

Luteolin's positive inhibition of melanoma cell lines.

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SUMMARY

If not treated early, melanoma, a form of skin cancer, can lead to death in patients. Currently the few treatments for melanoma include surgical removal, chemotherapy, or immunotherapy without any treatment based on natural small molecules currently available. Luteolin (3',4',5,7-tetrahydroxyflavone) is a flavonoid that occurs in fruits, vegetables, and herbs. Research suggests that luteolin is effective against various forms of cancer by triggering apoptosis pathways. In addition, luteolin was consistently shown to have marginal cytotoxicity against normal cells. Thus, luteolin is currently being researched as a possible anticancer agent. This experiment was performed using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to test cellular cell viability. Each sample was administered varying doses of luteolin by 2-fold serial dilution. These samples were later administered an MTT solution and scanned using an absorbance microplate reader to measure cell viability. The results of our study demonstrate that increased luteolin dosage limited melanoma cell survival rate by as much as 98% *in vitro*. Although promising, further research is needed to accept luteolin as a clinical drug. This experiment analyzes the effects of luteolin on the cell viability of malignant melanoma cells using an *in vitro* experiment to research alternative melanoma treatments and hopefully to help further cancer research as a whole.

INTRODUCTION

Historically, almost 34% percent of medicines found between 1981 and 2010 are either natural compounds themselves or are derivatives of natural compounds (1). Luteolin, or 3',4',5,7-tetrahydroxyflavone, is a flavonoid that commonly occurs in many different types of fruits, vegetables, and medicinal herbs (2). In plants, Luteolin is used as a protection against microorganisms, infection, and UV radiation (3). Plants rich in luteolin have been used in traditional Chinese medicine to reduce inflammation, treat disease, and battle cancer (3). With the lower risk of breast, colon, and prostate cancer with populations in Asia compared to the West, researchers have raised the question of whether luteolin has anticancer effects (4).

In 2019, advanced stages of melanoma caused around 7000 deaths in the United States alone (5). Melanoma is caused by mostly UV radiation damage to melanocytes,

skin cells that produce the skin pigment melanin (6). Some potential indicators of melanoma include moles that have asymmetrical shape, uneven borders, different colors, large size, and the tendency to change size, shape, or color over time (6). The severity of melanoma depends upon its stage, which are ranked 0 to IV. While Stage 0 to Stage I are not dangerous, Stage II can easily spread to important tissues such as the lymph nodes (7). Stage III tumors have spread to the local lymph nodes and Stage IV melanomas have advanced to major organs such as the brain, heart, and liver (7). The last two stages both cause deaths due to organ failure, with Stage IV melanomas being terminal for most patients (7). Although early stage melanomas can be easily removed with surgery, late stage melanomas are more difficult to treat (7). Immunotherapy, radiation therapy, and chemotherapy may not always work and can leave many side effects (8). Since early stages are not commonly detected and late stages can result in many complications, it is thus useful to find alternative methods of combating melanoma (8).

Recent evidence has established a positive effect of luteolin against various forms of cancer (9). Luteolin may primarily have its antioncogenic effect due to its ability to block cell-survival pathways and instead trigger apoptosis by primarily suppressing MMP-2, and MMP-9 proteins (1). In research performed by Mano Horinaka et al, luteolin was shown to have marginal cytotoxicity against normal cells even at high doses (9). Additionally, long term exposure did not show any apparent toxicity in rats (30 mg/kg, p.o. for 20 days) (4). Both research indicates that high dosages of luteolin have little effect in healthy cells for both *in vitro* and *in vivo*. A combination of its proven effects against several forms of cancer, low toxicity in healthy cells, and prevalence in history allows for this compound to be a prime candidate for testing for alternative forms of melanoma treatment.

The goal of this research is to establish the relationship between luteolin and melanoma cell death to develop an alternative drug candidate to melanoma by treating melanoma cells under *in vitro* conditions with luteolin. Though healthy cells have a high tolerance to luteolin, at high enough doses it can still be toxic to some degree. Therefore, it is desirable to find the highest dose that effectively inhibit cancer cells while remaining nontoxic to normal cells.

