tip and slide the plate top to uncover column #7 of the plate.
6. From the microfuge tube labeled 1, which is the 20 µM sumac drug dilution, pipet 50 µL each into rows A, B and C of column #7.
7. Repeat steps 8- 9 for the appropriate Sumac concentration to columns #3-#6 and rows A-C. Make sure to mix the contents gently and change pipet tips in between different columns.
8. Add 50 µL of RPMI media to rows A-C in column #2. Remember that there is no drug in this column.
9. Add 50 µL of RPMI media to rows A-C in column #1. Remember that there is no drug or cells in this column. This will bring the total volume of media in these wells up to 100 µL.
10. Repeat steps 7-12 for CPT. Fill the CPT drugs to rows D-F in the same columns under the Sumac herb wells.
11. Cover the plate with the lid and place it back into the tissue culture incubator set at optimum $CO2$).
12. Record the time of placement of the cells. The cells should be incubated for 48 hours.
13. Clean up.

[30] http://en.wikipedia.org/wiki/Serial_dilution (Retrieved April 9, 2012)
[31] http://www.bio.umass.edu/micro/immunology/elisa/serial.htm (Retrieved April 15, 2012)
[32] Brock, Thomas D., pg. 67.
[33] http://rothlab.ucdavis.edu/protocols/M04.html (Retrieved April 19, 2012)

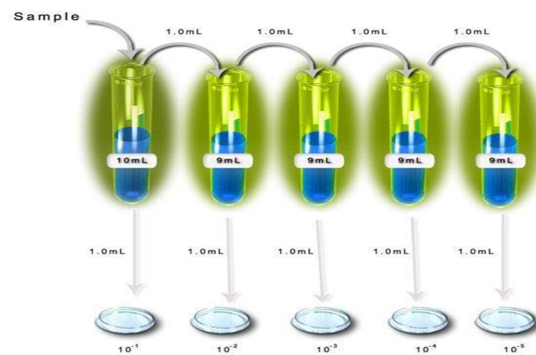


Figure 3: This picture depicts the steps of serial dilution and how the method was used in the experiment.

Appendix 3: The Use of RPMI

Roswell Park Memorial Institute medium, commonly referred to as **RPMI**, is a form of medium used in cell culture and tissue culture. It has traditionally been used for growth of Human lymphoid cells. This medium contains a great deal of phosphate and is formulated for use in a 5% carbon dioxide atmosphere. RPMI 1640 has traditionally been used for the serum-free expansion of human lymphoid cells.[34]

Figure 4: The RPMI media used in the investigation.

[34] http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-Culture/Mammalian-Cell-Culture/Classical_Media/RPMI.html (Retrieved April 16, 2012)

Appendix 4: The ATCC Catalog

The American Type Culture Collection (ATCC) is a private, not-for-profit biological resource center whose mission focuses on the acquisition, authentication, production, preservation, development and distribution of standard reference microorganisms, cell lines and other materials for research in the life sciences. Established in 1914 and originally incorporated by scientists in 1925 to serve as a worldwide repository and distribution center for cultures of microorganisms, the ATCC has developed into the global leader in research and development expertise for identifying, characterizing, preserving and distributing a wide range of cell lines and microbes.[35]

ATCC's collections include a wide range of biological materials for research, including cell lines, molecular genomics tools, microorganisms and bioproducts. The organization holds a collection of more than 4,000 human, animal and plant cell lines and an additional 1,200 hybridomas. The molecular genomics collection at ATCC contains 8 million cloned genes from a host of species, including human, mouse, soybean, rat, monkey, zebrafish and several disease vectors. ATCC's microorganism collection includes a collection of more than 18,000 strains of bacteria from 900 genera, as well as 2,000 different types of animal viruses and 1,000 plant viruses. In addition, ATCC maintains collections of protozoans, yeasts and fungi with over 49,000 yeast and fungi strains from 1,500 genera and 2,000 strains of protists.[35]

The ATCC catalog was used to determine the specific number of the 'hepatocellular carcinoma' disease that the *Homo sapiens* liver cancer cells had. It was found that 'hepatocellular carcinoma' was numbered as CRL-2233 in the ATCC catalog.[35]

Appendix 5: The Use of CPT (Camptothecin)

CPT was discovered in 1966 by M. E. Wall and M. C. Wani in systematic screening of natural products for anticancer drugs and showed remarkable anticancer activity in preliminary clinical trials but also low solubility and (high) adverse drug reaction.