RESULTS

To evaluate luteolin's effect on melanoma cells, cells were

a

Dosage (µM)	Col 1 0 µM	Col 2 0.0313 µM	Col 3 0.0625 µM	Col 4 0.125 µM	Col 5 0.25 µM	Col 6 0.5 µM	Col 7 1 µM	Col 8 2 µM	Col 9 4 µM	Col 10 8 µM	Col 11 16 µM	Col 12 32 µM
570nm	1.4235	1.2783	1.2296	1.2736	1.3163	1.1313	1.1331	1.131	0.9461	0.4469	0.14378	0.0562
650nm	0.1629	0.1558	0.1532	0.1534	0.1592	0.1398	0.1415	0.1456	0.12445	0.0758	0.049	0.0403
Absolute Difference	1.2605	1.1224	1.0763	1.1202	1.157	0.9914	0.9915	0.9855	0.8216	0.3711	0.0946	0.01586

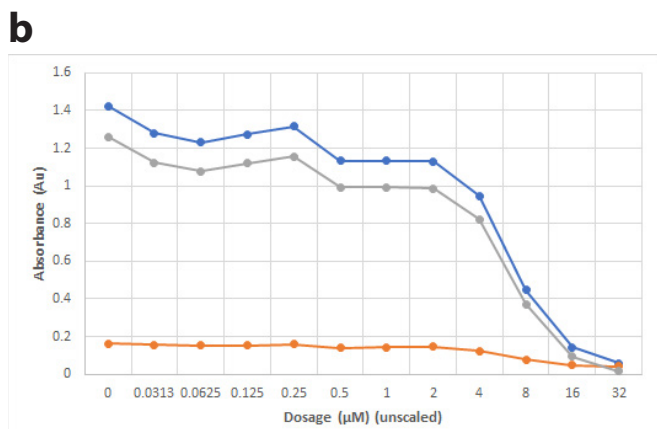


Figure 1: Higher dosage results in lower absorbances. (a) Absorbance of each wavelength (n=4). (b) Data points indicate the mean absorbances of each dosage.

administered dosages varying from 0–32 µM. These dosages were chosen because it was outside of the toxicity which would otherwise affect normal cells and these dosages have been shown to result in a range of responses from almost no termination to complete termination of melanoma cells (4)(14). These cells were then evaluated using spectrophotometry of an MTT Assay. MTT is a dye that when in contact with NAD(P)H oxido-reductases enzymes in cells reduces into a formazan which gives off a purple hue. When cells do not metabolize NAD(P)H, such as when they are undergoing apoptosis, MTT is not reduced and solution remains clear. Therefore, a sample with higher cell viability will have a greater absorbance of a longer wavelength compared to a sample with a low cell viability, which will have more light pass

Dosage (µM)	0	0.0313	0.0625	0.125	0.25	0.5	1	2	4	8	16	32
Absorbance deviation	0.0842	0.1052	0.0704	0.1003	0.0608	0.0904	0.0623	0.0852	0.0608	0.0423	0.0177	0.0047
Survival Rate Deviation	6.678	4.183	5.583	7.959	5.826	7.169	4.940	6.758	4.825	3.358	1.400	0.371
Survival Rate Percent Deviation %	6.678	4.697	6.538	8.956	5.257	9.114	6.281	8.645	7.404	9.405	9.658	10.449
Final Average Percent Deviation	7.757											

Table 1: Absorbance deviation, survival rate deviation, and the mean percentage deviation of the survival rate.

a

Dosage (µM)	Col 1 0 µM	Col 2 0.0313 µM	Col 3 0.0625 µM	Col 4 0.125 µM	Col 5 0.25µ M	Col 6 0.5µ M	Col 7 1µM	Col 8 2µM	Col 9 4µM	Col 10 8µM	Col 11 16µM	Col 12 32µM
Trial 1	88.41	86.28	90.15	87.36	93.37	83.82	77.23	73.30	66.74	25.35	5.88	0.83
Trial 2	98.26	81.70	81.06	74.46	84.42	79.56	81.75	77.61	63.50	36.10	9.42	1.98
Trial 3	102.58	89.01	91.797	99.56	99.86	86.91	85.46	91.69	73.29	29.50	8.39	0.95
Trial 4	110.79	82.48	78.548	94.11	89.51	64.32	70.20	70.11	57.22	26.82	6.33	1.28
Average	100.00	89.05	85.389	88.87	91.79	78.65	78.66	78.18	65.19	29.44	7.51	1.26

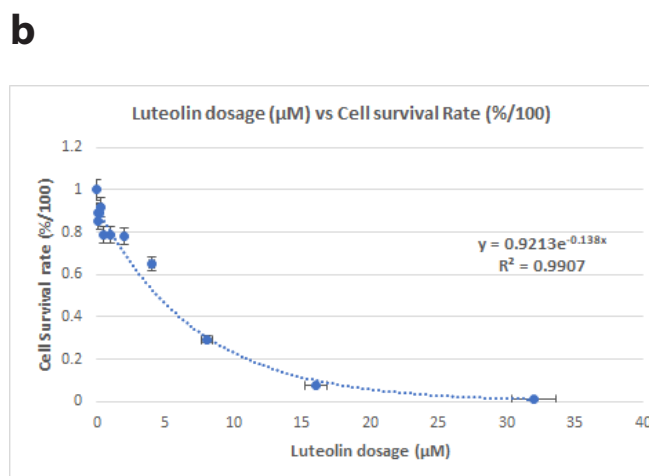


Figure 2: Luteolin inhibits melanoma cell survival rates in a dose dependent manner. (a) Survival rate of cells relative to the control. (b) Data points indicated the mean survival rate for each dose. The survival rate decreased exponentially as dosage increased, with the highest tested dosage of 32 µM resulting in only about 1% of cells surviving.

through instead of being absorbed (**Figure 1**).

After each trial was analyzed using spectrophotometry, the reference absorbance was subtracted by the foreground to avoid any skewed data due to the environment (**Figure 1**). Then using the average control as a baseline for 100% survival rate, a dilution curve was calculated to test the dose-dependent effect of luteolin on cell survival (**Figure 2b**).

The overall trend showed the percentage of survival

Levine's Test (homogeneity of variance)				
Total Degree of Freedom	Sum of Squares	Mean Square	F-Statistic	P-value
47	0.08676	0.001845	1.18969	0.330119
ANOVA Summary				
Total Degree of Freedom	Sum of Squares	Mean Square	F-Statistic	P-value
47	5.1897	0.005	89.987	0.01

Table 2: Dosage is statistically significant to cell survival rate. The Levine's Test (left) tested for eligibility of the data for a one-way ANOVA (right). The one-way ANOVA returned the p-value, or the statistical significance, of the data. The low p-value indicates a high statistical significance of the data.

exponentially decreased as the dosage increased, with the full dose bringing an average 98% termination rate (**Figure 2a**). This shows that as the dosage increases the survival rate exponentially decreases, which means that luteolin similarly induces apoptosis in malignant melanoma cells.

The overall percentage deviation was relatively low with average percent deviation of 7.757% amongst all samples (**Table 1**) and a R^2 of 0.99 (**Figure 2b**). This suggests the experiment is very consistent and can be replicated in similar laboratory settings. The data also fits well with the line of best fit, meaning that the data was not heavily skewed by outliers. After performing a Levine's test on the data, we got a p-value of 0.33, and since that value is higher than 0.05, it was eligible for a one-way ANOVA. This gave us a p-value of 0.01 (**Table 2**). Therefore, luteolin is shown to significantly affect melanoma's survival rate.

DISCUSSION

This research's purpose was to demonstrate the effects of luteolin, a chemical compound commonly found in fruits and vegetables, on the cell viability of cancerous melanoma cells in hopes of contributing towards the use of luteolin in the treatment of disease. The overall trend showed the percentage of survival exponentially decreased as the dosage increased, with the highest dose bringing an average 98% elimination rate (**Figure 2a**). With a significant statistical significance and This further supports our hypothesis that luteolin can be used to effectively reduce the cell viability of melanoma cells. Thus, luteolin may be a viable drug candidate for the treatment of melanoma.

Although the overall trend showed a decrease in cell viability as the dosage increased, 0.125 μM and 0.25 μM instead seemed to have a slight increase in cell proliferation from lower doses (**Figure 2a**). Due to the high statistical significance of the data, it may be likely that simply at lower doses, luteolin has a relatively similar effect. The effectiveness of a drug generally is similar in low doses but as the dosage increases its effectiveness increases dramatically, until it plateaus

past a certain point. This can be seen in **Figure 2b**, where the cell elimination rate dramatically increases at dosages greater than 2 μM (**Figure 2b**). It is entirely possible that a difference of dosage at 0.0625 μM or 0.125 μM has little effect on the elimination rate, which will cause these results to display about the same cell proliferation, if not a bit higher even for a higher dose. Still, we cannot rule out the possibility of an error due to serial dilution. Serial dilution was chosen in that it is flexible, quick, and can give an even range of values. One weakness of serial dilution is that it may be prone to error propagation at low doses. This may result in some of the doses being a bit lower than the expected, hence the slight increase in cell propagation.