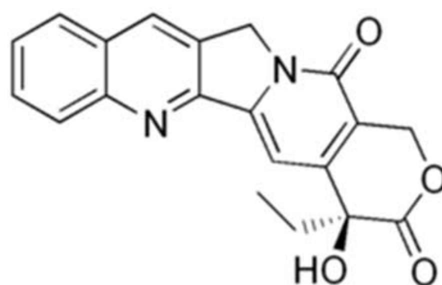


Figure 5: The molecular structure of CPT.

[35] http://www.atcc.org (Retrieved on January 9, 2012)

Appendix 6: Trypan Blue Exclusion Assay and Countess Cell Counter

Trypan blue is a vital stain used to selectively color dead tissues or cells blue. Live cells or tissues with intact cell membranes are not colored by this stain. Since cells are very selective in the compounds that pass through the membrane, trypan blue is not absorbed in a viable cell; however, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue color under a microscope. Since live cells are excluded from staining, this staining method is also described as a dye exclusion method.[36]

These cells are loaded on a **countess slide**, a microscope slide that is especially designed to enable cell counting, and the cell counter counts the cells based on the number of cells stained and gives an estimate of total, living and dead cells, along with the percent viability. The depth of the sink is predefined, thus the volume of the counted culture can be calculated and with it the concentration of the cells. [37]

Procedure for Trypan Blue Exclusion Staining:

1. Obtain a tube with 3 mL of human liver cancer cells and gently mix them up and down by a sterile 2 mL pipet.
2. Take a sterile 1.5 or 2 mL microfuge tube and transfer 200 µL of the cell suspension to the microfuge tube. Then discard the pipet.
3. Label a tube 'COUNT'
4. Remove 20 µL of cells from the tube with 200 µL of cells and transfer the 20 µL to the 'COUNT' tube.
5. Add 20 µL of the 0.4% Trypan Blue stain to the 'COUNT' tube and mix the sample gently by a sterile pipet.
6. Let the cells stain for 5 minutes.

Procedure for counting cells using the Countess™ Cell Counter:

1. After the time period, acquire a countess cell counting chamber slide and pipet 10 µL into each of the two chamber ports of the slide. Label these ports 'A' and 'B'.
2. Discard the 'COUNT' tube into a biohazard bag.
3. Turn on the cell counter and insert the slide into the slide port.
4. Count the cells by pressing 'COUNT CELLS'.
5. Record the cell count.

[36] http://groups.molbiosci.northwestern.edu/morimoto/research/Protocols/II.%20Eukaryotes/A.%20Cell%20Culture%20Trypan%20Assay (Retrieved April 24, 2012)
[37] http://celeromics.com/en/resources/docs/Articles/Cell-counting-Neubauer-chamber.pdf (Retrieved April 24, 2012)

Procedure for diluting cells to the desired concentration:

alculate the volume of original cells needed using the values of the original cell 106cells/mL), the desired final solution volume (5 mL) and a desired final concentration (105 cells/mL) by a dilution calculator.

2. Record the volumes of cells (530 μL) and RPMI media (447 μL) that need to be combined to achieve the desired cell concentration.
3. Remove the counting chamber slide and discard into the biohazard bag.

Figures 6 and 7: The Trypan Blue Stain and the Countess Cell Counter used in the investigation.

Appendix 7: The Use of SRP

The signal recognition particle (SRP) is an abundant, cytosolic, universally conserved ribonucleoprotein (protein-RNA complex) that recognizes and targets specific proteins to the endoplasmic reticulum in eukaryotes and the plasma membrane in prokaryotes. It stains desired cells and discerns them.[38] It is a volatile liquid and is sensitive to sunlight.

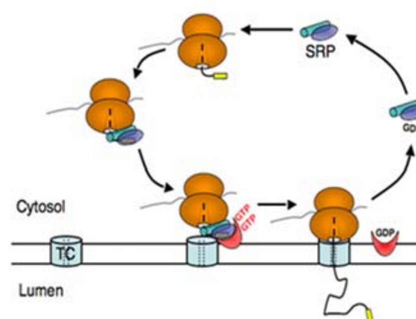


Figure 8: SR particle targeting proteins and transferring the ribosome nascent chain complex (RNC) to the protein-conducting channel, which produces stain.