In future experiments, there are some practices that can be done to improve this experiment's accuracy. One alternative method to serial dilution is to create multiple existing stock concentrations instead of having one stock concentration to perform serial dilution across. This allows for the concentrations to have fewer errors, but at the cost of having less variability, less ease of use, and uneven spacing. Additionally, it is labor and resource intensive to create enough stock concentrations to have enough conclusive data, but the option was available to us it would be the ideal method. The 4 trials do help to reduce inconsistencies, but more trials should be done in the future to mitigate the effect of random errors and bias on the overall data. Though it was established that luteolin has little effect on healthy cells, the dosages tested in this experiment should be tested on healthy cells *in vitro* to be certain. Other chemical compounds that have similar properties need to be studied in case they have similar properties for manufacturing additional medical treatments.

After this experiment, there is still much more testing before luteolin can be considered an official treatment, however. Preclinical studies such as *in vivo* experiments in animals are already being done to determine toxicity and optimal dosage (9). This is even before considering clinical studies with real patients, which may take upward 10 years before luteolin can be approved as an optimal drug. It may

take decades before luteolin can be available to the public. Ultimately, as research is still being performed on luteolin, this experiment has identified an effective dosage for melanoma cells, contributing towards the use of luteolin as a new treatment for disease.

MATERIALS AND METHODS

Setup

To test for cell viability among the melanoma cell lines, the independent variable was the dosage of luteolin (micromolars) applied to the samples and the dependent variable was the cell survival rate. This experiment was done by using an MTT assay, an investigative procedure to determine cellular cell viability by using the dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), or MTT.

All cell cultures came from the same SK-mel-28 cell line (vendor: ATCC, catalog number: ATCC® HTB-72™), maintained at a temperature of 37°C inside of a 5% CO₂ incubator, and were treated with the same amount of medium and growth factor. The medium used, Eagle's minimum essential medium (EMEM) (vendor: Lonza, catalog number: 12-611F), was supplemented with 5% FBS and 5% NBS (fetal bovine serum, newborn bovine serum) and 1% Penicillin/Streptomycin antibiotics. No other growth factors were used. The passage number was estimated to be 50. Cells are expected to grow to about 60-70% confluence level, with the control not exceeding 90%. To avoid contamination, all experimentation was performed in a BSL2 biosafety hood that was cleaned regularly with UV light. All laboratory equipment was properly cleaned or disposed of to mitigate any contamination (with approved IBC protocol, UCI 2013-1458).

Cell Culture

Cells were detached using 1 ml 0.25% trypsin (vendor: Lonza, catalog number: 17-161E). After leaving the sample in the CO₂ incubator for 5 minutes, 4 ml of EMEM added to the dish. Approximately 5000 cells were seeded into each well of a 96-well plate, about 100 µl in each well for this experiment, which was then placed back into the incubator. After waiting 24 hours, the cell medium was disposed of and 500 µl of EMEM was added to all the wells except for the 12th column, which had 1000 µl medium. 2 µl of 16 mM luteolin was added to the 12th column of the cell culture and a 2-fold serial dilution was performed across the plate. The cells were not disturbed as they remain adhered to the plate. The first column, the control, was not diluted. The culture was then placed in the incubator for 72 hours.

MTT Assay

After 72 hours, the MTT was prepared by mixing 0.5g of solid powdered MTT with 10ml of PBS to create a liquid solution, and 100 µl was placed inside each well for 1 hour. Then 100 µl DMSO is used to dissolve the MTT. The data was analyzed using a Synergy HTX, multimode reader (BioTek) to find the cell viability in each well. The experiment was

repeated 4 times in total, using a different well plate each time.

Statistical Analysis

When analyzing an MTT assay, the Synergy multimode reader will give two tables of data, one absorbance of 570 nanometers, and another of reference wavelength of 650 nanometers. 650nm must be subtracted by 570nm to get the actual absorbance by the samples (**Figure 1a**).

The cell survival rate is calculated by dividing the mean control absorbance difference with every cell well then finding the mean survival rate for said dosage by dividing the sum of all the trials for said dosage by the number of total trials. (**Figure 2a**).

Deviations are calculated by finding the absolute difference from the mean. The survival percentage deviation is the survival deviation divided by the corresponding average

$$\text{Survival rate} = \frac{\sum \frac{\text{Experimental}}{\text{Mean Control}}}{\text{Number of trials}}$$

of that dosage and the final average percentage deviation is the average of all percentage deviations (**Table 1**).

The data ran through Levine's test (homogeneity of variance test) to find if the data was eligible for a one-way ANOVA. After checking if the p-value was over 0.05, the data was put through a one-way ANOVA with the null hypothesis being the dosage having no effect of cell survival. A p-value was calculated from the result, which was used to find statistical significance. A p-value of under 0.05 was listed as statistically significant while a p-value over 0.05 was listed as not significant (**Table 2**).

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