[38] Gilmore R, Blobel G, Walter P., pg. 463–9.

Appendix 8: Microtiter Plates, Absorption Values and iMark Microplate Absorbance Reader

A microtitre plate (spelled microtiter in the United States) or microplate or microwell plate is a flat plate with multiple "wells" used as small test tubes. The microplate has become a standard tool in analytical research and clinical diagnostic testing laboratories. A very common usage is in the enzyme-linked immunosorbent assay (ELISA), the basis of most modern medical diagnostic testing in humans and animals.[39]

A microplate typically has 6, 24, 96, 384 or even 1536 sample wells arranged in a 2:3 rectangular matrix. Some microplates have even been manufactured with 3456 or even 9600 wells, and an "array tape" product has been developed that provides a continuous strip of microplates embossed on a flexible plastic tape.[40]

Each well of a microplate typically holds somewhere between tens of nanolitres to several millilitres of liquid. The enormous growth in studies of whole live cells has led to an entirely new range of microplate products which are "tissue culture treated" especially for this work. The surfaces of these products are modified using a plasma discharge to make them easier for adherent cells to grow on.[41]

Procedure for setting up a 96-well microtiter plate with cells:

1. Pipet 447 µL RPMI media into a sterile 15 mL conical centrifuge tube.
2. Suspend the stock solution of cancer cells by pipetting up and down a couple of times and then add 530 µL of the solution into the conical centrifuge tube.
3. Mix the media and the cells by pipetting gently.
4. Obtain a 96-well sterile microtiter plate. Label the rows and the columns. (eg. A-F, 1-7)
5. Uncover the first column of the plate, and using a P200, pipet 50 µL of RPMI media only (no cells) into rows A through F for that column, column #1.
6. Move the lid of the plate over one column and pipet 50 µL of the diluted cancer cell solution from the conical centrifuge tube into each of the rows A through F for that column, column #2.
7. Repeat step 6 for columns #3-#7 of the plate.

   CO2 tissue culture incubator. It should stay for 24 hours before the drug addition step.
9. Clean up.

Microplate Readers (also known as plate readers) are laboratory instruments designed to detect biological, chemical or physical events of samples in microtiter plates. They are widely used in

[39] http://www.corporeality.net/museion/category/medical-scientific-instruments/ (Retrieved May 4, 2012)
[40] Elaine May, p. 22.
[41] Farkas E., pg. 913.

research, drug discovery, bioassay validation, quality control and manufacturing processes in the pharmaceutical and biotechnological industry and academic organizations. [42]

The use of the reader in this investigation is to detect absorbance. **Absorbance** (also called **optical density**) of a material is a logarithmic ratio of the radiation falling upon a material, to the radiation transmitted through a material.[43]

Procedure for performing the Cell Viability Assay:

1. Obtain the 96-well plate from the tissue culture incubator after 48 hours.
2. Pipet 20 μL of SRP reagent (Appendix 7) into each well of the 96-well assay plate. There should be 48 wells (rows A-F and columns 1-8) to fill. Use a clean pipet tip for each addition. Do not forget to mix the reagent well with the cells by pipetting up and down several times after each addition.
3. After adding the reagent, return the plates to the incubator, where they will incubate for 1 hour at 37°C.
4. After the time period, read the absorbance values at 490 nm using the BioRad iMark microtiter plate reader. Then record and process the data.

Absorbance detection has been available in microplate readers for more than 3 decades, and is used for assays such as cell viability, protein and nucleic acid quantification or enzyme activity assays. A light source illuminates the sample using a specific wavelength selected by an optical filter and a light detector located on the other side of the well measures how much of the initial (100 %) light is transmitted through the sample: the amount of transmitted light will typically be related to the concentration of the molecule of interest.[44]

Figure 9: The iMark Microplate Absorbance Reader used in the investigation.

[42] http://en.wikipedia.org/wiki/Plate_reader (Retrieved April 24, 2012)
[43] Zitzewitz, Paul W., pg. 395.
[44] Mohamed-Bassem, A. Ashour, pg. 353–360.

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